ONLINE SUPPLEMENT

Leukocyte-dependent responses of the microvasculature to chronic angiotensin II exposure.

Alper Yildirim, Janice Russell, Li-Sue S. Yan, Elena Y. Senchenkova,

and D. Neil Granger

Department of Molecular & Cellular Physiology, LSU Health Sciences Center, Shreveport, Louisiana 71130

Materials and Methods

Production of bone marrow chimeras: Bone marrow cells were isolated from the femurs and tibias of donor mice. WT→WT chimeras were produced by transplanting marrow from WT C57BL/6 mice expressing CD45.2 into WT congenic recipients that express CD45.1. $gp91^{phox-/-}$→WT, and $AT_1r^{-/-}$→WT mice were generated by transplanting marrow from either $gp91^{phox-/-}$ or $AT_1r^{-/-}$ mice (both of which express CD45.2) into WT (CD45.1) recipients. WT→ $gp91^{phox-/-}$ mice were also generated by transplanting bone marrow from WT mice into $gp91^{phox-/-}$ recipients. Recipient mice were irradiated with two doses of 500-525 Rads, 3 h apart, after which 5 x 10^6 donor bone marrow cells were injected into the femoral vein. The chimeras were kept in autoclaved cages, with 0.2% neomycin in drinking water for two weeks, after which normal drinking water was used. Flow cytometry used to verify chimera reconstitution (usually requiring 6-8 weeks) by staining for CD45.1 (CD45 congenic mice) and CD45.2 (expressed by C57BL/6 mice or knockout mice on C57BL/6 background) expression on circulating leukocytes with a FITC-conjugated anti-CD45.2 antibody and a PE-conjugated anti-CD45.1 antibody (eBioscience). This procedure normally yields greater than 90% penetrance of the transferred marrow at 6 weeks or longer after transplant. These bone marrow transfer protocols allow for the creation of mice wherein the genetic deficiency ($gp91^{phox}$ and AT_1r) is confined to the circulating blood cells.

Intravital microscopic methods: Ketamine/xylazine anesthetized mice were placed in a semi-upright position on a Plexiglas microscope stage.[17] Then, 100 x 10^6 CFSE- labeled platelets were infused over 5 min and were allowed to circulate for 5 min before intravital observation. Venules with diameters between 20 – 40 μm and wall shear rates >500 sec^{-1} were selected for study. An optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, TX) was used to measure red blood cell velocity (V_{RBC}) in the microvessels and wall shear rate (WSR) was calculated from the product of mean red blood cell velocity [$V_{mean} = V_{RBC}/1.6$] and microvascular cross-sectional area, assuming cylindrical geometry. Wall shear rate (WSR) was calculated on the

basis of the Newtonian definition: WSR = $8(V_{mean}/D_v)$. Adherent leukocytes in postcapillary venules were determined, over a 2 min period, by light microscopy, while the adhesion of CFSE-labelled platelets was monitored under fluorescence microscopy for a period of 1 min. The microscopic images were recorded on DVD and blood cell adhesion was analyzed offline. Adhesion values were recorded in 100 μm length vessel segments at 300 μm intervals along the length of the vessel, beginning as near to the source of the venule as possible. The mean value of each variable within a single venule was calculated, and comparisons were made between the experimental groups. Platelets (number per millimeter square) were considered adherent if they arrested for ≥30 s. A leukocyte was considered adherent if it remained stationary for ≥30 s (number per millimeter square). Leukocyte emigration was measured online at the end of each observation period and expressed as the number of interstitial leukocytes per high-powered field of view adjacent to the observed vascular segment (number per field).

Flow cytometric analysis of leukocyte subpopulations: Different circulating leukocyte populations were monitored using flow cytometry. Mouse blood was collected by tail-vein bleed and mixed with heparin (20U/mL). The erythrocytes were lysed, and leukocytes were pelleted by centrifugation, resuspended in FACS buffer. Samples were first incubated with FcR block (CD16/CD32), and then stained with a combination of 6 antibodies to identify total leukocytes (CD45.2-FITC), monocytes (CD115-PE), B-lymphocytes (CD45R-PE/Cy5), T-lymphocytes (CD3-PE/Cy7), neutrophils (Gr-1-eFluor660), and macrophage (F4/80-eFluor450) (all from eBioscience). Appropriate isotype controls were used to determine non-specific binding. Gating was performed for live, $CD45.2^+$ events constituting the total leukocyte population.

Four-color flow cytometry utilizing a combination of CD41-APC, CD45.2-FITC, Gr-1-PE, F4/80-eFluor450 and isotype control (eBioscience) were used to detect and quantify aggregates of leukocytes, neutrophils, and monocytes with platelets. Neutrophils and monocytes were distinguished from other cells by their size and complexity. All samples were analyzed on BD LSR II (BD Biosciences). Data analysis was performed using BD FACSDiva software (version 6.0)

Spinning disk confocal microscopy: An Olympus BX51WI upright microscope (Olympus, Center Valley, PA) with a 40XW (LUMPLFLN) objective and equipped with a 3i LaserStack laser launch (3i; Denver, CO), Yokogawa CSU-X1-A1N-E spinning disk confocal unit (Yokogawa Electric Corporation, Tokyo, Japan) and electron multiplier CCD camera (C9100-13; Hamamatsu, Bridgewater, NJ) was used for these experiments. Anti-mouse CD45.2 FITC or AF568 conjugated (4 μg/mouse; eBiosiences; San Diego, CA), anti-mouse CD115 (10 μg/mouse; Biolegend; San Diego, CA) conjugated with Alex Fluor 568 protein labeling kit (Molecular Probe; Eugene, OR) and anti-mouse Ly-6G (Gr-1) eFluor 660 or AF488 conjugated (2 μg/mouse; eBiosiences), anti-mouse CD3 e660 (2 μg/mouse; eBiosiences) and anti-mouse B220 e660 (2 μg/mouse; eBiosiences) were injected intravenously to image total leukocytes, monocytes and neutrophils, T-lymphocytes and B-lymphocytes, respectively. 488-, 561 and 640-nm laser excitation were used in rapid sequence and visualized with the appropriate filters (Semrock, Rochester, NY). Typical exposure times for all excitation wavelengths were 200 ms. Slidebook software (3i Denver, CO) was used to drive the confocal system and capture images for offline analysis. Leukocytes were considered adherent if they remained stationary for 30 seconds or longer.

Results

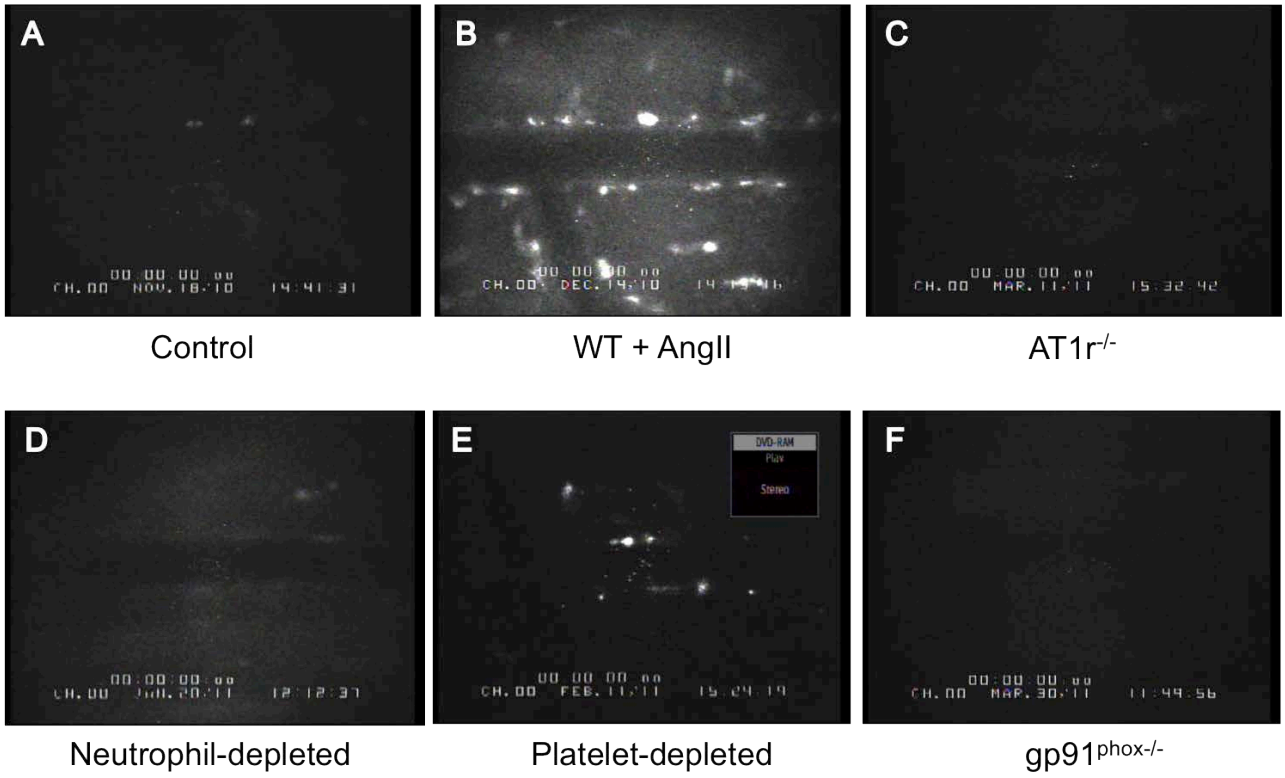

Figure S1. Florescence microscopic images of DHR oxidation within and surrounding cremaster muscle venules in the following experimental groups: WT controls (panel A), WT-AngII (panel B), angiotensin II-type 1 receptor deficient ($AT1r^{-/-}$)-AngII (panel C), WT-neutropenic-AngII (panel D), WT-thrombocytopenic-AngII (panel E) and $gp91^{phox}$ deficient mice ($gp91^{phox-/-}$)-AngII (panel F) mice.

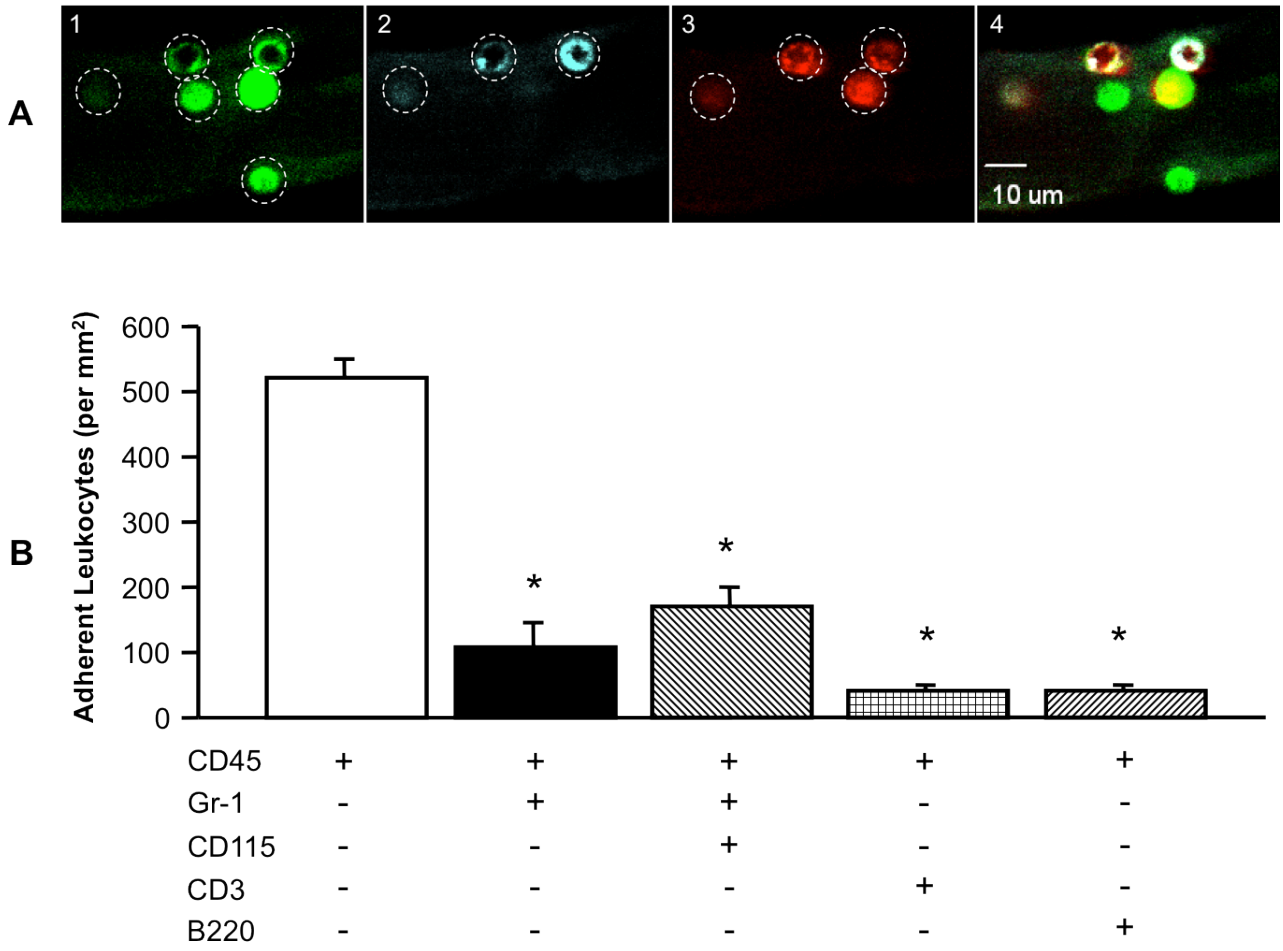

Figure S2. Panel A: Spinning-disk confocal-microscopic imaging of different leukocyte populations recruited into cremaster postcapillary venules of AngII treated mice. In-vivo Ab labeling was used to identify adherent CD45+ cells (green) A1, CD115+ monocytes (blue) A2, Gr-1+ neutrophils (red) A3 and CD45, CD115, Gr-1 positive cells (multi-channel overlay) A4. Panel B: Number of cells in each leukocyte subset recruited into cremaster muscle postcapillary venules of AngII treated mice (per mm2). CD45 represents total leukocyte population, CD115 shows monocytes, Gr-1 represents neutrophils, CD3 represents T-lymphocytes and B220 corresponds to B-lymphocytes. *, indicates $p<0.05$ relative to CD45+ cells.

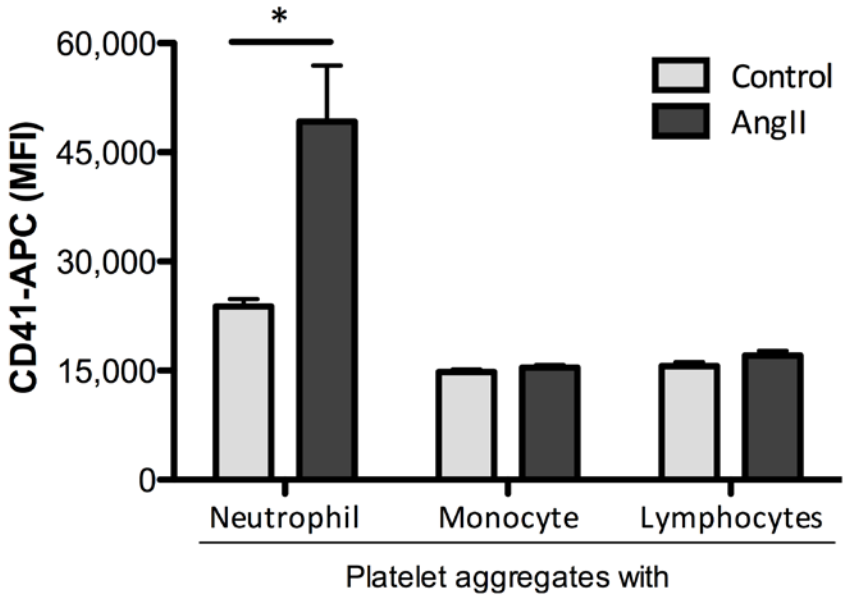

Figure S3. Formation of platelet aggregates with neutrophils (PNA), monocytes (PMA) and lymphocytes (PLyA) in WT and AngII treated mice, as detected by flow-cytometry. CD41 MFI indicates the relative amount of platelets attached per leukocyte. *, indicates $p<0.05$ relative to WT control.