SUPPLEMENTAL MATERIALS

Oxidative stress product, 4-hydroxy-2-nonenal, induces the release of tissue factor-positive microvesicles from perivascular cells into circulation

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MATERIALS AND METHODS

Reagents

HNE was purchased from Cayman Chemical (Ann Arbor, MI). Recombinant human factor VIIa (FVIIa) was provided by the late Walter Kisiel, University of New Mexico, Albuquerque, NM. Purified human factor X and Xa were purchased from Enzyme Research Laboratories (South Bend, IN). Rat monoclonal antibody against mouse TF (1H1) was kindly provided by Daniel Kirchhofer, Genentech Inc. (South San Francisco, CA, USA). Mouse anti-VE cadherin and rabbit anti-HNE antibodies were from Santa Cruz Biotechnology, Inc (Dallas, TX) and Abcam (Cambridge, MA), respectively. Cell culture medium EBM-2 and growth factor supplements were purchased from Lonza (Walkersville, MD), DMEM and fetal bovine serum were from GIBCO (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Fluorescent secondary antibodies were from Life Technologies (Thermo Fisher Scientific, Waltham, MA).

Hematological Analysis

For analysis of blood cell count, blood was collected via the submandibular vein in EDTA (2.7 mM) anticoagulant, and hematological parameters were analyzed using HEMAVET-HV950FS (Drew Scientific, Inc., CT) analyzer. Blood was collected in citrate anticoagulant for determining TF procoagulant activity and thrombin-antithrombin (TAT) complex levels in plasma.

Saphenous Vein Bleeding

The saphenous vein bleeding model has been described in detail in our earlier study.[1] Briefly, following the treatments, an approximately 1-mm longitudinal distal cut was made in the saphenous vein of the ventral hind limb of the anesthetized mice (ketamine, 100 mg/kg plus xylazine, 8.5 mg/kg, i.p. in 100 to 125 µl volume). Bleeding from the incision was allowed for 30 min, and the blood from the injury site was adsorbed onto Kimwipes. The time taken to achieve hemostasis and the number of hemostatic plugs formed during the 30-min period was recorded by disrupting the plug formed each time using a blunted 30-G needle. The amount of blood loss was evaluated by extracting the hemoglobin from the Kimwipes and recording the O.D. in a spectrophotometer at 550 nm and extrapolating from the values of known standards.

Nanoparticle Tracking Analysis (NTA)

MVs size distribution and concentration were analyzed in Malvern Panalytical NanoSight 300 using NTA software. Parameters used for NTA are as follows. Capture settings: Camera type, sCMOS; Laser type, Blue 488; Camera level, 13 (NTA 3.0); Slider shutter, 800; Slider gain, 350; FPS, 25.0; Temperature, 22.2-22.4°C; Viscosity, Water (0.943-0.948 cP); Analysis settings: Detection threshold , 5; Blur size, Auto; Max jump distance, Auto 11.4-13.9 pix.

Myeloperoxidase Activity Assay

Mouse blood from saline-, HNE (6 h or 24 h) or LPS (6 h) was drawn in citrate anticoagulant and plasma harvested as described above. The plasma samples either diluted or undiluted in 50 mM phosphate buffer (pH 6.0) were subjected to myeloperoxidase activity assay using hexadecyltrimethylammonium bromide method.

Immunohistochemistry

Mice were perfused with saline to remove blood in tissues as described earlier,[2] and the inflated lungs were fixed in Excel fixative (StatLab, McKinney, TX) for 48 h. Tissues were processed using graded alcohol and xylene, embedded in paraffin, 5 µm-thin sections were cut, and de-paraffinized using standard procedures. Rehydrated sections were immunostained with rat anti-mouse Ly6G (Novus, St. Charles, MO) or isotype control IgG (10 µg/ml each). Immunostained sections were developed using AEC

(aminoethyl carbazole, Sigma Aldrich) chromogen and counterstained with hematoxylin. Tissue sections were visualized in Olympus microscope equipped with 4x/0.13, 10x/0.30, 20x/0.50, 40x/0.75 objective lenses. Images were captured with an Olympus DP27 camera using Olympus Cellsens software.

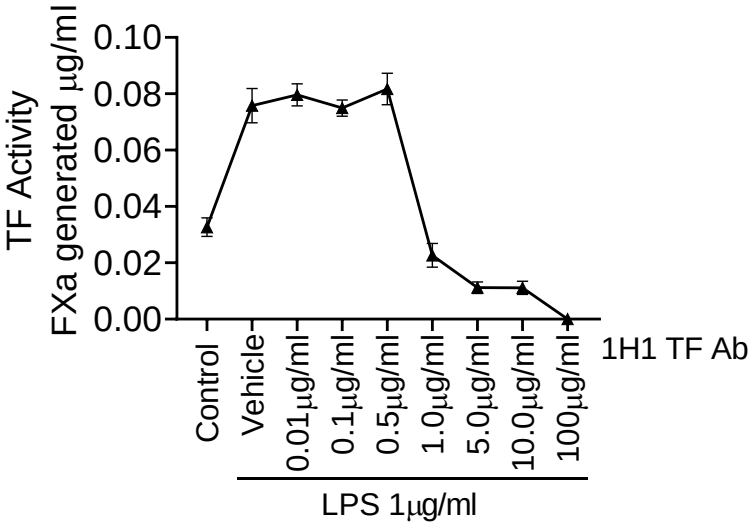
Barrier Permeability Assays

Endothelial cell barrier permeability *in vitro* was analyzed as described in our earlier study.[3] Briefly, endothelial cells were cultured to confluency in a Transwell permeable support insert (12 mm diameter and 3.0 µm pore size). The cells were treated with HNE or a control vehicle in the complete culture medium for varying times. Following the treatment, the medium was removed from the apical chamber, and 0.5 ml of Evans Blue dye (0.67 mg/mL) in 4% BSA in the serum-rich medium was added to the apical chamber. The amount of dye leaked into the lower chamber in 10 min was measured by taking an aliquot from the lower chamber and measuring absorbance at 650 nm in a spectrophotometer. Vascular permeability *in vivo* was measured using either Evans blue dye or fluorescein dextran as described in our recent publication.[4]

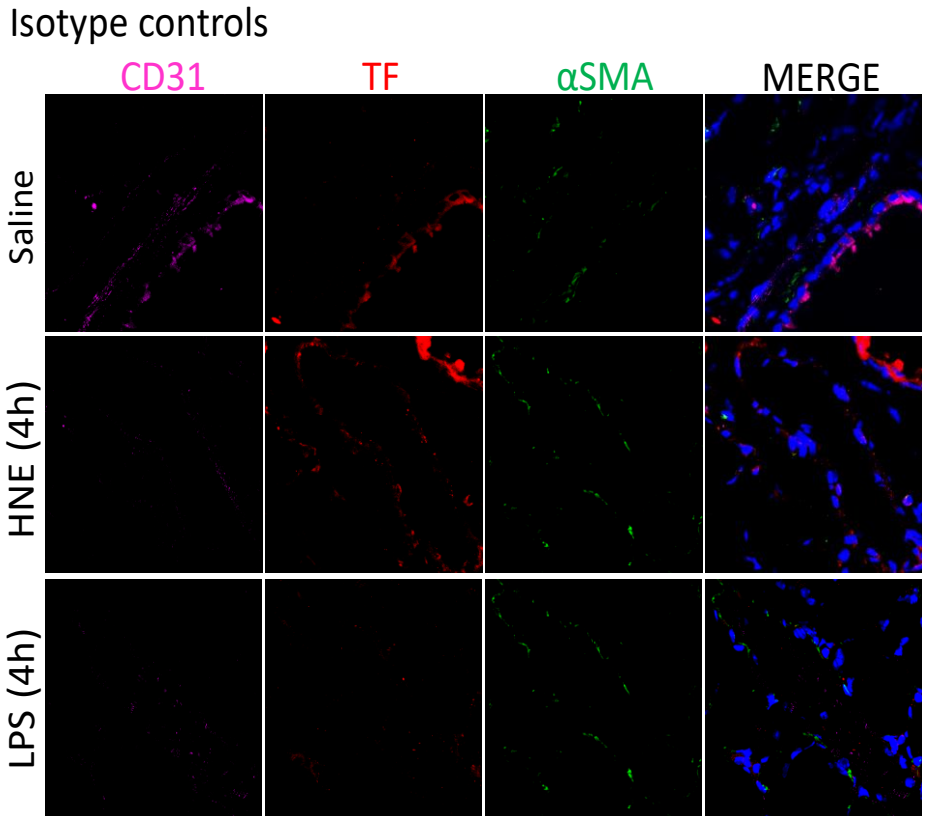
References

1. Keshava S, Sundaram J, Rajulapati A, Pendurthi UR and Rao LV. Pharmacological concentrations of recombinant factor VIIa restore hemostasis independent of tissue factor in antibody-induced hemophilia mice. *J Thromb Haemost*. 2016;14:546-50.
2. Gopalakrishnan R, Hedner U, Ghosh S, Nayak R, Allen TC, Pendurthi UR and Rao LV. Bio-distribution of pharmacologically administered rFVIIa. *J Thromb Haemost*. 2010;8:301-310.
3. Sen P, Gopalakrishnan R, Kothari H, Keshava S, Clark CA, Esmon CT, Pendurthi UR and Rao LV. Factor VIIa bound to endothelial cell protein C receptor activates protease activated receptor-1 and mediates cell signaling and barrier protection. *Blood*. 2011;117:3199-208.
4. Magisetty J, Pendurthi UR, Madhunapantula SV, Grandoni J and Rao LVM. Increased Accumulation and Retention of rhFVIIa (eptacog beta) in Knee Joints of Hemophilia A Mice Compared to Wild-Type Mice. *Thromb Haemost*. 2019;119:1283-1294.

Supplementary Figure I. NIH3T3 cells were incubated with varying concentrations of 1H1 murine TF mAb (10 ng/ml to 100 μg/ml) for 30 min. Cell surface tissue factor activity was measured in factor X activation assay.



Supplemental Figure II. Lung tissue sections from saline-, HNE-, or LPS-challenged mice were subjected to immunostaining with control IgG in parallel with immunostaining for TF, αSMA and CD41 (Fig 6D in the main manuscript). The gain settings for image capturing were identical for tissue sections stained with control IgG and specific antibodies.



Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Mouse	The Jackson Laboratory	C57 Wild-Type	M/F	https://www.jax.org/strain/000664

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Parent - Male	NA	NA	NA	NA	NA
Parent - Female	NA	NA	NA	NA	NA

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
Rat anti-TF (1H1)	Daniel Kirchhofer, Genentech Inc.	NA	In vitro: 10 µg/ml		NA
Rat anti-TF (1H1)	Daniel Kirchhofer, Genentech Inc.	NA	In vivo: 2 mg/kg b.w. twice		NA
Rabbit anti-TF	Novo	NA	10 µg/ml for IP		NA
Rabbit anti-TF	Novo	NA	5 µg/ml for IF		NA
Mouse anti-hEPCR	Dr Charles T Esmon	NA	5 µg/ml for IF		NA
Goat anti-TF	R&D Systems	AF3178	0.2 µg/ml for WB		https://www.rndsystems.com/products/mouse-coagulation-factor-iii-tissue-factor-antibody_af3178
Rabbit anti-HNE	Abcam	ab46545	1:500 for WB		https://www.abcam.com/4-hydroxynonenal-antibody-ab46545.html
Rabbit anti-HNE	Abcam	ab46545	1:50 for IHC		https://www.abcam.com/4-hydroxynonenal-antibody-ab46545.html
Mouse anti-VE cadherin	Santa Cruz Biotechnology	sc-9989	1:500 for WB		https://www.scbt.com/p/ve-cadherin-antibody-f-8
Goat anti-VE cadherin	Santa Cruz Biotechnology	sc-6458	5 µg/ml for IF		https://www.scbt.com/p/ve-cadherin-antibody-c-19
Rabbit anti-αSMA	Cell Signaling Technology	19245	1:1000 for WB		https://www.cellsignal.com/products/primary-antibodies/a-smooth-muscle-actin-d4k9n-xp-rabbit-mab/19245

DOI [to be added]

Mouse anti-αSMA	R&D Systems	MAB 1420	5 µg/ml for IF		https://www.rndsystems.com/products/human-mouse-rat-alpha-smooth-muscle-actin-antibody-1a4_mab1420
Mouse anti-Endosialin	Millipore Sigma	MAB2626	1:1000 for WB		https://www.emdmillipore.com/US/en/product/Anti-Endosialin-Antibody-clone-B1-35,MM_NF-MAB2626
Mouse anti-Endosialin	Millipore Sigma	MAB2626	5 µg/ml for IF		https://www.emdmillipore.com/US/en/product/Anti-Endosialin-Antibody-clone-B1-35,MM_NF-MAB2626
Sheep anti-GFAP	R&D Systems	AF2594	0.2 µg/ml		https://www.rndsystems.com/products/human-rat-gfap-antibody_af2594
Rat anti Ly-6G	Biolegend	127602	10 µg/ml		https://www.biolegend.com/en-us/search-results/purified-anti-mouse-ly-6g-antibody-4767
Rat anti-CD31	Biolegend	102502	5 µg/ml		https://www.biolegend.com/en-us/products/purified-anti-mouse-cd31-antibody-380

DNA/cDNA Clones

Clone Name	Sequence	Source / Repository	Persistent ID / URL
NA	NA	NA	NA

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
HUVEC	LONZA	NA	https://bioscience.lonza.com/lonza_bs/CH/en/Primary-and-Stem-Cells/p/000000000000184655/HUVEC-%E2%80%93-Human-Umbilical-Vein-Endothelial-Cells%2C-Single-Donor%2C-in-EGM-2
BEND3	ATCC	NA	https://www.atcc.org/Products/All/CRL-2299.aspx
THP-1	ATCC	NA	https://www.atcc.org/products/all/TIB-202.aspx
WI38	ATCC	NA	https://www.atcc.org/products/all/CCL-75.aspx
PASMC	LONZA	NA	https://bioscience.lonza.com/lonza_bs/CH/en/Primary-and-Stem-Cells/p/000000000000185155/PASMC---Human-Pulmonary-Artery-Smooth-Muscle-Cells

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
NA	NA	NA

Other

Description	Source / Repository	Persistent ID / URL
Recombinant human factor VIIa (FVIIa)	Provided by late Walter Kisiel, University of New Mexico, Albuquerque, NM	NA
Purified human factor X (FX)	Enzyme Research Laboratories	https://enzymeresearch.com/product/human-factor-x/

DOI [to be added]

Purified human activated factor X (FXa)	Enzyme Research Laboratories	https://enzymeresearch.com/product/human-factor-xa/#:~:text=Human%20Factor%20Xa%20is%20prepared,is%20observed%20by%20SDS%2DPAGE
4-hydroxy-2-nonenal (HNE)	Cayman Chemical	https://www.caymanchem.com/product/32100/4-hydroxy-nonenal
LPS from E. coli O111:B4	Millipore Sigma	https://www.sigmaaldrich.com/catalog/product/sigma/l2630?lang=en&region=US
Mouse IL-6 ELISA Kit	eBioscience	https://www.thermofisher.com/elisa/product/IL-6-Mouse-Uncoated-ELISA-Kit-with-Plates/88-7064-22
Mouse KC ELISA Kit	RayBiotech	https://www.raybiotech.com/mouse-kc-cxcl1-elisa-kit-for-serum-plasma-and-cell-culture-supernatant/
Mouse Thrombin-Antithrombin assay kit	Assaypro	https://assaypro.com/Products/Details/EMT1020-1

DOI [to be added]