Deficiency of Superoxide Dismutase Impairs Protein C Activation and Enhances Susceptibility to Experimental Thrombosis

Sanjana Dayal, Sean X. Gu, Ryan D. Hutchins, Katina M. Wilson, Yi Wang, Xiaoyun Fu, Steven R. Lentz

Objective—Clinical evidence suggests an association between oxidative stress and vascular disease, and in vitro studies have demonstrated that reactive oxygen species can have prothrombotic effects on vascular and blood cells. It remains unclear, however, whether elevated levels of reactive oxygen species accelerate susceptibility to experimental thrombosis in vivo.

Approach and Results—Using a murine model with genetic deficiency in superoxide dismutase-1 (SOD1), we measured susceptibility to carotid artery thrombosis in response to photochemical injury. We found that SOD1-deficient (Sod1−/−) mice formed stable arterial occlusions significantly faster than wild-type (Sod1+/+) mice (P<0.05). Sod1−/− mice also developed significantly larger venous thrombi than Sod1+/+ mice after inferior vena cava ligation (P<0.05). Activation of protein C by thrombin in lung was diminished in Sod1−/− mice (P<0.05 versus Sod1+/+ mice), and generation of activated protein C in response to infusion of thrombin in vivo was decreased in Sod1−/− mice (P<0.05 versus Sod1+/+ mice). SOD1 deficiency had no effect on the expression of thrombomodulin, endothelial protein C receptor, or tissue factor in lung or levels of protein C in plasma. Exposure of human thrombomodulin to superoxide in vitro caused oxidation of multiple methionine residues, including critical methionine 388, and a 40% decrease in thrombomodulin-dependent activation of protein C (P<0.05). SOD and catalase protected against superoxide-induced methionine oxidation and restored protein C activation in vitro (P<0.05).

Conclusions—SOD prevents thrombomodulin methionine oxidation, promotes protein C activation, and protects against arterial and venous thrombosis in mice. (Arterioscler Thromb Vasc Biol. 2015;35:1798-1804. DOI: 10.1161/ATVBAHA.115.305963.)

Key Words: catalase • protein C • superoxides • thrombomodulin • thrombosis

Thrombotic complications, including myocardial infarction, stroke, pulmonary embolism, and deep vein thrombosis, are common causes of morbidity and mortality in patients with vascular disease. A high incidence of thrombotic events is also seen in patients with cancer and in the aged population.2,4 Despite these well-established clinical observations, the mechanisms by which distinct pathological states contribute to thrombosis remain poorly understood. One commonality among many of the prothrombotic conditions is an increase in vascular oxidative stress,4 which may generate excess reactive oxygen species (ROS). In vitro studies have suggested that ROS such as superoxide can have prothrombotic effects on vascular and blood cells, including enhanced platelet activation,5 increased expression or activity of tissue factor (TF),2 and dysregulation of anticoagulant pathways,4 all of which may predispose to arterial and venous thrombosis.

Findings from clinical studies have identified multiple sources for increased ROS in cardiovascular disease, including an increase in the expression and activity of pro-oxidant enzymes5 such as NADPH oxidase, NO synthase, and xanthine oxidase and a decrease in antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase.9–14 It remains unclear to what extent these oxidative mechanisms contribute to thrombosis in vivo.

The objective of this study was to determine the contribution of superoxide to the increased susceptibility to experimental thrombosis in vivo, using mice genetically deficient in SOD1. SOD1 is a copper- and zinc-containing enzyme that is the major cytosolic form of SOD, which catalyzes the conversion of superoxide to hydrogen peroxide. SOD1 is the predominant isoform of SOD expressed in the vasculature.15 Mice with homozygous deficiency of SOD1 (Sod1−/− mice) have increased superoxide in vascular tissue.16 Our new
findings demonstrate that the loss of SOD1 causes increased susceptibility to arterial and venous thrombosis in mice, and that SOD protects from superoxide-mediated oxidation of thrombomodulin and impairment of the protein C anticoagulant system.

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

Results

Vascular ROS Are Increased in Mice With SOD1 Deficiency
To confirm that mice deficient in SOD1 have increased vascular ROS, we measured tiron-quenchable lucigenin-enhanced chemiluminescence, a relatively selective indicator of superoxide,17 in the aorta. We detected a significant increase in fluorescence in aortic sections from $Sod1^{-/-}$ mice when compared with $Sod1^{+/+}$ mice ($P=0.035$; Figure 1). This finding is consistent with a previous report of increased superoxide levels in the vascular wall.16

Deficiency of SOD1 Enhances Susceptibility to Both Arterial and Venous Thrombosis
We next examined the effect of SOD1 deficiency on susceptibility to arterial thrombosis. Baseline prothrombin time, partial thromboplastin time, and platelet count were similar in $Sod1^{+/+}$ and $Sod1^{-/-}$ mice (Table). After photochemical injury of the carotid artery, the time to first occlusion was $\approx 3$ times faster in $Sod1^{-/-}$ mice (8.5±1.9 minutes) than in $Sod1^{+/+}$ mice (24.5±8.4 minutes; $P=0.010$; Figure 2A). Similarly, the time to stable occlusion was significantly faster in $Sod1^{-/-}$ mice than in $Sod1^{+/+}$ mice (16.2±3.3 versus 34.0±9.1 minutes, respectively; $P=0.032$; Figure 2B). An inferior vena cava ligation method was used to assess susceptibility to stasis-induced venous thrombosis. $Sod1^{-/-}$ mice developed significantly heavier (14.8±4.4 mg in $Sod1^{-/-}$ mice versus 3.9±2.3 mg in $Sod1^{+/+}$ mice; $P=0.047$) and longer (5.5±1.5 mm in $Sod1^{-/-}$ mice versus 1.6±0.8 mm in $Sod1^{+/+}$ mice; $P=0.044$) inferior vena cava thrombi when compared with $Sod1^{+/+}$ mice (Figure 2C and 2D). These data demonstrate that deficiency of SOD1 increases susceptibility to both arterial and venous thrombosis.

SOD1 Deficiency Does Not Enhance Platelet Activation in Response to Thrombin
In previous work, we have demonstrated that $H_2O_2$, which is generated by the action of SOD1 on superoxide, contributes to increased thrombotic susceptibility and thrombin-stimulated platelet activation.18 We therefore asked whether deficiency of SOD1 alters platelet activation. However, in response to thrombin, we did not observe a difference in the surface expression of P-selectin or fibrinogen binding to $Sod1^{-/-}$ platelets as compared with $Sod1^{+/+}$ platelets.
(Figure 3). These data suggest that deficiency of SOD1 in platelets does not influence α granule release or activation of platelet integrin α2bβ3.

**Deficiency of SOD1 Does Not Influence TF Expression or Activity**

We next investigated the effect of SOD1 deficiency on the expression and activity of TF, a major trigger of coagulation and thrombosis.19 Because TF expression has been reported to be redox-sensitive,7 real-time quantitative polymerase chain reaction was performed to quantitatively measure levels of TF mRNA in lung homogenates. We found, however, that the expression of TF mRNA was not altered in Sod1−/− mice (P=0.3 versus Sod1+/+ mice; Figure IA in the online-only Data Supplement). Because de-encryption of TF leading to its activation is also redox-regulated, we measured TF activity in a factor Xa activation assay. Again, we did not detect any difference in TF activity between Sod1−/− and Sod1+/+ mice (P=0.5; Figure IB in the online-only Data Supplement). These results suggest that accelerated thrombosis in Sod1−/− mice is not likely caused by oxidative upregulation of TF gene expression or activity.

**SOD1 Deficiency Impairs Generation of Activated Protein C**

Thrombomodulin, an endothelial transmembrane glycoprotein, can undergo methionine oxidation, limiting its anticoagulant activity to support thrombin-mediated generation of activated protein C (APC) from protein C.8 Therefore, to determine whether the activation of endogenous protein C is altered in SOD1-deficient mice, circulating plasma levels of APC were measured after intravenous injection of thrombin. Plasma levels of APC were 45% lower in Sod1−/− mice than in Sod1+/+ mice (Figure 4A; P=0.035). The decreased thrombin-induced generation of APC in Sod1−/− mice was not because of lower baseline levels of protein C, because plasma protein C levels tended to be higher, rather than lower, in Sod1−/− mice than in Sod1+/+ mice (P=0.07; Table).

Next, we measured generation of exogenous APC by thrombin in lung homogenates from Sod1−/− and Sod1+/+ mice. The data revealed a 40% decrease in thrombomodulin-dependent APC generation in Sod1−/− mice as compared with Sod1+/+ mice (Figure 4B; P=0.030). These findings suggest that deficiency of SOD1 leads to impaired thrombomodulin anticoagulant activity (decreased thrombomodulin-dependent activation of protein C by thrombin).

Another endothelial transmembrane protein, the endothelial protein C receptor (EPCR), can act in concert with thrombomodulin to promote protein C activation.20 Therefore, we further investigated whether SOD1 deficiency affects the expression of thrombomodulin or EPCR. No differences in thrombomodulin mRNA (measured by real-time quantitative polymerase chain reaction) or thrombomodulin protein (measured by Western blotting) were detected in the lungs of Sod1−/− mice when compared with Sod1+/+ mice (P=0.4 and P=0.2, respectively; Figure 5). Similarly, EPCR mRNA levels were comparable in the lungs of Sod1−/− and Sod1+/+ mice (P=0.6; Figure II in the online-only Data Supplement). These data suggest that the decreased generation of APC with SOD1 deficiency is not because of loss of expression of thrombomodulin or EPCR.

**Exposure of Human Thrombomodulin to Superoxide Leads to Oxidation of Methionine 388 and Loss of Thrombomodulin Anticoagulant Activity**

To determine whether SOD can protect thrombomodulin from methionine oxidation, we exposed recombinant human thrombomodulin to hypoxanthine plus xanthine oxidase to generate superoxide in vitro. Exposure to superoxide inhibited thrombomodulin anticoagulant activity, measured as thrombomodulin-dependent APC generation, by 40% (P=0.0002; Figure 6A). The inhibitory effect of superoxide was partially prevented by the addition of SOD or catalase and almost completely prevented by coinoculation with both SOD and catalase (P=0.0003 versus catalase alone; Figure 6A).

Human thrombomodulin contains 4 methionine residues in its extracellular domain, and it has been demonstrated previously that oxidation of methionine 388 to methionine sulfoxide (Met388(O)) causes loss of thrombomodulin anticoagulant activity.5,21,22 We therefore performed nano-liquid chromatography–mass spectrometry/mass spectrometry to quantify the extent of thrombomodulin methionine oxidation at Met388. We found that exposure to superoxide resulted in a 2.6-fold
increase in Met388(O) ($P=0.0003$; Figure 6B). Oxidation of Met388 was partially prevented by SOD ($P=0.042$) and almost completely prevented by catalase or coincubation with SOD and catalase ($P=0.0001$; Figure 6B). The other 3 extracellular methionine residues in human thrombomodulin (Met42, Met205, and Met291) also underwent oxidation after exposure to superoxide (Figure III in the online-only Data Supplement), but these methionine residues are unlikely to affect thrombomodulin anticoagulant activity because they are outside of the critical epidermal growth factor (EGF)-like domains of thrombomodulin required for protein C activation (Figure IIID in the online-only Data Supplement).21

Taken together, these data suggest that superoxide induces loss of thrombomodulin anticoagulant activity at least in part via increased oxidation of Met388. The protective effects of both SOD and catalase suggest that both superoxide and H2O2 contribute to methionine oxidation and inhibition of thrombomodulin anticoagulant activity.

Discussion

Despite a large body of literature demonstrating activation of oxidant pathways and increased production of ROS in vascular diseases,23 relatively little is known about the role of ROS in driving thrombosis in vivo. Several studies have demonstrated increased levels of superoxide in blood vessels of humans and animals with atherosclerosis and other vascular conditions.24–26 Previous studies using SOD1-deficient mice have implicated superoxide in the mechanism of endothelial dysfunction27 and cerebral hypertrophy.28 One major finding of the current study is that genetic deficiency of SOD1 increases susceptibility to both arterial and venous thrombosis in mice. This finding suggests that superoxide, when present in excess, can be considered to be a prothrombotic mediator in vivo. Another key finding from our study is that deficiency of SOD1 is associated with an impaired protein C anticoagulant response to thrombin. These findings suggest that elevation of superoxide because of SOD1 deficiency may lead to thrombosis in part by impairing thrombomodulin-dependent protein C activation.

Thrombomodulin is a cell surface glycoprotein expressed on the luminal surface of endothelial cells.29 When bound to thrombin, thrombomodulin functions as a potent anticoagulant by converting circulating protein C to APC. APC then proteolytically cleaves activated factors V and VIII to prevent thrombin generation. Disruption of the thrombomodulin/APC anticoagulant pathway in mice results in a prothrombotic phenotype. For example, Weiler et al30 demonstrated that mice with thrombomodulin deficiency have increased susceptibility to carotid artery thrombosis. Mice with endothelial-specific deficiency of thrombomodulin exhibit severe thrombosis at an early age and die because
of consumptive coagulopathy. Similarly, mice deficient in EPCR or partially deficient in PC also are prothrombotic. Our new findings that SOD1-deficient mice with decreased APC generation have increased susceptibility to arterial and venous thrombosis are consistent with these previous observations.

Thrombomodulin contains a critical redox sensitive methionine residue (Met388) that, when oxidized, limits its ability to activate protein C. Therefore, we hypothesized that increased vascular superoxide in Sod1−/− mice would promote the oxidative inactivation of thrombomodulin and thereby decrease APC generation. After thrombin infusion, we observed a significant decrease in the levels of plasma APC in Sod1−/− mice when compared with Sod1+/− mice. Similarly, exogenous generation of APC by thrombin also was decreased in lung tissue from Sod1−/− mice. Importantly, no differences in the expression of thrombomodulin mRNA or protein, EPCR mRNA, or plasma levels of protein C were detected between Sod1+/− and Sod1−/− mice, which is consistent with a post-translational oxidative modification of thrombomodulin as the likely mechanism of decreased APC generation in Sod1−/− mice.

Using nano-liquid chromatography–mass spectrometry/mass spectrometry to quantify methionineoxidation in human thrombomodulin, we found that superoxide-induced inhibition of recombinant human thrombomodulin anticoagulant activity in vitro was associated with significantly increased levels of thrombomodulin Met388(O). Both oxidation of Met388 and loss of thrombomodulin anticoagulant activity were partially prevented by incubation with SOD. Interestingly, coinoculation with SOD and catalase conferred an even greater degree of protection, with almost complete prevention of superoxide-induced inhibition of thrombomodulin anticoagulant activity and Met388 oxidation, which suggests a role for both superoxide and H2O2 in the redox regulation of thrombomodulin. An important limitation of these findings is that the oxidation status of thrombomodulin was assessed in a cell-free system. Additional work is needed to determine the specific role of SOD in protecting against thrombomodulin Met388 oxidation in vivo, particularly because SOD1 is an intracellular enzyme and thrombomodulin is a cell-surface protein with extracellular oxidation sites. We speculate that deficiency of cytosolic SOD1 may cause the accumulation of not only superoxide (a charged anion that cannot cross biological membranes) but also other uncharged ROS that can diffuse across the plasma membrane to interact with surface proteins such as thrombomodulin.

Three additional thrombomodulin methionine residues (Met42, Met205, and Met291) also underwent oxidation after exposure to superoxide. However, these oxidized methionine residues are less likely to affect thrombomodulin anticoagulant activity because they are outside of the critical EGF-like domains of thrombomodulin required for protein C activation, and it has been demonstrated that thrombomodulin Met388 is the key methionine residue involved in redox regulation of its anticoagulant activity. Thrombomodulin also has antifibrinolytic effects through its ability to enhance thrombin-mediated activation of thrombin activatable fibrinolysis inhibitor. However, oxidation of Met388 on thrombomodulin has no effect on thrombin activatable fibrinolysis inhibitor activation despite producing a significant decrease in protein C activity.

In a previous study, we reported that H2O2 contributes to a prothrombotic phenotype in aged mice. Unlike the effects of superoxide seen in Sod1−/− mice, however, the increased thrombotic susceptibility induced by H2O2 in aged mice was associated with platelet hyperactivation rather than an effect on protein C activation. In the current study, we did not observe enhanced activation of platelets from Sod1−/− mice, which suggests that the platelet SOD1 does not directly protect from platelet hyperactivation. It remains possible, however, that indirect antioxidant effects of SOD1, such as protection from endothelial dysfunction and diminished NO bioavailability, may help blunt platelet activation and protect from thrombosis in vivo. We also considered the possibility that the protective antithrombotic effect of endogenous SOD1 may be mediated through decreased TF activity, because both the expression and activity of TF are known to be redox regulated. However, we did not detect any changes in TF mRNA expression or TF activity in Sod1−/− mice.

Clinical studies demonstrate a clear link between increased oxidative stress and vascular complications in several disease settings. SOD1 gene variants have been associated with increased risk of cardiovascular death in patients with type 2 diabetes mellitus. Relevant to our studies in Sod1−/− mice, increased oxidative stress in patients with type 2 diabetes mellitus has been reported to be associated with decreased levels of APC, which may lead to a prothrombotic phenotype. Gene variants in other SOD enzymes also have been linked with cardiovascular events. For example, the SOD3 (the gene encoding extracellular SOD) polymorphism R213G is associated with an increased risk for ischemic heart disease. Moreover, the expression of this variant has been linked to increased cardiovascular and cerebrovascular death in diabetic patients. Similarly, an A16V polymorphism in SOD2 is associated with increased cardiovascular events. Together with the data from Sod1−/− mice reported herein, these studies strongly suggest that oxidative stress leads to increased thrombotic susceptibility and that SOD is required for redox regulation of hemostatic pathways. It is likely that endogenous SOD protects against thrombosis by modulating multiple oxidation-sensitive pathways, including the superoxide-mediated oxidation of thrombomodulin and impairment of APC generation.

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Disclosures
None.

References


**Significance**

Using mice deficient in superoxide dismutase, this study provides in vivo evidence that superoxide modulates the thrombomodulin/protein C anticoagulant pathway and protects against thrombosis. These data have important clinical implications for vascular diseases characterized by increased oxidative stress and thrombotic susceptibility. Strategies to reduce the inhibitory effect of oxidative stress on thrombomodulin anticoagulant activity may represent an approach to prevent thrombosis.
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Supplemental Figure I. Deficiency of SOD1 does not influence TF mRNA expression or activity in mice. (A) TF mRNA levels in lung homogenates were determined by qRT-PCR and normalized to GAPDH, and data are displayed as fold-change relative to control (Sod1+/+ mice). Comparisons of normalized expression values (\(\Delta\text{Ct}\)) employed the conventional \(\Delta\Delta\text{Ct}\) fold change method. Ct values: 24.46 ± 0.09 for Sod1+/+ vs. 24.21 ± 0.07 for Sod1−/− mice. \(P = 0.3\) vs. Sod1+/+ mice (n = 5 to 6 mice per group). (B) TF activity in lung homogenates was determined using a TF chromogenic activity assay. Data were quantified based on a TF standard curve. Values are mean ± SEM; \(P = 0.5\) (n = 4 mice per group).
Supplemental Figure II. Deficiency of SOD1 does not alter mRNA levels of EPCR. EPCR mRNA levels in lung were determined by qRT-PCR. Levels of mRNA were normalized to GAPDH, and data are displayed as fold-change relative to control (Sod1+/+ mice). Comparisons of normalized expression values (ΔCt) employed the conventional ΔΔCt fold change method. The Ct values for EPCR were 24.34 ± 0.14 in Sod1+/+ vs. 24.16 ± 0.28 in Sod1/- mice. P = 0.6 vs. Sod1+/+ mice (n = 5 to 6 mice per group).
Supplemental Figure III. Superoxide-induced oxidation of TM Met42, Met 205, and Met291.
Recombinant human TM was incubated with or without 5 mU/ml xanthine oxidase and 1 mM hypoxanthine (X-XO) in the presence or absence of 50 U/ml PEG-SOD and/or 250 U/ml PEG-catalase. The content of (A) Met42(O), (B) Met205(O), and (C) Met291(O) was determined by nano-LC-MS/MS (n=4). *P < 0.05 vs. untreated control, † P < 0.05 vs. X-XO treatment, # P < 0.05 vs. PEG-catalase only. (D) Schematic representation of TM domain structure. The extracellular portion of TM contains a lectin-like domain, six epidermal growth factor (EGF)-like domains, and a serine/threonine (S/T)-rich domain. The region of TM involved in thrombin binding and protein C activation are EGF-like domains 4, 5, and 6. The location of the four extracellular methionine residues are indicated (arrows) with the redox active regulatory methionine 388 (M388) highlighted. TM also contains a transmembrane domain and a cytoplasmic tail (CYTO).
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. 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. 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. 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. 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. 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. 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. 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. 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.

Activation of exogenous protein C by thrombin was measured in lung lysates using a two-stage assay described previously. 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 37°C, activation of
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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°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 MgCl₂, 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. 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°C. Plasma was collected in aliquots and stored frozen at -80°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 CaCl₂ 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