Materials and Methods

Mice and experimental protocol

Breeding pairs of heterozygous *Sod1*-deficient (SOD1tm1Leb) mice on a mixed B6/129S background were obtained from The Jackson Laboratory. Littermate offspring were genotyped for the targeted and wild type *Sod1* alleles in the University of Iowa Genome Editing Core Facility as described previously.[1] Homozygous *Sod1*-deficient mice (*Sod1*-/-) and wild-type littermates (*Sod1+/+)* were studied at the age of 5-10 months. All animal protocols were approved by the University of Iowa Animal Care and Use Committee.

Detection of vascular ROS

Vascular ROS were detected in the proximal aorta using lucigenin-enhanced chemiluminescence. Briefly, fresh sections of proximal aortae were incubated with 5 µM lucigenin (*N,N′*-dimethyl-9,9′-biacridinium dinitrate, Sigma) in a FB 12 luminometer (Titerek Berthold), followed by addition of tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid, Sigma, 10^{-2} M final concentration). The rate of generation of ROS was determined as tiron-quenchable lucigenin-enhanced chemiluminescence, which is a selective indicator of superoxide.[2] Values were normalized to the weight of the aortic sections.

In vivo thrombosis

Carotid artery thrombosis: Carotid artery thrombosis was induced by photochemical injury as described previously.[3] Briefly, mice were anesthetized with sodium pentobarbital (70-90 mg/kg intraperitoneally) and ventilated mechanically with room air and supplemental oxygen. The right common carotid artery was transilluminated continuously with a 1.5-mV, 540-nm green laser (Melles Griot), and rose Bengal (35 mg/kg) was injected via a femoral vein catheter. Blood flow was monitored continuously for 90 minutes or until stable occlusion occurred, at which time the experiment was terminated. Stable occlusion was defined as the time at which blood flow remained absent for ≥ 10 minutes.

Inferior vena cava (IVC) thrombosis: Susceptibility to thrombosis in the venous system was measured as described previously.[3] Briefly, mice were anesthetized using ketamine/xylazine (87.5 mg/kg ketamine and 12.5 mg/kg xylazine, intraperitoneally). The IVC was ligated inferiorly to the left renal vein and harvested two days later for measurement of the length and weight of thrombus.

Platelet activation

Platelet activation was determined as described previously.[3] Briefly, washed platelets were activated with human thrombin (0.5 U/mL; Haematological Technologies), for 2 minutes at 37°C, incubated with either FITC-conjugated sheep anti-human fibrinogen antibody (Novus Biologicals) or rat anti-mouse CD62P (P-selectin) antibody (BD Biosciences), and analyzed on a Becton Dickinson FACScan flow cytometer.

Real time PCR
Levels of mRNA for tissue factor (TF), thrombomodulin (TM), endothelial protein C receptor (EPCR), and GAPDH were measured by quantitative real-time PCR as described previously.[4] Total RNA was isolated from lung tissue using Trizol reagent (Invitrogen). Reverse transcribed cDNA was incubated with TaqMan Universal PCR mix, PCR primers and 6-carboxy fluorescein-labeled probes (Applied Biosystems) as described.[4] The comparative threshold cycle ($\Delta\Delta C_T$) method was used for quantification with values normalized to GAPDH and expressed relative to levels in *Sod1+/+* lungs. Validation experiments were performed to confirm equal amplification efficiency for all primers sets.

Protein C activation
Activation of endogenous protein C was measured in response to injection of thrombin as described previously.[4] Mice were anesthetized by inhalation of 75% carbon dioxide/25% oxygen, and 50 μl of either saline or human α-thrombin (80 U/kg) (Haematological Technologies Inc.) was administered by retroorbital injection. After 10 minutes, blood was collected by cardiac puncture. The concentration of activated protein C (APC) in citrate/benzamidine-treated plasma was measured using an enzyme capture ELISA.[4, 5]

Activation of exogenous protein C by thrombin was measured in lung lysates using a two-stage assay described previously.[4] To prepare lung lysates, tissue was homogenized in 0.02 mol/L Tris (pH 8.0) containing 0.1 mol/L NaCl. Triton X-100 was added to a concentration of 1.0%, and the samples were incubated at room temperature for 10 minutes. The lysates were then centrifuged at 3500 rpm for 15 minutes at room temperature and the supernatant fraction was flash frozen and stored at -80°C for later assays of protein C activation. Protein C activation was initiated by adding lung lysate supernatant to a Tris buffered (pH 7.4) solution of 5.2 nM human α-thrombin (Enzyme Research, HT3564) and 150 nM human protein C (Haematologic Technologies Inc, HCPA-0070). After incubation for 30 minutes at 37°C, the reaction was stopped by addition human antithrombin (Haematologic Technologies Inc, HCATH-012) and heparin. The amount of APC was quantified by measuring the change in absorbance at 405 nm using a chromogenic APC substrate, S-2366 (Chromogenix, 82-1090 3a). Reference curves were generated using rabbit lung thrombomodulin (American Diagnostica). One unit of activity was defined as the amount of activated protein C generated in the presence of 1.0 nmol/L rabbit thrombomodulin. The total protein concentration of each sample was quantified by the Bradford method (Bio-Rad Protein Assay Kit 500-0001).

Activation of exogenous protein C by thrombin was measured in the presence of recombinant human TM (R&D Systems) after exposure to exogenous superoxide. The generation of superoxide was initiated by the addition of 5 mU/ml xanthine oxidase (Sigma) and 1 mM hypoxanthine (X-XO) (Sigma) to a PBS buffered (pH 8.0) solution of 100 nM recombinant human TM in the presence or absence of 50 U/ml PEG-SOD (Sigma) and/or 250 U/ml PEG-catalase (Sigma), or 100 mU/ml recombinant human MsrA and/or MsrB1 (Prospec). Following a 30-minute incubation at 37ºC, activation of

protein C was measured using the two-stage assay as described above. The activity of TM in absence of X-XO was defined as 100%.

Mass spectrometry

Human recombinant TM containing only the extracellular domain (R&D Systems) was incubated in the presence or absence of X-XO, PEG-SOD and/or PEG-catalase as described for *in vitro* activation of protein C. Following 30 minutes of incubation, samples were flash frozen and stored at -80^{0}C until analysis. Samples were reduced with 5 mM DTT, alkylated with 15 mM iodoacetamide, digested with AspN and deglycosylated by PNGase F in a buffer containing 50 mM Tris (pH 8.0), 1 mM $MgCl_2$, 5 mM methionine and 5% acetonitrile. The resultant peptides were analyzed using nano-LC-MS/MS in the positive ion mode with a Thermo Scientific LTQ Orbitrap Velos mass spectrometer coupled to a Waters nanoACQUITY Ultra Performance liquid chromatography system. Peptides were separated at a flow rate of 300 nL/minute on a nanoUPLC BEH130 C18 column (100 × 0.075 mm, 1.7 µm, Waters), using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Peptides were eluted using a linear gradient of 5%–35% solvent B over 50 minutes. Percent oxidation of individual Met residues was determined by dividing the peak area of the Met oxidized peptide by the sum of the peak areas of both oxidized and unoxidized peptides.

Western blotting

Western blotting was performed as described previously.[6] Samples of lung lysates containing 20 µg protein (determined by the Bradford method (Bio-Rad) were run on 10% polyacrylamide Tris-HCl gels (Bio-Rad) under non-reducing conditions. Membranes were probed with 1 µg/ml of monoclonal antibody raised against mouse TM (R&D) or with 0.5 µg/ml monoclonal antibody raised against β-actin (Abcam ab8226) for 2 hours at room temperature. Secondary antibodies used were HRP-conjugated goat-anti-rat for TM and goat-anti-rabbit for β-actin (Thermo, 10 ng/ml, 1 hour at room temperature). Immunoreactive bands were visualized using SuperSignal West Femto (Pierce) detection system and quantified by densitometry.

Baseline platelet count, clotting assays and protein C levels

Blood was collected by cardiac puncture into 3.8% sodium citrate (9:1, v/v), centrifuged at 8000 rpm for 20 minutes at 4^{0}C. Plasma was collected in aliquots and stored frozen at -80^{0}C until analyzed for clotting assays and protein C level. Prothrombin time (PT) and activated partial thromboplastin time (PTT) were measured in an automated BCS-XP instrument (Siemens). PT was performed by adding Innovin reagent to plasma and PTT was performed by adding Actin FSL reagent and 25 mM $CaCl_2$ to the plasma. Protein C levels in mouse plasma were measured using a mouse protein C ELISA (BlueGene Biotech). For platelet count, blood was collected through retro orbital sinus in an EDTA coated glass capillary tube, diluted 2X with PBS and analyzed using a Hemavet 850 FS instrument (Drew Scientific).

TF activity assay
TF activity in lung was measured using a chromogenic activity assay (Innovative Research). This assay measures the ability of TF to promote the activation of factor X (FX) to factor Xa (FXa) by factor VIIa. The amount of FXa produced is quantified using a specific chromogenic FXa substrate releasing a yellow para-nitroaniline (pNA) chromophore. The change in absorbance of the pNA at 405 nm is directly proportional to TF enzymatic activity.

Statistical analysis
One-way analysis of variance (ANOVA), followed by Tukey's post-hoc test was used to compare data for thrombus size after IVC ligation and qPCR for TM and EPCR. One-way ANOVA on ranks was performed to compare data for ROS measurements, occlusion time, platelet activation, protein C activation, and qPCR for TF. A value of $P < 0.05$ was used to define statistical significance. Values are reported as mean ± SEM.

References

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