Molecular imaging reveals rapid reduction of endothelial activation in early atherosclerosis with apocynin independent of anti-oxidative properties

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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_{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 GPIbα on platelets (MB_{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_{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_{Ctr}, 2.8±0.2 for MB_{VCAM}, 2.9±0.2 for MB_{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_{VCAM}, MB_{Pl} or MB_{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 phosphate buffered saline through a left ventricular puncture at an infusion pressure ≤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 $Na_2HPO_4 \cdot 2H_2O$, 0.4mM KH_2PO_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_2O for HPLC analysis (1100 Series (Agilent, Palo Alto, Ca). Hydroethidine and its two oxidized products, i.e., superoxide-specific 2-hydroxyethidium and ethidium [4] 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.

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