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Thrombospondin-1 Regulates Blood Flow via CD47 Receptor-Mediated Activation of NADPH Oxidase 1

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Abstract

Objective—Although the matricellular protein thrombospondin-1 (TSP1) is highly expressed in the vessel wall in response to injury, its pathophysiological role in the development of vascular disease is poorly understood. This study was designed to test the hypothesis that TSP1 stimulates reactive oxygen species (ROS) production in vascular smooth muscle cells (VSMCs) and induces vascular dysfunction by promoting oxidative stress.

Methods and Results—Nanomolar concentrations of TSP1 found in human vascular disease robustly stimulated superoxide ($O_2^{\bullet-}$) levels in VSMCs at both cellular and tissue level as measured by cytochrome *c* and electron paramagnetic resonance. A peptide mimicking the C-terminus of TSP1 known to specifically bind CD47 recapitulated this response. Transcriptional knockdown of CD47 and a monoclonal inhibitory CD47 antibody abrogated TSP1-triggered $O_2^{\bullet-}$ *in vitro* and *ex vivo*. TSP1-treatment of VSMCs activated phospholipase C and protein kinase C, resulting in phosphorylation of the NADPH oxidase (Nox) organizer subunit $p47^{phox}$ and subsequent Nox1 activation, leading to impairment of arterial vasodilatation *ex vivo*. Further, we observed that blockade of CD47 and Nox1 gene silencing *in vivo* in rats improves TSP1-induced impairment of tissue blood flow following ischemia reperfusion.

Conclusion—Our data suggest a highly-regulated process of ROS stimulation and blood flow regulation promoted through a direct TSP1/CD47-mediated activation of Nox1. This is the first

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report to our knowledge of a matricellular protein acting as a ligand for Nox activation and through specific engagement of integrin-associated protein CD47.

Keywords

Thrombospondin-1; CD47; NADPH oxidase; reactive oxygen species; vascular disease

Matricellular proteins are non-structural proteins integrated into the structural extracellular matrix that regulate important cell signaling pathways via binding to specific cell-surface receptors and modulating the activity of growth factors and inflammatory cytokines[1]. Matricellular proteins are present at low levels in the wall of healthy blood vessels and are highly upregulated in the vessel wall in various vascular disorders[2, 3]. However, though increasing evidence supports the role of matricellular proteins in vascular disease[3, 4], their potential to stimulate reactive oxygen species (ROS) production in vascular tissue and their pathological significance in oxidative stress-mediated vascular dysfunction remain unknown.

CD47 is a ubiquitously expressed transmembrane receptor of the immunoglobulin superfamily that also functions as a receptor for the matricellular protein thrombospondin-1 (TSP1). TSP1 was originally isolated from platelets[5] and has been long known for its pro-aggregatory and anti-angiogenic effects[6]. More recent studies showed that TSP1 expression is markedly increased in the vessel wall in cardiovascular disorders, including diabetes mellitus, atherosclerosis and ischemia-reperfusion injury (IRI)[2, 3, 7]. Although these findings suggest that TSP1 plays a role in vascular disease, its effect via cognate receptor CD47 appears limited to adenylate cyclase activation[8] and stimulation of apoptosis[9]. More recently, TSP1-CD47 signaling has emerged as an important mediator of vascular dysfunction by interfering with nitric oxide (NO) bioavailability and action[10, 11]. Despite the fact that TSP1 levels surrounding arterial VSMCs rise sharply in response to injury[12]; the ability of TSP1 to stimulate VSMC ROS, the source of ROS, the mechanism of stimulation, and its role in oxidative stress-mediated vascular dysfunction are entirely unknown.

NADPH oxidase (Nox) enzyme complex is a major source of ROS in the vascular wall and important mediator of vascular disease[13]. The Nox family consists of seven members (Nox1-5, and DUOX1/2), which differ in tissue distribution, subcellular localization, and regulation [14, 15]. Of the major vascular Nox isozymes, Nox2 produce primarily superoxide anion ($O_2^{\bullet -}$), while Nox4 generates hydrogen peroxide (H_2O_2)[16]. Nox2 has been shown to be activated by growth factors[17], lipid mediators[18], cytokines[19], and mechanical forces[20] and its role in vascular disease is relatively well characterized. In contrast, Nox4 is constitutively active[16] and has been reported to have a protective role in cardiovascular tissue although this is still somewhat controversial[21, 22]. Interestingly, the role of another major vascular isoform of Nox, Nox1, in vascular disease is less well characterized. Angiotensin II (AngII) has been shown to stimulate Nox1-derived $O_2^{\bullet -}$ generation in VSMCs[16]. Recent studies demonstrated that Nox1-derived ROS play an important role in the pathogenesis of atherosclerosis and diabetes[23, 24].

We postulated that TSP1, through interaction with its cognate receptor CD47, stimulates Nox-derived ROS production, leading to arterial dysfunction. The results of the present

study provide seminal mechanistic insight into how matricellular protein TSP1 can induce Nox1 assembly and stimulates ROS generation. In addition, the data presented herein demonstrate the involvement of a new signaling axis TSP1-CD47-Nox1 in the regulation of vascular tone and control of blood flow. The actions of TSP1 described herein have the potential to bring to the forefront an entirely new class of compounds, namely matricellular proteins, which may regulate vascular Nox, thus having broad implications for vascular diseases and therapies targeting the same.

Materials and Methods

Detailed methodologies for cell culture, measurement of $O_2^{\bullet -}$ using L-012 chemiluminescence and cytochrome *c*, assessment of SOD activity and expression, EPR and Laser Doppler analysis are provided in the Supplemental Methods.

Animals

All animal studies were performed under a protocol approved by the IACUC of the University of Pittsburgh. $Nox1^{y/-}$ mice on a C57BL/6 background and C57BL/6 mice were kindly provided by Dr. Kathy K. Griendling (Emory University) from her breeding colonies. $Nox1^{y/-}$ mice were originally generated and approved for our use by Dr. Karl-Heinz Krause (University of Geneva, Switzerland)[25]. Additional wild type C57BL/6 male mice were purchased from Jackson Labs (Bar Harbor, ME, USA). Male Sprague-Dawley rats were purchased from Charles River (Wilmington, MA, USA).

Gene silencing

Gene suppression in VSMCs—Nox1 was gene suppressed using siRNA against Nox1 (Invitrogen, Carlsbad, Ca, USA) according to the recommendation of the manufacturer. CD47 (5′-CGTCACAGGCAGGACCCACTGCCCA-3′) morpholino oligonucleotides (GeneTools LLC, Philomath, OR, USA) were used to gene silence CD47. Knockdown of Nox1 and CD47 was confirmed by qPCR and Western blot, respectively.

Whole animal gene silencing using Nox1 vivo-morpholino oligonucleotides—Male Sprague-Dawley rats (200–225 g) were injected with a Nox1 targeting vivo-morpholino oligonucleotide (5′-ACCAGCCAGTTTCCCATTGTCAAAT-3′) on days 1 (10 mg/kg) and 3 (5 mg/kg) via the tail vein and used for experiments on day 5 according to the manufacturer's instructions (Gene Tools). Control rats were injected with the solvent (PBS) of the morpholino oligonucleotide (Morpholino-vehicle). Knockdown of Nox1 was confirmed by Western blot.

Vessel myography

Aortic rings from control (Morpholino-vehicle) and antisense Nox1 morpholino-treated (Nox1 morpholino) rats were denuded and placed on myograph stirrups (Danish Myo Technology, Atlanta, GA). Rings were incubated with vehicle or TSP1 (2.2 nM, 60 min), precontracted with phenylephrine (PE, 3×10^{-7} M) and after obtaining a stable plateau phase, endothelium-independent vasorelaxation was evoked by the NO donor sodium nitroprusside (SNP, 10^{-10} to 10^{-5} M) as described[26].

Statistical analysis

All results are expressed as means ± SEM. Significance of the differences were assessed by two-way ANOVA followed by Bonferroni post hoc test. Vasodilator responses are expressed as a percentage of PE-induced preconstriction. A value of $p < 0.05$ was considered to be statistically significant.

Results

TSP1 is a Rapid and Potent Stimulator of Smooth Muscle $O_2^{\bullet-}$

As TSP1 levels are elevated in vascular disease[3, 7], we evaluated the ability of TSP1 to induce $O_2^{\bullet-}$ generation in vascular cells. Human aortic VSMCs were challenged with exogenous human TSP1 and $O_2^{\bullet-}$ generation was measured using cytochrome *c*. At concentrations found in the human circulation in patients with vascular disease (2.2 nM)[27], TSP1 significantly stimulated $O_2^{\bullet-}$ production in human VSMCs (Fig. 1A). EPR data demonstrate that pathophysiologically relevant concentrations of TSP1 (1.1, 2.2 and 11 nM) stimulates $O_2^{\bullet-}$ production in rat aortic VSMCs (Fig. 1B) with a peak response occurring after stimulation with 2.2 nM of TSP1. Fig. 1B also shows that TSP1 at concentrations found in healthy individuals (0.22 nM, 60 min) did not stimulate $O_2^{\bullet-}$ (Fig. 1B). Moreover, $O_2^{\bullet-}$ production rapidly and robustly rose as early as 30 min after administration of TSP1 (Fig. 1C) as measured by an independent luminol-based method. TSP1 stimulated $O_2^{\bullet-}$ by ∼2-fold (5.2±0.9 vs. 10.6±1.2 pmol $O_2^{\bullet-}$/mg protein/min for vehicle- vs. TSP1-treatment, respectively, $*p < 0.05$) and at far lower concentrations than prototype ROS inducers phorbol myristate acetate (PMA) and AngII (Fig. 1D). These data demonstrate for the first time that TSP1 rapidly and potently stimulates ROS in VSMCs.

Suppression of SOD is Not Involved in TSP1-mediated Elevations in $O_2^{\bullet-}$

Increased $O_2^{\bullet-}$ levels and oxidative stress-mediated vascular dysfunction can occur secondary to decreased expression or activity of SOD[28]. We investigated whether the elevation in $O_2^{\bullet-}$ in response to TSP1 was, in part, attributable to reduced SOD expression or activity. Treatment of VSMCs with 2.2 nM TSP1 for 60 and 180 min did not alter SOD1 (Cu/ZnSOD) expression or total SOD activity (Supplemental Fig. 1A and B).

TSP1-Stimulated $O_2^{\bullet-}$ Requires CD47

Our group previously reported that vascular cells express CD47[29] and that TSP1 avidly binds CD47 in vascular smooth muscle[30]. To test whether CD47 could participate in TSP1-stimulated $O_2^{\bullet-}$ production, CD47 expression was suppressed by 46% using antisense CD47 morpholino oligonucleotides (Fig. 2A and Supplemental Fig. 2) and $O_2^{\bullet-}$ production was monitored using cytochrome *c* reduction. Suppression of CD47 in VSMCs reduced TSP1-stimulated $O_2^{\bullet-}$ production (Fig. 2B). In addition, blockade of CD47 signaling using a CD47 monoclonal inhibitory antibody (clone OX101), but not a monoclonal CD36 antibody (clone JC63.1), dramatically decreased TSP1-stimulated $O_2^{\bullet-}$ (Fig. 2C and Supplemental Fig. 3). Conversely 7N3, a peptide activator of CD47 derived from the C-terminus of TSP1[30], significantly stimulated $O_2^{\bullet-}$ in VSMCs (Fig. 2D). Moving to the tissue level, we examined whether TSP1, via CD47, stimulates $O_2^{\bullet-}$ production in mouse arteries *ex vivo*.

Endothelium-free aortic segments were treated with a monoclonal antibody against CD47 (clone MIAP301) or an isotype-matched immunoglobulin $IgG_{2\alpha}$ and TSP1-stimulated $O_2^{\bullet-}$ was determined using the membrane permeable electron paramagnetic resonance (EPR) spin probe CMH. TSP1 rapidly stimulated $O_2^{\bullet-}$ production in aortae treated with control antibody. Treatment with the CD47 blocking antibody completely abolished TSP1-stimulated $O_2^{\bullet-}$ production (Fig. 2E and F). These results are the first to demonstrate that physiologically-relevant concentration of TSP1 stimulates $O_2^{\bullet-}$ production and via its cognate receptor CD47.

Activated CD47 Increases $O_2^{\bullet-}$ Production via Nox1

Next, we determined the source TSP1-stimulated $O_2^{\bullet-}$ in VSMCs using pharmacological inhibitors of several classes of oxidases. Preincubation with the mitochondrial electron transport inhibitor rotenone (50 μM) and the nitric oxide synthase (NOS) inhibitor L-NAME (100 μM) did not inhibit TSP1-stimulated $O_2^{\bullet-}$ production in VSMCs (Supplemental Fig. 4). In contrast, DPI (50 μM), a promiscuous inhibitor of flavin-containing oxidases, significantly inhibited $O_2^{\bullet-}$ production (Supplemental Fig. 4). These data suggested the source of TSP1-stimulated $O_2^{\bullet-}$ in VSMCs is Nox.

Since Nox1 is a major source of $O_2^{\bullet-}$ in VSMCs[15], we hypothesized that TSP1 stimulates $O_2^{\bullet-}$ via Nox1. To test the hypothesis, $O_2^{\bullet-}$ production in membrane fractions from Nox1-suppressed vs. control VSMCs was measured by cytochrome *c* reduction. TSP1 elevated $O_2^{\bullet-}$ production by ∼ 4-fold in the membrane fraction of scrambled (Scrmb) siRNA-transfected VSMCs (Fig. 3A). In contrast, siRNA knockdown of Nox1 completely ablated TSP1-stimulated $O_2^{\bullet-}$ (Fig. 3A). Confirming the effectiveness of Nox1 siRNA, qPCR showed levels of Nox1 mRNA were decreased by 67% in Nox1 siRNA-treated vs. Scrmb siRNA-treated VSMCs. In contrast, we observed no change in Nox4 mRNA levels. Nox2 and Nox5 proteins are undetectable in rat aortic VSMCs[15].

Nox4 is another major Nox isoform in VSMCs that produces mainly H_2O_2[16]. To investigate whether TSP1 stimulates H_2O_2 production in VSMCs via Nox4, we utilized siRNA to gene silence Nox4, treated cells with TSP1 (2.2 nM TSP1, 60 min) and measured H_2O_2 production using Amplex Red fluorescence. qPCR showed levels of Nox4 mRNA were decreased by ∼70 % in Nox4 siRNA-treated vs. Scrmb siRNA-treated VSMCs. As shown in Supplemental Fig. 5, TSP1 did not stimulate H_2O_2 production in Scrmb siRNA-treated VSMCs. Gene silencing of Nox4 did not affect H_2O_2 production in TSP1-treated VSMCs. These data suggest that TSP1 under these conditions does not stimulate H_2O_2 in VSMCs via Nox4.

To ensure that Nox1 was indeed stimulated by TSP1, we measured TSP1-stimulated $O_2^{\bullet-}$ levels in endothelium-free aortic rings from wild type and $Nox1^{y/-}$ mice. Superoxide increased ∼ 4-fold in aortae from wild type mice as detected by the membrane permeable EPR spin probe CMH (Fig. 3C). Conversely, TSP1 did not elicit $O_2^{\bullet-}$ production in $Nox1^{y/-}$ aortae (Fig. 3B and C). Fig. 3C shows cumulative EPR $CM^{\bullet}$ radical intensities in vehicle- and TSP1-treated aortic rings. Proof that $O_2^{\bullet-}$ was the ROS responsible for the $CM^{\bullet}$ radical was provided by inhibition of the $CM^{\bullet}$ signal on exposure to superoxide dismutase (SOD). Finally, we corroborated Nox1 stimulation through CD47 receptor by employing the

receptor-specific peptide 7N3. Peptide 7N3 significantly stimulated $O_2^{\bullet-}$ production in aortic rings from control (morpholino-vehicle treated) rats. This effect was reduced in aortic rings from Nox1 gene-suppressed (Nox1 morpholino-treated) rats (Fig. 3D). Nox1 suppression was confirmed by Western blot (Fig. 3D inset and Supplemental Fig. 6A and B). These results suggest that TSP1 through activation of CD47 receptor stimulates Nox1-derived $O_2^{\bullet-}$ production.

TSP1 Promotes Nox Activation via PKC & p47^{phox}

Phosphorylation of the Nox organizer subunit p47^{phox} is a critical initiating step in Nox assembly and activation[31]. Protein kinase C (PKC), phospholipase C (PLC) and phospholipase D (PLD) have been implicated in p47^{phox} phosphorylation and subsequent Nox activation[32, 33]. As shown in Fig. 4A, the PLC inhibitor U-73122 and the PKC inhibitor calphostin C (but not the phospholipase D inhibitor VU0155069) inhibited TSP1-induced $O_2^{\bullet-}$, suggesting that PLC and PKC, but *not* PLD, mediate TSP1-induced Nox1 activation. To explore whether TSP1 induces p47^{phox} phosphorylation in VSMCs, p47^{phox} was immunoprecipitated and probed with an anti-phosphoserine antibody. TSP1 treatment of VSMCs significantly increased serine phosphorylation in p47^{phox} (Fig. 4B). Taken together, these data suggest that TSP1-mediated Nox1 stimulation occurs via PKC (and not PLD) activation and subsequent phosphorylation of the Nox organizer subunit p47^{phox}. Next we tested the effect of TSP1 on classical downstream signaling molecules of vascular dysfunction, including p38 mitogen-activated protein kinase (MAPK) and c-jun N-terminal kinases (JNK). Interestingly, concentrations of TSP1 that increased $O_2^{\bullet-}$ production (2.2 nM) did not stimulate p38 MAPK or JNK phosphorylation in VSMCs (Supplemental Fig. 7A and B).

TSP1 Attenuates Vasodilatation and IRI-blood flow via Nox1

Scavenging of the physiologic vasodilator NO by $O_2^{\bullet-}$ is one way by which ROS inhibits vasodilatation. We tested the effects of TSP1 on NO-mediated vasodilatation of rat thoracic aortae. TSP1 (2.2 nM) significantly inhibited relaxation by the NO donor sodium nitroprusside (SNP) (Fig. 5A). However, in aortae in which Nox1 was silenced, SNP-mediated vasodilatation was insensitive to TSP1. These data suggest that TSP1 can limit NO-mediated vasodilatation through Nox1.

Increased ROS production secondary to IRI inhibits tissue blood flow[34]. The results in isolated aortae suggested that plasma TSP1 might acutely control blood flow *in vivo* via Nox1. We tested this in a pre-clinical rat model of hindlimb IRI. Intravenous TSP1 acutely decreased recovery of hindlimb blood flow following IR as measured by real time laser Doppler (Supplemental Fig. 8). However, in Nox1 gene silenced animals intravenous TSP1 did not decrease hindlimb reperfusion following IR (Fig. 5B and C). Next, we investigated whether CD47 activation by TSP1 limits tissue blood flow *in vivo* following IR. Ninety minutes before ischemia, the animals were treated with a CD47 monoclonal antibody (clone OX101; 0.4 μg/g body weight) via a single intraperitoneal injection as we reported previously[35]. As shown in Fig. 5D, limiting CD47 activation with an antibody that prevents TSP1 binding inhibits TSP1's ability to limit tissue blood flow following IR. Supplemental

Fig. 9 demonstrates that rat femoral artery and skeletal muscle express CD47. These data suggest that circulating TSP1 limits tissue blood flow following IR via CD47 and Nox1.

Discussion

Here we show for the first time that the secreted matricellular protein TSP1 is a dynamic and robust regulator of tissue ROS, whose mechanism of action includes a role for CD47, PLC, PKC, and Nox1. Blockade of TSP1-CD47 interaction hinders a chain of signaling events involving $p47^{phox}$ and Nox1 activation, restoring blood flow in IRI. These findings are supported by multiple *in vitro* and *in vivo* gene silencing and biologics approaches. CD47-, and Nox1-silenced VSMC, $Nox1^{y/-}$ vessels, and aortae from Nox1 morpholino-treated animals all displayed reduced ROS in response to TSP1. Moreover, taking a systems approach, suppression of Nox1 abrogated TSP1's regulatory effect on tissue blood flow. These findings support TSP1 as an important modulator of blood perfusion in injured organs and may suggest a broader role of matricellular proteins in Nox activation and tissue homeostasis.

Results initially obtained in human and rat arterial VSMCs were translated to the tissue level in multiple murine models, suggesting conservation of our discovery that TSP1, via mechanisms involving $p47^{phox}$ and Nox1 activation, leads to increased $O_2^{\bullet-}$ generation. Indeed, these are the first studies to show Nox activation by a matricellular protein. Moreover, there are very limited data on the effect of TSP1, or any matricellular protein, for that matter on ROS production in any cell type[36, 37] and the source of ROS in those reports was indeterminate or linked to mitochondrial membrane potential shifts. It is important to note that concentrations of TSP1 used in these earlier studies were 10 to 20 fold higher[36, 37] than the concentrations applied in the present study. Moreover, ROS production induced by the high concentrations of TSP1 employed in those studies was associated with cell death. In the present work, we show that TSP1, at concentrations found in human vascular disease (2.2 nM)[27], significantly increased VSMC $O_2^{\bullet-}$ generation in the absence of cytotoxic effects and TSP1 at concentrations found in the circulation of healthy individuals (0.22 nM) did not stimulate $O_2^{\bullet-}$.

TSP1 is the only known soluble ligand of cell receptor CD47[10]. 7N3, a peptide activator of CD47 derived from the C-terminus of TSP1, increased $O_2^{\bullet-}$ production, and blockade of CD47 activation by multiple corroborating methods completely abrogated TSP1-stimulated $O_2^{\bullet-}$ production. TSP1 is known to bind CD36, though modulation of cellular responses through this receptor require 50 to 100 fold greater concentrations of TSP1 than that for CD47[38], suggesting further propensity for Nox1 activation via CD47. Indeed, blockade of CD36 signaling did not inhibit the ability of TSP1 to stimulate $O_2^{\bullet-}$ production in VSMCs.

Recently we reported that TSP1 limited endothelial-dependent arterial vasodilatation through inhibition of endothelial NOS activation[39]. New work presented herein shows that TSP1 targets the VSMC compartment of thoracic aorta to stimulate Nox1-based $O_2^{\bullet-}$ production and further inhibits NO-mediated vasodilatation. These findings suggest that TSP1, which is markedly upregulated in the vessel wall in experimental and clinical peripheral vascular disease[3, 7], contributes to NO insufficiency through multiple

mechanisms including direct inhibition of NO production and concurrent generation of the potent NO scavenger $O_2^{\bullet-}$. TSP1-induced impairment of vascular relaxation is also likely to include NO-independent effects elicited by ROS on the VSMC contractile apparatus[40], although that work is outside the scope of this manuscript. It is important to note at this juncture that mechanisms regulating the vasoreactivity of conduit vs. microvessels are distinct and that findings obtained from aortic rings may only approximate mechanism of blood pressure regulation and blood flow changes with regard to TSP1. Nevertheless, the data in conjunction with our findings on femoral blood flow are consistent with NO and $O_2^{\bullet-}$ playing an important role in impaired microvessel responses.

The present study demonstrated that soluble TSP1 limits tissue perfusion following hindlimb IR in a Nox1-dependent manner. Thus, along with its previously established pro-aggregatory effects, the physiological role of TSP1 could be to prevent blood loss by reducing flow to an injured organ under less severe conditions. On the other hand, when TSP1 levels rise dramatically (i.e. vascular disease) an unabated constrictor response is likely to be deleterious. The present study identifies the TSP1-CD47-Nox1 axis as a proximate promoter of vascular ROS and dysfunction and provides novel targets for enhancing tissue perfusion under conditions in which TSP1 is upregulated. Indeed, pharmacological blockade of CD47 *ex vivo* abrogated TSP1-stimulated ROS, and gene silencing of Nox1 and CD47 blockade *in vivo* significantly improved TSP1-induced impairment of hindlimb perfusion following IR. It is, however, important to note that the protective role of Nox1 suppression in our pre-clinical model may involve several mechanisms. TSP1 not only stimulates VSMC $O_2^{\bullet-}$ as shown in the present study, but increases platelet adhesion and activation and affects endothelial cell function[39, 41]. As both endothelial cells[15] and platelets[42] express Nox1 and CD47, it is tempting to speculate that plasma TSP1 via activation of CD47-Nox1 signaling in endothelial cells and platelets contributes to the observed impairment of tissue reperfusion in this model.

In conclusion, our findings identify a novel, significant and unexpected mechanism of blood flow regulation by the TSP1-CD47-Nox1 signaling axis that is likely to have broad implications for multiple cardiovascular diseases no less multiple other organ systems and pathologies. We have demonstrated that blockade of CD47 as well as transcriptional knockdown of CD47 inhibits TSP1-stimulated ROS production. On a systems level, gene silencing of Nox1 or antibody blockade of CD47 activation completely abrogates TSP1-mediated inhibition of arterial vasodilatation; and improves blood flow in a preclinical model of IRI. These findings establish a fundamental role for TSP1 as an important modulator of tissue ROS. They also define a regulatory role for TSP1 via CD47, Nox1, and ROS in tissue injury and reperfusion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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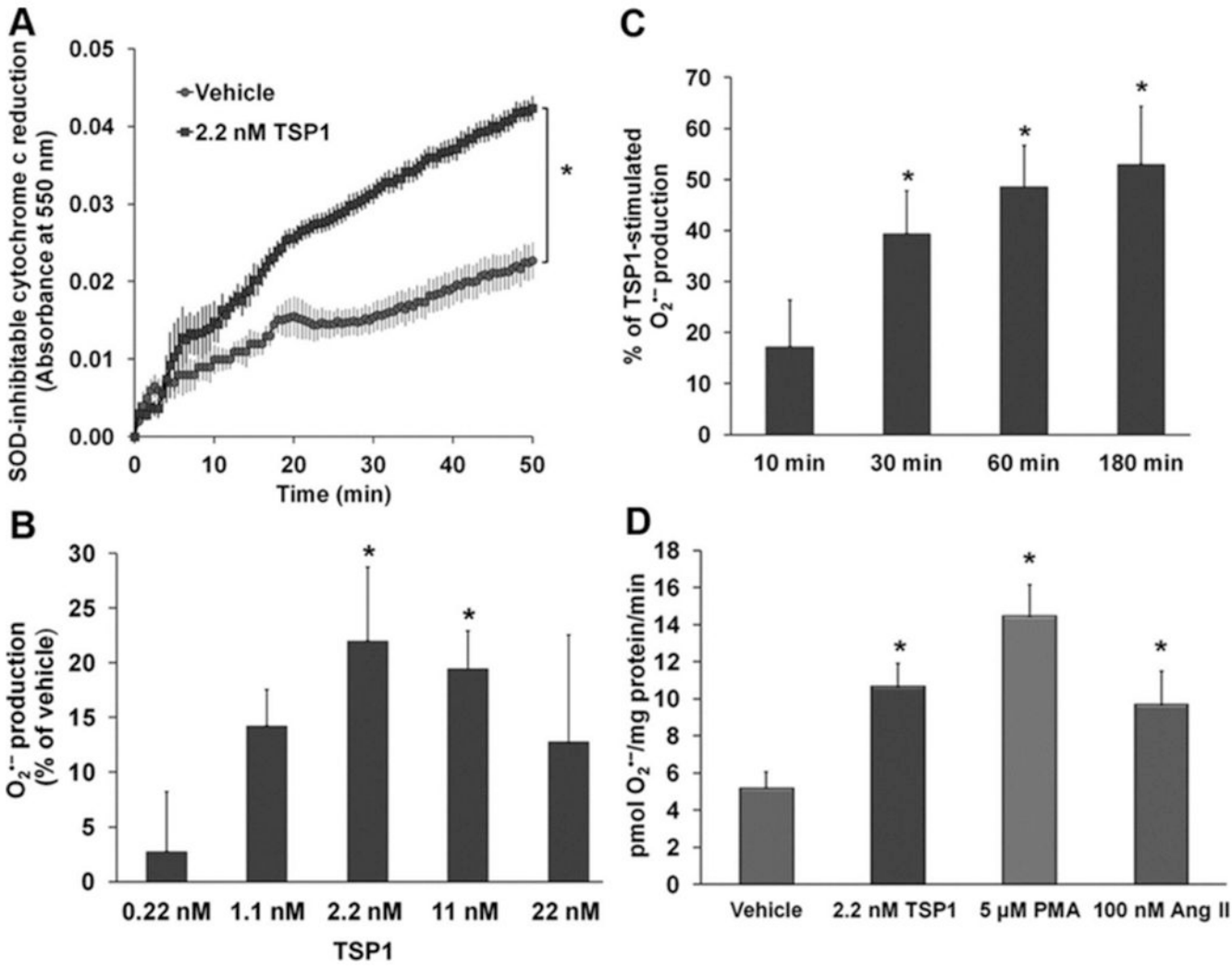

Figure 1.

TSP1 is a rapid and potent stimulator of VSMC $O_2^{\bullet-}$. **A)** Human aortic VSMCs were treated with vehicle or TSP1 (2.2 nM, 60 min). Superoxide production was measured in the 28,000 g membrane fraction using cytochrome *c* reduction (n=8). **B)** Rat aortic VSMCs were incubated with vehicle or TSP1 (0.22, 1.1, 2.2, 11 or 22 nM) for 60 min. Superoxide production was measured using EPR (n=4). **C)** Rat aortic VSMCs were incubated with vehicle or 2.2 nM TSP1 for 10, 30, 60, and 180 min. Superoxide production was measured using L-012 chemiluminescence (n=4). **D)** Rat aortic VSMCs were treated with vehicle, TSP1 (2.2 nM, 60 min), PMA (5 μM, 60 min) or AngII (100 nM, 120 min). Superoxide production was measured using cytochrome *c* (n=6-7). Data represent the mean ± SEM. **p* < 0.05 indicates significant difference between vehicle- vs. TSP1-/PMA-/AngII-treatment.

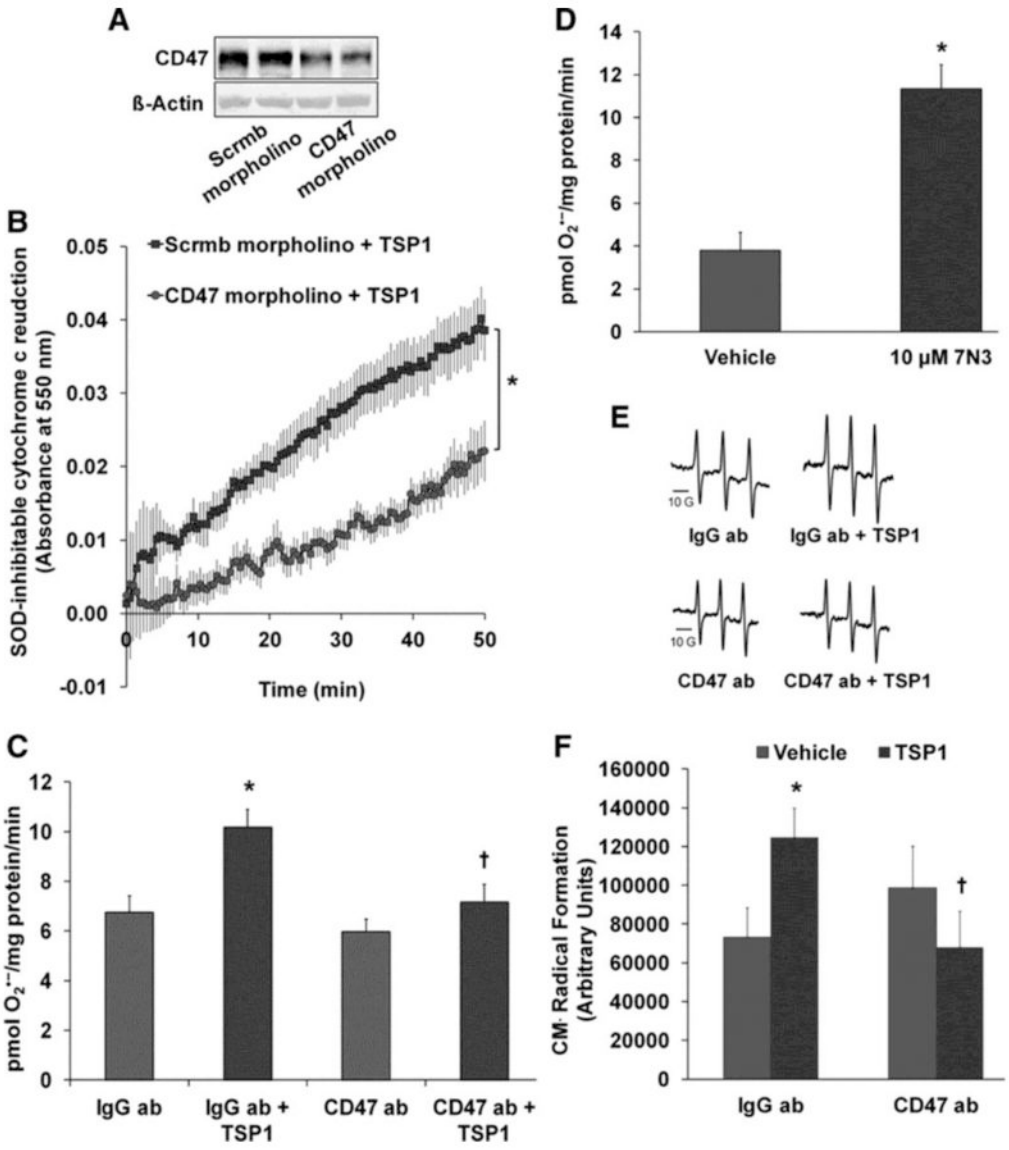

Figure 2.

TSP1-stimulated $O_2^{\bullet-}$ production requires CD47. **A)** Rat aortic VSMCs were treated with a CD47 or scrambled (Scrmb) morpholino (10 μM) as per the manufacturer's instructions (GeneTools). Western blot images are representative of 6 independent experiments. **B)** CD47 and Scrmb morpholino-treated VSMCs were incubated with TSP1 (2.2 nM, 60 min) and $O_2^{\bullet-}$ production was measured using cytochrome *c* (n=3). **C)** VSMCs were treated with a CD47 monoclonal antibody [2 μg/ml, CD47 (OX101), Santa Cruz Biotechnology] or isotype control immunoglobulin IgG_1 (2 μg/ml, Santa Cruz Biotechnology) for 30 min, and treated with vehicle or TSP1 (2.2 nM, 60 min). Superoxide production was measured using cytochrome *c* (n=4). **D)** VSMCs were stimulated with a TSP1-based peptide (7N3) that binds CD47 (10 μM, 60 min) and $O_2^{\bullet-}$ production was measured was measured using cytochrome *c* (n=5). **E)** Endothelium-denuded wild type mouse aortic rings were pre-treated with a CD47 monoclonal antibody [2 μg/ml, CD47 (MIAP301), Santa Cruz Biotechnology]

or isotype control immunoglobulin $IgG_{2\alpha}$ (2 μg/ml, Santa Cruz) for 30 min, and treated with vehicle or TSP1 (2.2 nM, 60 min). $CM^{\bullet}$ radical formation was measured for 60 min at 37 °C using EPR. Representative $CM^{\bullet}$ spectra are presented. **F)** Cumulative and averaged $CM^{\bullet}$ radical formation in vehicle- and TSP1-treated endothelium-denuded aortic rings (n=7). Data represent the means ± SEM. $^{*}p < 0.05$ indicates significant difference between vehicle- and TSP1/7N3-treatment. $^{\dagger}p < 0.05$ indicates significant difference between control IgG- and CD47 antibody-treatment.

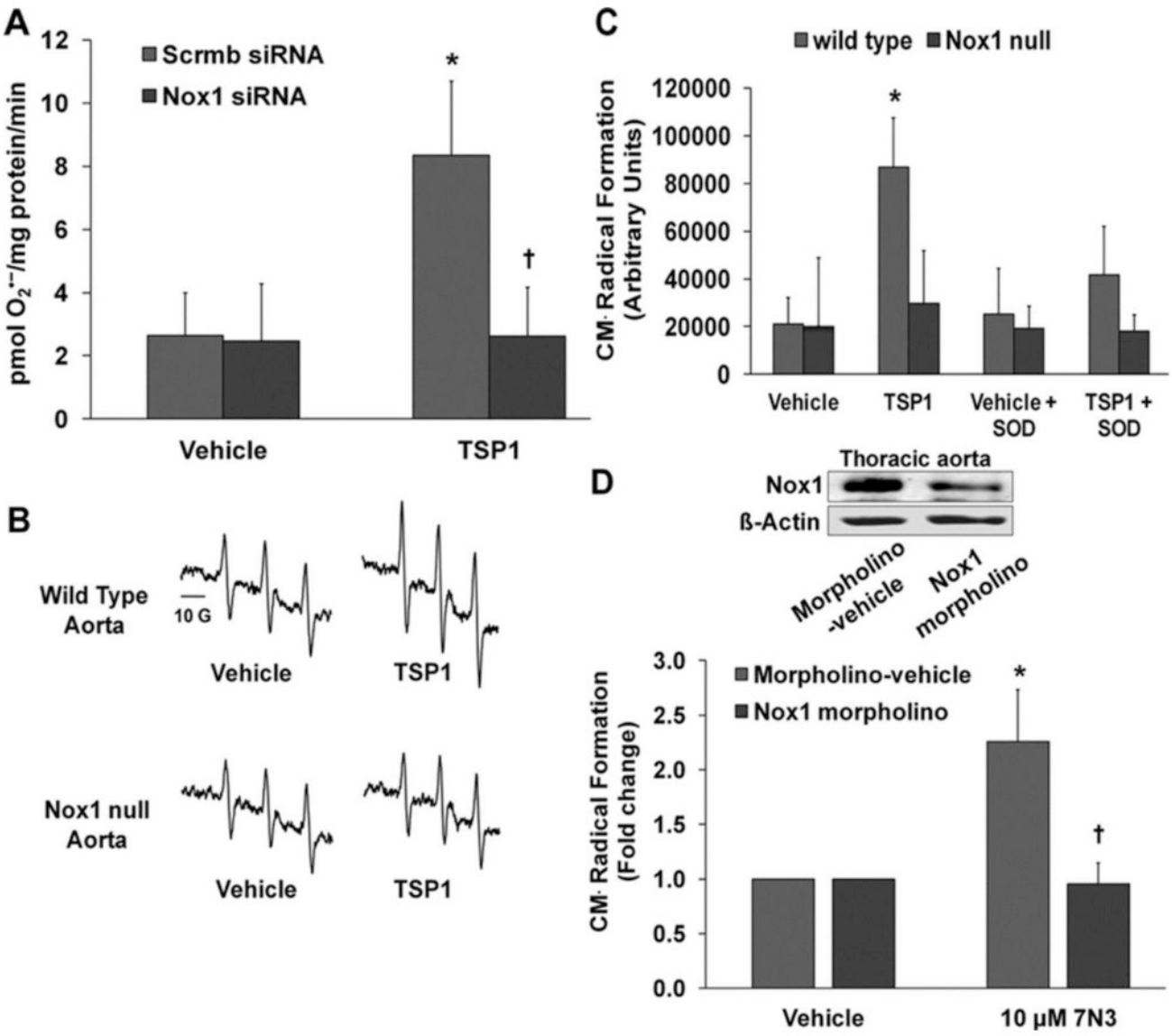

Figure 3.

Activated CD47 increases $O_2^{\bullet-}$ production via Nox1 stimulation. **A)** VSMCs were transfected with Nox1 or Scrmb siRNA and treated with vehicle or TSP1 (2.2 nM, 60 min). Superoxide production was measured using cytochrome *c* (n=3). **B)** Endothelium-denuded wild type and Nox1 null aortic rings were treated with vehicle or TSP1 (2.2 nM, 60 min) and $CM^{\bullet}$ radical formation was measured using EPR. Images are representative of 3 independent experiments. **C**) Cumulative and averaged $CM^{\bullet}$ radical formation in vehicle- and TSP1-treated endothelium-denuded aortic rings. The specificity of CMH for $O_2^{\bullet-}$ was confirmed by the addition of SOD (150 U/ml) (n=3). **D**) Endothelium-denuded aortic rings from control (Morpholino-vehicle) and Nox1-morpholino treated rats were treated with vehicle or 7N3 (10 µM, 60 min). $CM^{\bullet}$ radical formation was measured for 60 min at 37 °C using EPR (n=3-4). Data represent the means ± SEM. *$p < 0.05$ indicates significant difference between

vehicle- and TSP1/7N3-treatment. $^{\dagger}p < 0.05$ indicates significant difference between control and Nox1 siRNA/morpholino treatment.

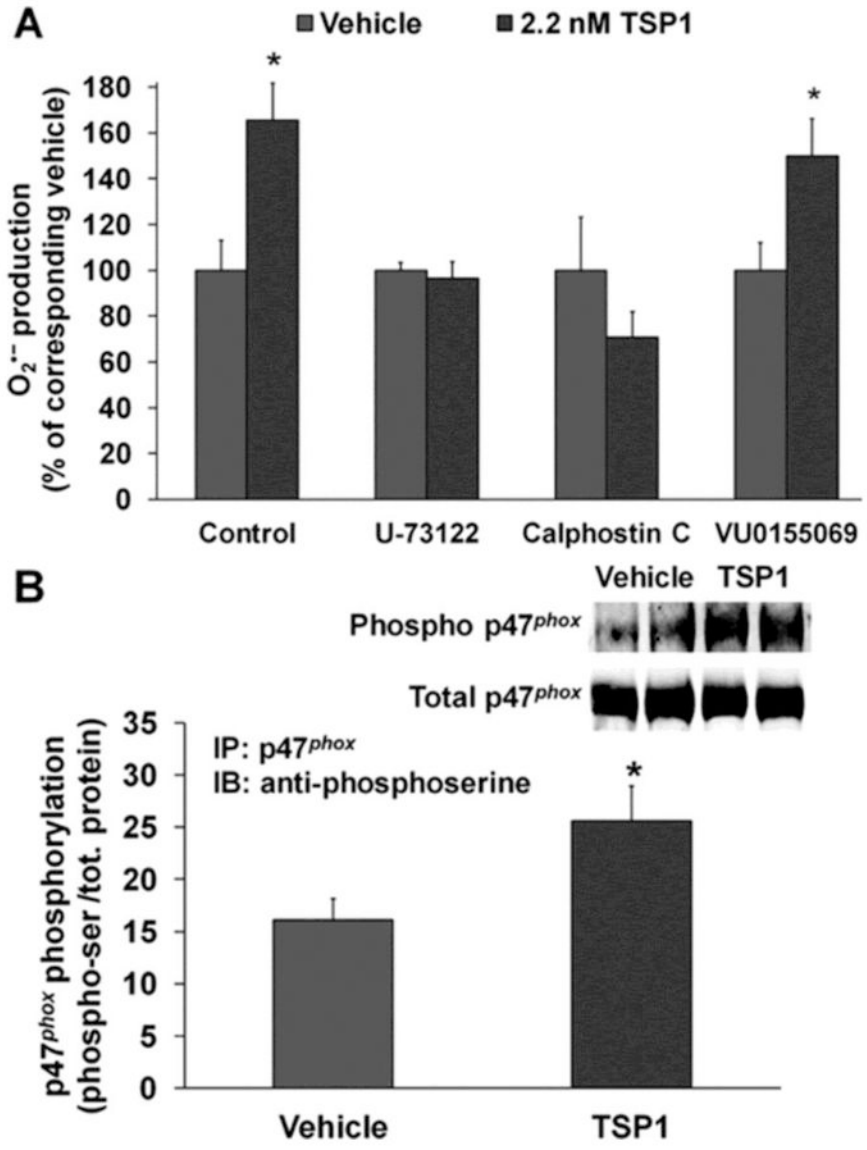

Figure 4.

TSP1 targets Nox subunit assembly via PKC activation and p47*phox* phosphorylation. **A)** VSMCs were incubated with vehicle (control), the PLC inhibitor U-73122 (1 μM, 1 min), the PKC inhibitor calphostin C (1 μM, 30 min) or the PLD inhibitor VU0155069C (10 μM, 30 min), and treated with vehicle or TSP1 (2.2 nM, 60 min) (n=5-6). Superoxide production was measured using cytochrome *c*. **B)** Cells were incubated with vehicle or TSP1 (2.2 nM, 60 min). Precipitated p47*phox* was probed with an anti-phosphoserine antibody (n=6). Data represent the means ± SEM. **p* < 0.05 indicates significant difference between vehicle- and TSP1-treatment.

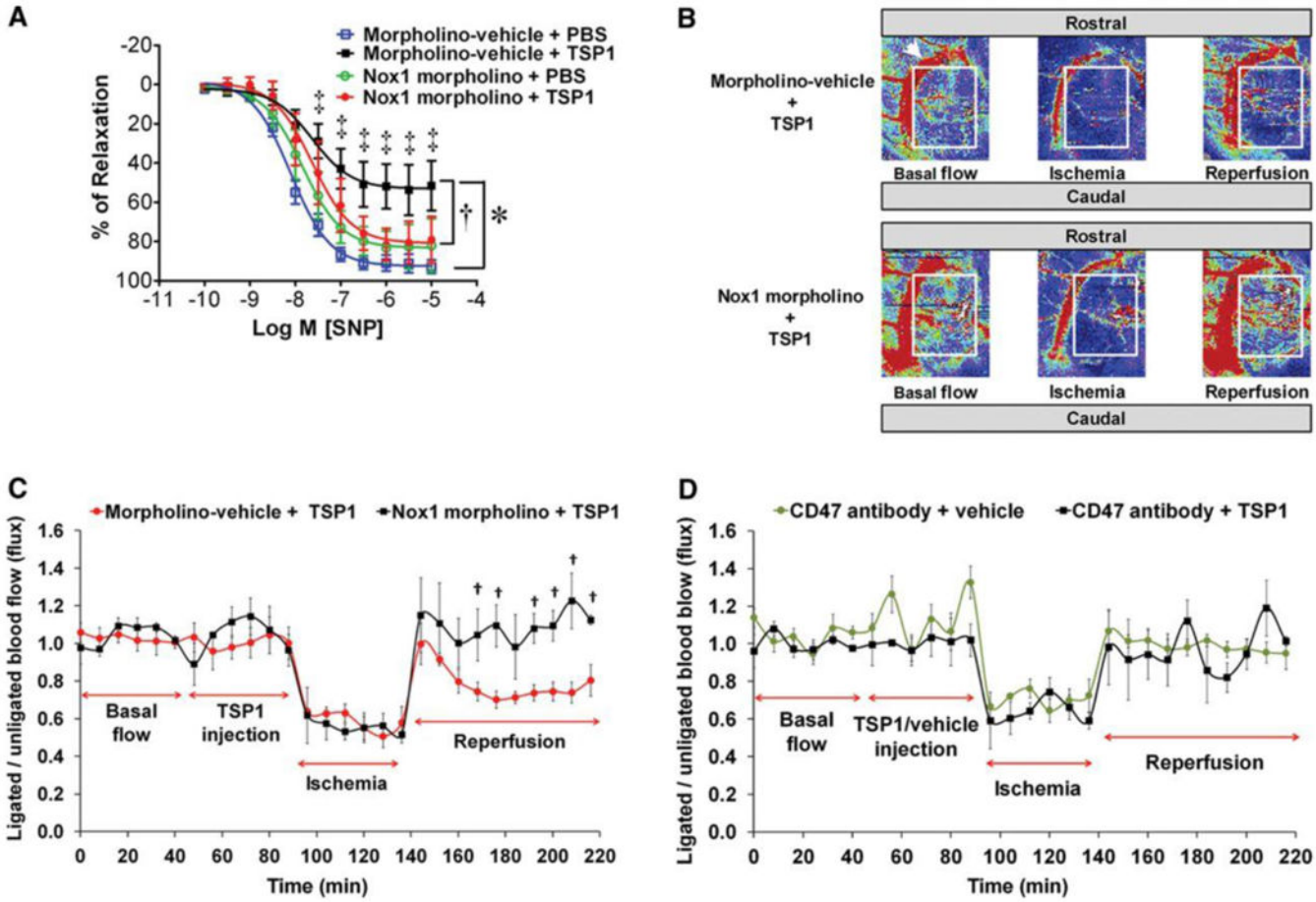

Figure 5.

TSP1 activates arterial Nox1 to inhibit vasodilatation and IRI-blood flow. **A)** Endothelium-denuded thoracic aortas from control (Morpholino-vehicle) and Nox1 vivo-morpholino-treated (Nox1 morpholino) rats were preincubated with vehicle (PBS) or TSP1 (2.2 nM, 60 min) and preconstricted with phenylephrine (PE, 3×10^{-7} M). Endothelium-independent vasorelaxation was stimulated by the NO donor SNP (10^{-10} to 10^{-5} M) (n=4). **B)** Laser Doppler analysis of hindlimb blood flow during ischemia (45 min) and subsequent reperfusion (80 min) was performed in control (Morpholino-vehicle) and Nox1 vivo-morpholino-treated rats. Fifty min prior to ischemia, rats were injected via the tail vein with TSP1 (60 μg/kg body weight). Representative color Doppler images of the ligated hindlimbs at baseline, following 20 minutes of ischemia and 40 minutes of reperfusion are presented. Red coloration of laser Doppler images indicates maximum and blue coloration minimum blood flow. Regions of interest (ROI) selected for blood flow analysis are defined by white-outlined boxes. White arrow indicates femoral artery. **C)** Changes in hindlimb perfusion at indicated time points are presented as the flux ratio between the ischemic and non-ischemic limbs (n=3-4). **D)** Ninety minutes before ischemia, the animals were treated with a CD47 monoclonal antibody (clone OX101; 0.4 μg/g body weight) via a single intraperitoneal injection. Laser Doppler analysis of hindlimb blood flow during ischemia (45 min) and subsequent reperfusion (80 min) was performed in vehicle- and TSP1-treated rats. Data

represent the means ± SEM. *$p < 0.05$ indicates significant difference in relaxation between Morpholino-vehicle+PBS and Morpholino-vehicle+TSP1. ‡$p < 0.05$ indicates significant differences at individual concentrations between Morpholino-vehicle+PBS and Morpholino-vehicle+TSP1. †$p < 0.05$ indicates significant difference between Morpholino-vehicle+TSP1 and Nox1 morpholino+TSP1.