Molecular Imaging Reveals Rapid Reduction of Endothelial Activation in Early Atherosclerosis With Apocynin Independent of Antioxidative Properties

Elham Khanicheh, Yue Qi, Aris Xie, Martina Mitterhuber, Lifen Xu, Michika Mochizuki, Youssef Daali, Vincent Jaquet, Karl-Heinz Krause, Zaverio M. Ruggeri, Gabriela M. Kuster, Jonathan R. Lindner, Beat A. Kaufmann

Objective—Antioxidative drugs continue to be developed for the treatment of atherosclerosis. Apocynin is a nicotinamide adenine dinucleotide phosphate oxidase inhibitor with anti-inflammatory properties. We used contrast-enhanced ultrasound molecular imaging to assess whether short-term apocynin therapy in atherosclerosis reduces vascular oxidative stress and endothelial activation.

Approach and Results—Genetically modified mice with early atherosclerosis were studied at baseline and after 7 days of therapy with apocynin (4 mg/kg per day IP) or saline. Contrast-enhanced ultrasound molecular imaging of the aorta was performed with microbubbles targeted to vascular cell adhesion molecule 1 (VCAM-1; MBVCAM-1), to platelet glycoprotein Ibα (MBp1), and control microbubbles (MBtr). Aortic vascular cell adhesion molecule 1 was measured using Western blot. Aortic reactive oxygen species generation was measured using a lucigenin assay. Hydroethidium oxidation was used to assess aortic superoxide generation. Baseline signal for MBVCAM-1 (1.3±0.3 AU) and MBp1 (1.5±0.5 AU) was higher than for MBtr (0.5±0.2 AU; *P*<0.01). In saline-treated animals, signal did not significantly change for any microbubble agent, whereas short-term apocynin significantly (*P*<0.05) reduced vascular cell adhesion molecule 1 and platelet signal (MBVCAM-1: 0.3±0.1; MBp1: 0.4±0.1; MBtr: 0.3±0.2 AU; *P*<0.05 between agents). Apocynin reduced aortic vascular cell adhesion molecule 1 expression by 50% (*P*<0.05). However, apocynin therapy did not reduce reactive oxygen species content, superoxide generation, or macrophage content.

Conclusions—Short-term treatment with apocynin in atherosclerosis reduces endothelial cell adhesion molecule expression. This change in endothelial phenotype can be detected by molecular imaging before any measurable decrease in macrophage content and is not associated with a detectable change in oxidative burden. (Arterioscler Thromb Vasc Biol. 2013;33:2187-2192.)

Key Words: acetovanillone ■ atherosclerosis ■ microbubbles ■ molecular imaging ■ oxidative stress

Endothelial activation is a key step both in the initiation of atherosclerotic lesions and in their progression to a late stage, where inflammatory cell burden and susceptibility to acute atherothrombotic complications are high. Oxidative stress plays a major role in supporting and amplifying the endothelial activation in atherosclerosis. The family of NOX nicotinamide adenine dinucleotide phosphate (NADPH) oxidase present in plaque macrophages and in native endothelial and smooth muscle cells is a major source of reactive oxygen species (ROS) and, therefore, represents a potential therapeutic target. Apocynin is a polyphenolic drug that has been isolated from plant extracts and inhibits assembly of the NOX-2 isoform of the NADPH oxidase enzyme complex. In mice with advanced atherosclerosis, long-term therapy with apocynin has been shown to reduce endothelial adhesion molecule expression, platelet adhesion, and plaque growth, whereas in hypercholesterolemic rabbits, apocynin started at a much earlier stage of disease has been shown to prevent development of atherosclerotic lesions. It is unknown whether the beneficial effects of apocynin occur early after initiation of therapy. With regards to mechanism, it is unknown whether apocynin’s effects are entirely caused by a reduction in oxidative stress because polyphenolic drugs, such as apocynin, have anti-inflammatory effects independent of their antioxidant...
properties. Direct anti-inflammatory action independent of antioxidant properties has been substantiated by the reduced adhesion molecule expression in cultured endothelial cells exposed to apocynin.

In this study, we addressed many of these knowledge gaps. We performed in vivo ultrasound molecular imaging to test the hypothesis that short-term administration of apocynin in a model of early atherosclerosis reduces endothelial activation and platelet adhesion, 2 factors that are recognized to play an important role in plaque progression. Ex vivo techniques were used to evaluate whether these effects were associated with a reduction in vessel oxidative stress.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Effect of Apocynin on Vascular Cell Adhesion Molecule 1 Expression and Endothelial Platelet Adhesion
Apocynin treatment for 1 week reduced total aortic wall expression of vascular cell adhesion molecule 1 (VCAM-1) on Western blot by 50% (Figure 1A). On immunohistochemistry, small regions of neointimal formation were observed in the aortic root and proximal ascending portion. VCAM-1 staining was present on the endothelial lining and on macrophages in fibrofatty lesions in nontreated animals. In apocynin-treated animals, VCAM-1 expression was reduced both on endothelial cells and on macrophages within lesions, whereas the total amount of macrophages present in plaques remained unchanged (Figure 2). The reduction in endothelial VCAM-1 expression was evident both in regions overlying plaques and on endothelium in regions without plaques (Figure 1B and 1E).

In vivo labeling of platelets with rhodamine-6G allowed visualization of platelet/leukocyte aggregates on the vascular endothelial surface. Platelet/leukocyte aggregates were present not only in regions with early plaques but also in regions of the aortic endothelial surface with a normal appearance. Both the number of platelet/leukocyte aggregates per square millimeter (5.0±0.44 in apocynin-treated animals versus 9.6±0.52 in nontreated animals; P<0.01) and the percentage of endothelial surface covered by platelet/leukocyte aggregates were significantly reduced in animals that were treated with apocynin (Figure 3).

Effect of Apocynin on Vascular Oxidative Stress
Lucigenin assays showed robust NADPH oxidase activity in whole aortic rings of nontreated mice. In mice treated with apocynin, NADPH-dependent lucigenin chemiluminescence was not different compared with nontreated mice. Given the potential of lucigenin to undergo redox cycling and generate artifactual signals, high pressure liquid chromatography of tissue extracts after exposure of aortic rings to hydroethidine was performed to directly assess tissue superoxide content. In accordance with the results of the lucigenin assays, hydroethidine oxidation to 2-hydroxyethidium was not different between the 2 animal groups (Figure 4).

Molecular Imaging of VCAM-1 Expression and Platelet Adhesion
High-frequency ultrasound imaging was not of sufficient quality for evaluation in 1 animal in each group. In the remaining animals, there were no differences in left ventricular ejection fraction, peak aortic flow velocity, or aortic diameter, indicating that apocynin treatment did not lead to hemodynamic differences that could potentially influence targeted microbubble adhesion (Table).

Contrast-enhanced ultrasound molecular imaging in the ascending aorta at baseline showed greater signal enhancement for VCAM-1–targeted and platelet-targeted microbubbles compared with control microbubbles (Figure 5). After 7 days of treatment with apocynin, signal for VCAM-1–targeted and platelet-targeted microbubbles was not different from control microbubble signal (Figure 6). In contrast, in animals treated with saline injections, the signal for VCAM-1 and platelets was elevated significantly over control signal to a degree that was similar to baseline. In the subgroup of animals that were imaged before and after treatment, apocynin leads to a significant decrease in VCAM-1–targeted signal (from 1.80±0.51 to 0.30±0.10; P=0.046) and a strong trend for decrease in platelet-targeted signal (2.21±0.80 to 0.35±0.07; P=0.078). In animals imaged before and after treatment with saline injections, signal for VCAM-1 (1.44±0.55 versus 1.00±0.23; P=1.00)
and platelet-targeted signal (1.84±0.88 versus 1.26±0.40; \(P=0.69\)) did not decrease significantly.

**Discussion**

Endothelial activation plays a crucial role in the initiation and progression of atherosclerotic plaque formation. In this study, short-term treatment with the polyphenol apocynin in a murine model of early mild atherosclerosis leads to a reduction in endothelial inflammatory phenotype and platelet adhesion. These relatively acute changes were not associated with a measurable reduction in vascular NADPH oxidase activity or superoxide content.

Endothelial adhesion molecule expression is an early and important step in the pathogenesis of atherosclerosis. Deposition of oxidized low-density lipoprotein in the vascular wall leads to endothelial expression of proinflammatory cytokines, such as interleukin-1\(\beta\) and tumor necrosis factor-\(\alpha\). Locally increased cytokine levels result in an upregulation of cell adhesion molecules, such as VCAM-1, mediated by the transcriptional factor–activated protein-1 and nuclear factor \(\kappa\)B, which in turn promotes the recruitment of leukocytes to the vessel wall.

ROS generated by NADPH oxidases both in endothelial cells and in leukocytes in nascent plaques are thought to amplify vascular inflammation throughout the pathogenesis of atherosclerosis. Accordingly, mouse models with knockouts of the NADPH oxidase isoforms, NOX-1 and NOX-2, or the cytosolic NADPH oxidase subunit, p47phox, have shown a reduction in atherosclerotic plaque formation.9–11 In humans,

### Table. Echocardiographic Data

<table>
<thead>
<tr>
<th></th>
<th>Saline Treated (n=8)</th>
<th>Apocynin Treated (n=9)</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejection fraction, %</td>
<td>58.4±13.1</td>
<td>61.6±8.8</td>
<td>ns</td>
</tr>
<tr>
<td>Aortic internal diameter, mm</td>
<td>1.54±0.09</td>
<td>1.50±0.12</td>
<td>ns</td>
</tr>
<tr>
<td>Aortic peak systolic velocity, m/s</td>
<td>0.58±0.15</td>
<td>0.68±0.03</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values represent mean±1 SD. ns indicates not significant.
functional deficiency of the GP91 \textit{phox} subunit is associated with smaller carotid intima-media thickness.\textsuperscript{12} These findings have generated interest in using inhibitors of NADPH oxidases for the treatment of atherosclerosis. Apocynin inhibits NADPH oxidase activity in leukocytes allegedly by impeding the assembly of the cytosolic subunits at the cell membrane.\textsuperscript{13} In cell culture experiments it has been noted that the inhibitory action of apocynin occurs with a delay, suggesting that it has to undergo activation before inhibiting ROS generation. In the presence of H$_2$O$_2$ and myeloperoxidase, apocynin is converted to an apocynin radical and subsequently forms apocynin dimers, which are thought to be the active compounds that result in inhibition of NADPH oxidase activity.\textsuperscript{14} Thus, the effect of apocynin on NADPH oxidase inhibition during the later stages of atherosclerosis may depend on its activation in vascular tissue and require a sufficient inflammatory cell and oxidative stress burden. Notably, however, apocynin also exerts anti-inflammatory effects that are independent of NADPH oxidase inhibition.\textsuperscript{6} Such effects may be of importance during the early stages of atherosclerosis, when inflammatory cell load and oxidative burden are low.

We examined the effects of treatment with apocynin in a murine model of early atherosclerosis in mice with fibrofatty lesions. Assessing treatment effects of potential drug candidates during the early stages of atherosclerosis is of clinical significance because interventions that are started early during the pathogenesis of atherosclerosis are thought to afford a larger risk reduction for cardiovascular events than interventions that are initiated when clinical atherosclerotic disease is established.\textsuperscript{15} Our data indicate that treatment with apocynin results in a rapid decrease in endothelial expression of VCAM-1. These results are in line with observations in cell culture showing a decrease in VCAM-1 expression in response to apocynin\textsuperscript{8} and extend observations made in advanced atherosclerosis to earlier stages of plaque development.\textsuperscript{4} The decrease in signal from VCAM-1–targeted microbubbles was more pronounced than differences in VCAM-1 expression determined with Western blot. We believe this to be because of the capability of ultrasound molecular imaging to specifically assess endothelial inflammatory phenotype\textsuperscript{16} and thus to detect a more pronounced decrease in endothelial VCAM-1 compared with Western blot which assesses expression in the whole vascular wall. In addition, the observation that the decrease in vascular inflammation was not associated with a reduction of vascular NADPH oxidase activity or tissue superoxide

![Figure 5](image-url)  
**Figure 5.** Molecular imaging of the ascending aorta before the start of treatment. \(A\), Mean±SEM background-subtracted signal intensity for microbubbles targeted to vascular cell adhesion molecule 1 (VCAM-1; \(\text{MB}_{\text{VCAM}}\)) to glycoprotein Ib\(\alpha\) on activated thrombocytes (\(\text{MB}_{\text{Pl}}\)), and control microbubbles (\(\text{MB}_{\text{ctr}}\)). *P<0.01 vs \(\text{MB}_{\text{ctr}}\), †P<0.05 vs \(\text{MB}_{\text{Pl}}\) (n=12). Examples of color-coded contrast-enhanced ultrasound (CEU) images after injection of \(\text{MB}_{\text{Pl}}\) (\(B\)), of \(\text{MB}_{\text{VCAM}}\) (\(C\)), and of \(\text{MB}_{\text{ctr}}\) (\(D\)). The color scale for the CEU images is shown at the bottom of each frame.

![Figure 6](image-url)  
**Figure 6.** Molecular imaging of the ascending aorta after 7 days of saline or apocynin treatment. \(A\), Mean±SEM background-subtracted signal intensity in saline-treated (n=9) and apocynin-treated (n=10) animals for microbubbles targeted to vascular cell adhesion molecule 1 (VCAM-1; \(\text{MB}_{\text{VCAM}}\)) to glycoprotein Ib\(\alpha\) on activated thrombocytes (\(\text{MB}_{\text{Pl}}\)), and control microbubbles (\(\text{MB}_{\text{ctr}}\)). *P<0.01 vs \(\text{MB}_{\text{ctr}}\), †P<0.01 vs \(\text{MB}_{\text{Pl}}\), ‡P<0.05 vs the same microbubble in apocynin-treated animals. Examples of color-coded contrast-enhanced ultrasound (CEU) images after injection of \(\text{MB}_{\text{Pl}}\) (\(B\)), of \(\text{MB}_{\text{VCAM}}\) (\(C\)), and of \(\text{MB}_{\text{ctr}}\) (\(D\)) in saline-treated animals. In the same order, examples of color-coded CEU images after injection of \(\text{MB}_{\text{Pl}}\) (\(E\)), of \(\text{MB}_{\text{VCAM}}\) (\(F\)), and of \(\text{MB}_{\text{ctr}}\) (\(G\)) in apocynin-treated animals are shown.
content indicates that the anti-inflammatory effect of apocynin observed in our study was probably mediated through a ROS-independent mechanism in the very early stages of atherosclerotic plaque development, possibly mediated instead by its effects on cytochrome P450 pathways.\(^\text{17}\)

In addition to endothelial cell inflammatory activation, platelet–endothelial interactions play a role in vascular inflammation and the pathogenesis of atherosclerosis. Platelet–endothelial interactions mediated by P-Selectin and von Willebrand factor–glycoprotein Ib/\(\alpha\) ligation in the absence of plaque rupture accelerate plaque formation in murine atherosclerosis.\(^\text{18–20}\) The interaction of activated platelets with the endothelial surface results in the secretion of pro-inflammatory cytokines CD40L and interleukin-1\(\beta\), as well as of chemokines such as RANTES (regulated on activation, normal T cell expressed and secreted) and platelet factor 4 as of chemokines such as RANTES (regulated on activation, normal T cell expressed and secreted) and platelet factor 4, which facilitate increased monocyte recruitment.\(^\text{21–23}\)

This study was supported by Swiss Clinicians Opting for Research (SCORE) grants from the Swiss National Science Foundation (SNSF) to Dr Kaufmann (SNSF 3232B_123819 and 3232B0-141603) and to Dr Kuster (SNSF 144208), as well as by grants from the US National Institutes of Health (R01-DK-063508, R01-HL-078610, and RCI1-HL-100659 to Dr Lindner, and R01 HL42846 to Dr Ruggeri). Dr Khanicheh is supported by an MD-PhD start up grant from the University Hospital Basel.

Disclosures

None.

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Antioxidative drugs continue to be developed for the treatment of atherosclerosis. In this study, we assessed whether short-term therapy with the nicotinamide adenine dinucleotide phosphate oxidase inhibitor apocynin reduces vascular oxidative stress, endothelial inflammatory activation, and platelet adhesion in a murine model of early atherosclerosis, and whether molecular imaging is capable of detecting these changes. Assessing the treatment effects of potential drug candidates during the early stages of atherosclerosis is of clinical significance because interventions that are started early during the pathogenesis of atherosclerosis are thought to afford a larger risk reduction for cardiovascular events than interventions that are initiated late. We show that ultrasound molecular imaging is capable of detecting reductions in endothelial inflammatory activation and in platelet adhesion at a stage before any measurable, treatment-associated decrease in macrophage content occurs. These phenotypic changes are not associated with measurable changes in oxidative stress.

**Significance**

Antioxidative drugs continue to be developed for the treatment of atherosclerosis. In this study, we assessed whether short-term therapy with the nicotinamide adenine dinucleotide phosphate oxidase inhibitor apocynin reduces vascular oxidative stress, endothelial inflammatory activation, and platelet adhesion in a murine model of early atherosclerosis, and whether molecular imaging is capable of detecting these changes. Assessing the treatment effects of potential drug candidates during the early stages of atherosclerosis is of clinical significance because interventions that are started early during the pathogenesis of atherosclerosis are thought to afford a larger risk reduction for cardiovascular events than interventions that are initiated late. We show that ultrasound molecular imaging is capable of detecting reductions in endothelial inflammatory activation and in platelet adhesion at a stage before any measurable, treatment-associated decrease in macrophage content occurs. These phenotypic changes are not associated with measurable changes in oxidative stress.
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Arterioscler Thromb Vasc Biol. 2013;33:2187-2192; originally published online August 1, 2013; doi: 10.1161/ATVBAHA.113.301710
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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METHODS

Mouse Model and Experimental Setup

All experiments were performed in accordance with Swiss Federal Legislation and with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, and were approved by the local Animal Care and Use Committee at Oregon Health & Science University and the Animal Care Committee of the Canton of Basel. Male mice with a double knockout for the LDL receptor and the Apobec-1 editing enzyme on a C57Bl/6 background were used. These mice develop atherosclerosis in a predictable, age-dependent fashion while on a normal chow diet. At 20 weeks of age, when the mouse model shows lesions that cover about 5% of the total aortic surface, and small fibrofatty lesions can be seen on histology\(^1\), the mice were treated with either apocynin (4mg/kg/d; acetovanillone, Sigma) (n=40) or 0.9% saline (n=40) daily by intraperitoneal route. Animals were studied after 7 d of therapy. A small subset of animals (n=6 for each treatment group) was also studied before initiation of therapy. For each imaging study aortic contrast-enhanced ultrasound for endothelial adhesion molecule expression and platelet adhesion was performed. Assessment of aortic oxidative stress, VCAM-1 expression and platelet adhesion were performed by a panel of histologic and tissue assay techniques.

Microbubble Preparation

Biotinylated, lipid-shelled decafluorobutane microbubbles were prepared by sonication of a gas saturated aqueous suspension of distearoylphosphatidylcholine
(2mg/ml; Avanti Polar Lipids, Alabaster AL), polyoxyethylene-40-stearate (1mg/ml; Sigma), and 1,2-distearoyl-sn-glycero-phosphoethanolamine-N-[biotinyl(polyethylene glycol)]-2000 (0.1mg/ml, Avanti Polar Lipids, Alabaster AL). Microbubbles targeted to VCAM-1 (MB\textsubscript{VCAM}) were prepared by conjugation of biotinylated rat anti-mouse VCAM-1 antibody (MK 2.7) to the microbubble surface using biotin-streptavidin-biotin linking as previously described \(^2\). Microbubbles targeted to GPI\textsubscript{b\alpha} on platelets (MB\textsubscript{Pl}) were prepared by conjugating a biotinylated dimeric recombinant A1 domain (amino acids 445 to 709) of mouse von Willebrand factor (VWF) to the microbubbles. It has been shown previously that these microbubbles attach specifically to stationary platelet complexes even at high shear rates and that competitive inhibition from plasma VWF, or interaction with circulating platelets is minimal \(^3\). Control microbubbles (MB\textsubscript{Ctr}) bearing a non-specific isotype control antibody (R3-34, BD Bioscience) were also prepared. Microbubble concentration and size were measured by electrozone sensing (Multisizer III, Beckman- Coulter). Microbubble mean size was not statistically different for the three microbubble preparations (2.8±0.2 for MB\textsubscript{Ctr}, 2.8±0.2 for MB\textsubscript{VCAM}, 2.9±0.2 for MB\textsubscript{Pl}).

**Contrast Enhanced Ultrasound Molecular Imaging**

Ultrasound imaging (Sequoia Acuson C512; Siemens Medical Systems USA Inc., Mountain View, CA) was performed with a high-frequency linear-array probe (15L8) held in place by a railed gantry system. The ascending aorta including the sinus of valsalva of the mouse was imaged in a long axis plane from a right parasternal window. Contrast enhanced ultrasound (CEU) was performed with power modulation and pulse inversion (Contrast Pulse Sequence) imaging at a centerline frequency of 7 MHz and a dynamic range of 50 dB. The gain settings were adjusted to levels just below visible noise speckle and held constant. MB\textsubscript{VCAM}, MB\textsubscript{Pl} or MB\textsubscript{Ctr} (1x10\(^6\))
microbubbles per injection) were injected intravenously in random order. Ultrasound imaging was paused from the time of injection until eight minutes later when imaging was resumed at a mechanical index of 0.87. The first acquired image frame was used to derive the total amount of microbubbles present within the aorta. The microbubbles in the ultrasound beam were then destroyed with several (>10) image frames. Several image frames at a long pulsing interval (10 sec) were subsequently acquired to measure signal attributable to freely circulating microbubbles. Data were log-linear converted using known dynamic range lookup tables, and frames representing freely circulating microbubbles were digitally subtracted from the first image to derive signal from attached microbubbles alone. Contrast intensity was measured from a region of interest encompassing the sinus of valsalva, the ascending aorta and the initial portion of the aortic arch, extending into the origin of the brachiocephalic artery. The selection of the region of interest was guided by fundamental frequency anatomic images of the ascending aorta acquired at 14MHz at the end of each individual imaging sequence.

**Echocardiography**

High frequency (30MHz) ultrasound imaging (Vevo 770, Visual Sonics Inc., Toronto, Canada) was performed for assessment of cardiac function. M-Mode images of the left ventricle at the height of the papillary muscles were used to calculate ejection fraction. The aortic arch was imaged to measure internal diameter and the centerline aortic peak flow velocity in the same location was measured on pulsed-wave spectral doppler tracing as an index of aortic shear.

**Assessment of VCAM-1 and Plaque Macrophage Content**
VCAM-1 expression was assessed in four apocynin-treated and four saline-treated animals by Western blot and histology. For Western blot, the ascending portion of the aorta was homogenized in lysis buffer (Cell Signaling) containing 80mmol/L Pefabloc SC plus (Roche). Protein concentration was measured using the Micro BCA (bicinchoninic acid) protein assay kit (Thermo Scientific). Ten microgram of protein were resolved on SDS-PAGE and transferred to Polyvinylidene fluoride (PVDF) membranes (Amersham). Membranes were probed with monoclonal rat anti-mouse VCAM-1 (Clone # 112702, R&D Systems) and monoclonal anti-α-tubulin (Clone DM1A, Sigma) antibodies. Blots were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research) and band intensities were detected by enhanced chemiluminescence (Western Lightening Plus; Perkin Elmer) and quantitated using NIH ImageJ software (http://rsbweb.nih.gov/ij/).

Fluorescent immunohistochemistry was performed to spatially characterize the endothelial expression of VCAM-1 and to quantify plaque macrophage content. Frozen aortic sections were mounted on glass slides, fixed in -20°C Acetone, air-dried, blocked with 10% goat serum in TBS/FSGO and incubated overnight at 4°C with monoclonal rat anti-VCAM-1 (CBL-1300, Millipore) and then for 1 hour at room temperature with goat anti-rat Alexa-633 (A21094, Invitrogen). Subsequently, Macrophages were labeled with a biotinylated rat anti Mac-2 (125403, Biolgegend). Fluorescent labeling of anti Mac-2 was accomplished with Alexa-594 labeled streptavidin (016-580-084, JacksonImmunoResearch). Sections were mounted with Prolong gold antifade mounting medium containing DAPI and imaged on a Zeiss LSM 710 confocal microscope. Alexa-633 representing VCAM-1 was detected with an excitation wavelength of 633nm and an emission wavelength of 650-740nm and Alexa-594 representing Mac 2 with 594 excitation wavelength and 580-630 emission
wavelength. Exposure time, averaging, and laser intensity were kept constant for all images. For spatial quantification of plaque macrophage content, the number of positively stained pixels was counted on thresholded pictures and normalized to the total number of pixels of the plaque using Image-J. Thresholds were defined as the mean background intensity plus 40 times the standard deviation in each individual picture. For each mouse at least two cross-sections on different slides were imaged and quantified for both the base and ascending aorta.

Assessment of endothelial platelet adhesion

After the termination of imaging experiments, apocynin-treated and non-treated mice (n=5 each) were injected intravenously with 50µg rhodamine-6G (Sigma Aldrich). Ten minutes after injection, a right atrial incision was made through an anterior thoracotomy. The blood volume was removed with 10 ml of 37°C phosphate buffered saline through a left ventricular puncture at an infusion pressure ≤100 mm Hg. The ascending aorta was then carefully removed, incised longitudinally, and pinned endothelial side facing up for en face fluorescent microscopy (×20 objective) with epi-illumination at an excitation wavelength of 490nm. The degree of platelet/leukocyte complex attachment to the endothelium was quantified in 20 randomly selected non-overlapping visual fields by thresholding at >10 SD above normal endothelial surface with Image-J (National Institutes of Health, Bethesda, MD) and expressing area with positive fluorescence normalized to total endothelial surface area.

NADPH oxidase activity and superoxide anion production

For the assessment of NADPH oxidase activity, a right atrial incision was made through an anterior thoracotomy. The blood volume was removed with 10 ml of 37°C
C phosphate buffered saline through a left ventricular puncture at an infusion pressure $\leq$100mmHg. The ascending aorta was carefully removed and cut into three circular segments of equal length, and the wet weight of the individual segments was measured. Aortic rings were incubated with 85µL of Jude Krebs Buffer (119mM NaCl, 20mM HEPES, 4.6mM KCl, 1mM MgSO$_4$, 0.15mM Na$_2$HPO$_4$ 2H$_2$O, 0.4mM KH$_2$PO$_4$, 5mM NaHCO$_3$, 1.2mM CaCl$_2$, 5.5mM Glucose) containing protease inhibitor (Roche) at 37°C for 30 min. NOX activity was measured with a luminescence assay in a microplate luminometer with 2.5µL DMSO, 10µM lucigenin (Sigma) and 100µM NADPH (Sigma) per well (final volume 100uL/well). Data were recorded as relative light units over time, and integrated and calculated as area under the curve using Image J software for statistical analysis.

For measurement of superoxide (O$_2^-$) anion production, the aortas were removed as described above. The ascending aorta was dissected, cleaned from surrounding tissue and cut into rings of approximately 3 millimeters. The rings were incubated 30 minutes in 300 µl HBSS (Invitrogen) containing 50µM Hydroethidine (HE) (Sigma Aldrich) at 37°C in the dark. The rings were then washed in phosphate buffered saline, snap frozen and kept at -80°C. The day of the experiment, the rings were homogenised in 370 µl methanol. The homogenate was centrifuged at 13,000 rpm for 5 min and 50 µl of supernatant was used for protein quantification using BIO-RAD protein assay (Bio-Rad Laboratories GmbH, München) while the rest was dehydrated using a speed vacuum concentrator. The resulting pellet was dissolved in 100 µL H$_2$O for HPLC analysis (1100 Series (Agilent, Palo Alto, Ca)). Hydroethidine and its two oxidized products, i.e., superoxide-specific 2-hydroxyethidium and ethidium were separated by HPLC equipped with a fluorescence detector with excitation at 510 nm and emission at 595 nm. The area under EOH peak was calculated and values were normalized to protein content of the rings.
Statistical Analysis

Data were analyzed on GraphPad Prism (version 5.0d). Data are expressed as mean±SEM unless stated otherwise. Single comparisons between the two animal groups were performed with a Mann-Whitney test. Kruskal-Wallis ANOVA with Dunn’s post hoc test was used to compare microbubble signals within and between animal groups. For assessing the effect of treatment on targeted signals in the subgroup of animals that were imaged before and after treatment, a Wilcoxon matched-pairs signed rank test was used. A p value <0.05 (2-sided) was considered statistically significant.

REFERENCES for SUPPLEMENTAL SECTION


Supplemental Figure I. Representative HPLC readouts illustrating measurement of HE oxidation after exposure to aortic tissue (bottom) versus HE alone (top). HE = hydroethidine, 2-OHE = 2-hydroxyethidium, E+ = ethidium.