Molecular Imaging of Inflammation and Platelet Adhesion in Advanced Atherosclerosis: Effects of Antioxidant Therapy with NADPH Oxidase Inhibition

Liu et al: Imaging of Antioxidant Therapy in Atherosclerosis

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Abstract

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 LDL 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/day orally). In vivo molecular imaging of VCAM-1, P-selectin and platelet GPIbα in the thoracic aorta was performed with targeted contrast-enhanced ultrasound (CEU) molecular imaging. Arterial elastic modulus and pulse wave transit time were assessed using ultra-high frequency ultrasound and invasive hemodynamic measurements. Plaque size and composition were assessed by histology. Molecular imaging in non-treated mice detected a 2-fold increase in P-selectin expression, VCAM-1 expression, and platelet adhesion between 30 and 40 wks 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.

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 \textit{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.\(^4,6,7\) 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.

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 \textit{p47phox}.\(^7,9\) Apocynin has been shown to reduce atherosclerosis when given early in disease in animal models.\(^7\) Since the production of ROS are 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 pro-inflammatory properties.\(^10,11\)

In this study we used advanced \textit{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 LDL-receptor and Apobec-1 on a C57Bl/6 background were studied.12 Wild-type mice and DKO mice were studied at 30 wks of age (n=5 each), and DKO mice were also studied at 40 weeks of age with a preceding 10 wks 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 transthoracic 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 wks.

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 (MB_{GPIb}), P-selectin (MB_{p}), or VCAM-1 (MB_{v}) were prepared by conjugating the following ligands to the shell surface as previously described: dimeric recombinant A1 domain (amino acids 445 to 709) of mouse von Willebrand factor (VWF), rat anti-mouse monoclonal IgG1 against P-selectin (RB40.34) or VCAM-1 (MK 2.7). Control microbubbles (MB_c) were prepared using rat isotype control antibody (R3-34, Pharmingen Inc.). Microbubble concentration was measured by electrozone sensing (Multisizer III, Beckman-Coulter).

**Flow-chamber Assessment of Microbubble-Platelet Interactions**

To validate targeted attachment of MB_{GPIb}, 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 hr then blocked with denatured BSA (5 mg/mL). Human whole blood anticoagulated with D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (40 μM) was drawn through capillary tubes for 3 minutes at a wall shear rate of 500s^{-1} to form platelet-rich thrombi. Suspensions (3×10^6 mL^{-1}) of MB_{GPIb} and control microbubbles (either MB_c or microbubbles bearing a non-binding mutant (substitution Gly561→Ser) of human VWF A1-domain of vWF [MB_{GPIb-mut}]) were differentially labeled with the fluorophores DiI or 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 min. Tubes were washed with PBS and the number of microbubbles attached was averaged from 10-15 randomly selected non-overlapping optical fields (0.09 mm^2) on fluorescent microscopy. Experiments were performed in triplicate for each condition.
Molecular Imaging

Mice were anesthetized with inhaled isoflurane (1.0 to 1.5%). Contrast-enhanced ultrasound (CEU) of the ascending aorta and proximal aortic arch was performed with a linear-array probe (Sequoia, Siemens Medical Systems) using a right parasternal window. Multipulse phase-inversion and amplitude-modulation imaging was performed at 7 MHz and a mechanical index (MI) of 0.97. Images were acquired 8 min after intravenous injection of targeted or control microbubbles ($1 \times 10^6$ per injection) performed in random order. Signal from retained microbubbles was determined from the first frame obtained. As previously described,$^{14}$ 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 (Supplemental Figure A). 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-D 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 (30MHz) ultrasound imaging (Vevo 770, VisualSonics Inc.) 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 mid-ascending aorta was performed and aortic strain was calculated as the change in systolic and diastolic diameter divided by diastolic diameter ($\Delta D/D_{\text{dia}}$). A micromanometer (SPR-1000,
Millar Instruments, Inc.) was inserted into the aorta via the carotid artery for arterial pressure measurement and aortic elastic modulus ($\varepsilon_A$) was calculated by:

$$\Delta P \times \frac{D_{\text{dia}}}{\Delta D}$$

where $\Delta P$ is pulse pressure expressed in $10^5$ 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 pudendoepigastric 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 percent area change were assessed in the mid-ventricular short-axis plane. Stroke volume was calculated by the product of left ventricle outflow tract (LVOT) area and LVOT 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 two sites, the mid-ascending aorta and arch just proximal to the brachiocephalic artery, were evaluated at 40 wks of age (n=5 animals for each treatment group). Masson’s 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) for identifying monocytes and macrophages; or against GPIIb integrin (sc20234, Santa Cruz)
for platelets; or rabbit polyclonal antibody against VCAM-1 (BS-0369r, Bioss Inc.). Species-appropriate ALEXAFluor-488, -555, or -594 secondary antibodies (Invitrogen) were used. Spatial extent of Mac-2 expression was quantified by using non-confocal microscopy which provided the optimal binary data for positive/negative staining using Image-J and a threshold of >2SD above of the tunica media intensity excluding the elastic lamina. Data were expressed as Mac-2-positive area within the internal elastic lamina and as a percentage of total plaque area determined using low-level green autofluorescence identification. Confocal fluorescent microscopy was used to identify platelets on the endothelial surface.

**Statistical Analysis**

Data were analyzed on SPSS (version 16.0). All data were normally distributed and expressed as mean (±SD) unless otherwise stated. Comparisons between 30 wk wild-type and DKO mice, and of the 40 wk groups to 30 wk DKO mice were made with unpaired Students \( t \)-test (two-tailed). Comparisons between 30 wk mice and all three 40 wk DKO cohorts were made with one-way ANOVA and, when significant (\( p<0.05 \)), post-hoc analysis with unpaired Students \( t \)-test and Bonferroni correction for multiple comparisons was performed. Comparisons of in vitro microbubble adhesion at different shear conditions were made with two-way ANOVA and, when significant (\( p<0.05 \)) post-hoc analysis with paired \( t \)-tests.

**Results**

**Blood Pressure and Left Ventricular Function**

Arterial systolic and diastolic pressures in DKO mice at 30 wks 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 wks of age. This increase was prevented by apocynin treatment in a dose-dependent fashion. Moreover, for the high-dose apocynin treatment group systolic arterial pressure and pulse pressure were lower at 40 wks than 30 wks of age.

On echocardiography left ventricular fractional shortening, LVEF and stroke volume were not significantly different for wild type and DKO mice at 30 wks of age (Table). For DKO mice, there was a non-significant trend for an increase in these parameters between 30 wks and 40 wks of age but all were similar between treatment groups at 40 wks. Aortic internal diameter at 30 wks of age was greater for DKO compared to wild type mice and progressively increased by 40 wks of age in all treatment groups. Aortic peak systolic flow velocities were not different between treatment groups at 40 wks 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 wks and significantly increased between 30 and 40 wks 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 wks was similar to that at 30 wks of age. Although PTT was not different between DKO and wild type mice at 30 wks 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 wks 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 control microbubbles 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 non-binding form of VWF A1-domain (MB_GPIb-mut) (Figure 2B). Attachment for MB_GPIb 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 wks of age (Figure 3). In untreated DKO mice, signal for all three targets had increased by more than 2-fold between 30 and 40 weeks of age. At 40 wks 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 wk DKO mice. Signal for all targeted agents in the untreated DKO mice at 30 and 40 wks was significantly greater (p<0.01) than signal from control non-targeted 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 wks 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 wks of age. In DKO mice at 40 wks, 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 wks.

Histology in untreated DKO mice at 40 wks 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 wk 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 non-invasive imaging methods for characterizing vascular molecular, ultrastructural, and elastic phenotype \textit{in vivo} were employed to evaluate the effect of 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.\textsuperscript{1,2} Oxidative stress is an important regulator of pro-inflammatory gene expression mediated largely through the transcription factor nuclear factor-κB (NF-κB).\textsuperscript{4} There is strong evidence that through these mechanisms oxidative stress will increase expression of endothelial cell adhesion molecules such as P-selectin, ICAM-1, and VCAM-1; and production of pro-inflammatory chemokines.\textsuperscript{4,9,18,19} It is also well established that inactivation of nitric oxide (NO) by ROS can lead to acceleration of atherosclerosis and susceptibility to atherothrombotic events by interfering with NO’s anti-inflammatory, anti-platelet, and anti-proliferative effects.\textsuperscript{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.\textsuperscript{6,7} Again, self-amplifying mechanisms can be identified whereby many pro-inflammatory cytokines, thrombin, and angiotensin II further activate NADPH oxidase in vascular cells.\textsuperscript{1,20-22} The role of NADPH oxidase specifically in the development of atherosclerosis is strongly supported by the observation that gene-deletion of the p47\textit{phox} subunit of the NADPH oxidase complex in apolipoprotein-E-deficient mice reduces atherosclerotic lesion size in the descending aorta.\textsuperscript{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 biologic effects which include the prevention of NADPH oxidase assembly by inhibiting membrane translocation of the p47\textit{phox} and gp91\textit{phox} subunits.\textsuperscript{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.\textsuperscript{9,23,24} Apocynin specifically has been shown to reduce selectin and VCAM-1 expression, and platelet adhesion \textit{in vivo} studies of acute inflammatory lung injury.\textsuperscript{25} 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.\textsuperscript{26}

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 ApoE-/- mice.\textsuperscript{19} 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 since the ratio of monocytes to plaque area was significantly lower statistically from controls only in the low-dose group.

Molecular imaging data was useful for defining at least one potential mechanism by which inflammatory cell accumulation was reduced. Apocynin appeared to prevent the progressive and marked increase in the expression of two 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.

In order 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 since its
interaction with the platelet GPIbα receptor can occur despite 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 pro-inflammatory factors including C-C motif chemokines (RANTES, macrophage inflammatory protein-1β) and platelet factor-4. Platelets may also contribute to endothelial activation in atherosclerosis by CD40L-CD40 signaling, 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 prevented in a dose-dependent fashion by apocynin, which may have also contributed to the slower progression of disease and reduced macrophage infiltrate in the treated animals.

The improvement in the arterial elastic properties and blood pressure with apocynin we observed is likely multifactorial. As reviewed elsewhere, oxidative stress in animal models and in humans, even in the absence of atherosclerosis, has been strongly linked to impaired arterial elastic properties and hypertension. ROS may contribute to hypertension and vascular stiffness by reducing NO bioavailability, direct vasoconstrictor effects, and promoting vascular remodeling and medial hyperplasia. It is also now recognized that angiotensin II exerts its effects on blood pressure and vascular remodeling in part through activation of NADPH oxidase, and that both angiotensin converting enzyme inhibitors and angiotensin receptor blocking agents
improve blood pressure in part through reducing ROS production.\textsuperscript{1,35} Hence, it likely that the improvements in hemodynamics and elastic properties with apocynin therapy did not simply reflect the beneficial vascular effects of reducing the atherosclerotic burden.

There are several limitations of this study that deserve attention. First, we did not measure plasma apocynin levels. Our doses were guided by previous studies examining cardiovascular effects of apocynin and were several orders of magnitude lower than the reported LD\textsubscript{50}.\textsuperscript{7} 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 since this molecule is stored pre-formed 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 p47\textit{phox/-} mice. On molecular imaging, although control microbubble 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.\textsuperscript{36,37} 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.\textsuperscript{14} 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


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BP, blood pressure; DKO, double knockout mice; LVEF, left ventricular ejection fraction; *p<0.05 vs wild type; †p<0.05 vs DKO 30 wk. ‡p<0.05 vs untreated DKO group at 40 wks.
**Figure Legends**

**Figure 1.** (A) Elastic modulus (mean ±SEM) of the thoracic aorta ($E_{Ao}$) in wild type mice and DKO mice at baseline (30 wks) and at 40 wks after 10 wks of either no treatment (control) or apocynin given at low- and high-dose. (B) Examples of source data for from a non-treated animal illustrating age-dependent worsening of $E_{Ao}$ as a result of both an increase in the aortic pulse pressure ($\Delta P$) and a reduction in the systolic aortic diameter change ($\Delta D$) from M-mode echocardiography. (C) Pulse transit time (mean ±SEM) in wild type mice and DKO mice at baseline and at 40 wks. (D) Examples of pulse-wave spectral Doppler data from an untreated and high-dose apocynin-treated DKO mouse at 40 wks illustrating differences in pulse transit time measured as the time delay ($\Delta t$) for the onset of systolic flow from the ascending aorta (top) to the femoral artery (bottom). *p<0.05 vs. 30 wk wild type; †p<0.05 vs.30 wk DKO; ‡p<0.05 versus untreated (control) 40 wk DKO.

**Figure 2.** Mean (±SEM) number of microbubbles attached to platelet complexes per optical field (OF) for flow chamber studies when comparing microbubbles targeted to GPIbα ($MB_{GPIb}$) to either (A) control microbubbles not bearing a ligand ($MB_{C}$) suspended in PBS; or (B) control microbubbles bearing a mutated non-binding ligand ($MB_{Ib-mut}$) suspended in whole blood. Examples show fluorescent $MB_{GPIb}$ attaching to platelet complexes at shear rates of (C) 300 s$^{-1}$ and (D) 1,000 s$^{-1}$.

**Figure 3.** (A to D) Molecular imaging signal (mean ±SEM) for P-selectin, VCAM-1, or GPIbα, and for control non-targeted microbubbles. *p<0.05 vs. 30 wk wild type; †p<0.05 vs.
30 wk DKO; ‡p<0.05 versus untreated (control) 40 wk DKO. (E) Example from an untreated 40 wk DKO mouse of a 2-D ultrasound image of the aortic arch used to define regions-of-interest; and CEU molecular imaging from the same mouse after injection of either (F) GP_{lba}-targeted or (G) control microbubbles (color scale at bottom). As previously described, 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 Supplemental Figure A.

**Figure 4.** Vessel thickness (mean ±SEM) measured by high-frequency 2-D ultrasound imaging of the aortic arch measured at (A) the lesser curvature of the aortic arch, and (B) at the origin of the brachiocephalic artery. Images illustrate examples obtained from DKO mice (C) at 30 wks, (D) at 40 wks without treatment, or (E) at 40 wks with high-dose apocynin. Arrows illustrate regions of thickening at the lesser curvature of the arch and at the origin of the brachiocephalic artery. *p<0.05 vs. 30 wk wild type; †p<0.05 vs 30 wk DKO; ‡p<0.05 versus untreated (control) 40 wk DKO.

**Figure 5.** (A and B) Mean (±SEM) plaque area in absolute terms and expressed as a ratio to the total vessel area for DKO mice at 40 wks of age. (C and D) Mean (±SEM) area staining positive for Mac-2 in absolute terms and expressed as a ratio to total plaque area. Histology illustrates plaque formation on Masson’s trichrome staining from: (D and E) two separate untreated 40 wk DKO mice, and (F) a high-dose apocynin-treated mouse. (G) Examples of Mac-2 staining illustrating differences in
monocyte/macrophage accumulation at 40 wks in the different cohorts. Control staining conditions are provided in Supplemental Figure B. *p<0.05 vs. untreated control; and †p<0.05 vs. untreated controls and low-dose.

**Figure 6.** (A and B) Histology with Masson’s trichrome illustrating inflammatory cells or platelets (arrows) adherent to the endothelial surface of plaques from two separate untreated 40 wk DKO mice. (C and D) Examples of immunofluorescent histology for GPIIb from two separate mice confirming the presence of platelets (arrows) on the endothelial surface and also within the plaque milieu. Composite images of VCAM-1 immunofluorescent staining from (E) an untreated 40 wk DKO mouse, and (F) a high-dose apocynin treated mouse.
Molecular Imaging of Inflammation and Platelet Adhesion in Advanced Atherosclerosis: Effects of Antioxidant Therapy with NADPH Oxidase Inhibition

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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.