**Thrombospondin-1 Activation of Signal-Regulatory Protein-α Stimulates Reactive Oxygen Species Production and Promotes Renal Ischemia Reperfusion Injury**

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**ABSTRACT**

Ischemia reperfusion injury (IRI) causes tissue and organ injury, in part, through alterations in tissue blood flow and the production of reactive oxygen species. The cell surface receptor signal-regulatory protein-α (SIRP-α) is expressed on inflammatory cells and suppresses phagocytosis, but the function of SIRP-α in IRI has not been determined. We reported previously that the matricellular protein thrombospondin-1 is upregulated in IRI. Here, we report a novel interaction between thrombospondin-1 and SIRP-α on nonphagocytic cells. In cell-free experiments, thrombospondin-1 bound SIRP-α. In vascular smooth muscle cells and renal tubular epithelial cells, treatment with thrombospondin-1 led to phosphorylation of SIRP-α and downstream activation of Src homology domain 2–containing phosphatase-1. Thrombospondin-1 also stimulated phosphorylation of p47phox (an organizer subunit for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1/2) and increased production of superoxide, both of which were abrogated by knockdown or antibody blockade of SIRP-α. In rodent aortic rings, treatment with thrombospondin-1 increased the production of superoxide and inhibited nitric oxide–mediated vasodilation in a SIRP-α–dependent manner. Renal IRI upregulated the thrombospondin-1–SIRP-α signaling axis and was associated with increased superoxide production and cell death. A SIRP-α antibody that blocks thrombospondin-1 activation of SIRP-α mitigated the effects of renal IRI, increasing blood flow, suppressing production of reactive oxygen species, and preserving cellular architecture. A role for CD47 in SIRP-α activation in these pathways is also described. Overall, these results suggest that thrombospondin-1 binding to SIRP-α on nonphagocytic cells activates NADPH oxidase, limits vasodilation, and promotes renal IRI.


Thrombospondin-1 (TSP1) is a secreted matricellular protein produced by platelets, endothelial and vascular smooth muscle cells (VSMCs), and nonvascular cells. TSP1 transduces signals from the extracellular to cellular components of tissues through binding to cell surface receptors, including the integrins, CD36 and CD47. We and others have shown that TSP1 levels are increased in plasma and in conditions associated with decreased blood flow, such as ischemia reperfusion injury (IRI), atherosclerosis, pulmonary hypertension, and sickle cell anemia.

Signal regulatory protein-α (SIRP-α) is a cell surface receptor expressed on phagocytic and neuronal cells and activated through interactions with
the cell surface protein CD47, by growth factors or integrin signaling.\textsuperscript{8,9} SIRP-\(\alpha\) controls cell responses through the recruitment and phosphorylation of Src homology domain 2–containing phosphatase-1 (SHP1) and -2 (SHP2).\textsuperscript{10} SIRP-\(\alpha\) is classified as an inhibitory cell receptor, and SIRP-\(\alpha\)-mediated signaling suppresses macrophage phagocytosis.\textsuperscript{11} However, little is known about the role of SIRP-\(\alpha\) in vascular cells and IRI.

Loss of nitric oxide (NO) signaling, including decreased NO bioavailability, is a major contributor to cardiovascular disease.\textsuperscript{12} NO reacts rapidly with the reactive oxygen species (ROS) superoxide anion (\(\text{O}_2^{-}\)) which dramatically limits its biologic effect.\textsuperscript{13} This interaction becomes important after ischemia reperfusion, where pathologic ROS production, including \(\text{O}_2^{-}\), is increased. We have shown that TSP1 inhibits NO signaling\textsuperscript{6} and limits blood flow,\textsuperscript{14–16} but the exact mechanisms are still unclear.

Our data demonstrate that TSP1 stimulates phosphorylation of nonphagocytic SIRP-\(\alpha\) and stimulates NADPH oxidase (Nox)–mediated \(\text{O}_2^{-}\) production and that SIRP-\(\alpha\) phosphorylation is absent upon CD47 deletion. In arteries, TSP1 inhibits NO-mediated vasodilation through SIRP-\(\alpha\)–dependent stimulation of ROS. IRI upregulates renal TSP1–SIRP-\(\alpha\) signaling, increases pathologic ROS production, and promotes cell death. Disruption of TSP1–SIRP-\(\alpha\) signaling inhibits \(\text{O}_2^{-}\) production, promotes vasodilation, improves blood flow, and limits IRI.

RESULTS

TSP1 Engages and Phosphorylates Nonphagocytic SIRP-\(\alpha\)

TSP1 can interact with several cell surface receptors,\textsuperscript{1} including CD47, as we have reported.\textsuperscript{17} However, it is not known whether TSP1 can interact with or signal through SIRP-\(\alpha\). In arterial VSMC lysates, SIRP-\(\alpha\) was coprecipitated by a TSP1 monoclonal antibody and, conversely, TSP1 was coprecipitated by a SIRP-\(\alpha\) monoclonal antibody (Figure 1A). An isotype-matched control IgG antibody did not coprecipitate SIRP-\(\alpha\) (Figure 1A) or TSP1. In cell-free preparations, low concentrations of immobilized TSP1 bound soluble human SIRP-\(\alpha\) (Figure 1B). In contrast, the signature domain of TSP1 (E123CaG1), which contains the C-terminal of TSP1 and binds CD47,\textsuperscript{17} did not bind to SIRP-\(\alpha\) (Figure 1C). Extending these observations to cell culture systems, where endogenous TSP1 production was minimized by restricting serum and growth factors, we treated arterial VSMCs with exogenous TSP1 (2.2 nmol/L) and assessed SIRP-\(\alpha\) phosphorylation. TSP1 phosphorylated SIRP-\(\alpha\) within 10 minutes, and this persisted for at least 60 minutes (Figure 1D). Because these experiments used a general phospho-tyrosine antibody, we confirmed our results by immunoprecipitating for SIRP-\(\alpha\) and then probing for changes in tyrosine phosphorylation (Figure 1E). Finally, TSP1 treatment under these conditions did not alter total SIRP-\(\alpha\) protein levels (Figure 1D, densitometry presented).

TSP1 Activates a Downstream Target of SIRP-\(\alpha\)

Upon phosphorylation, SIRP-\(\alpha\) activates the Src homology-2 (SH2) domain containing protein phosphatases SHP1 and/or SHP2.\textsuperscript{18} We tested whether TSP1 activates these downstream signal transducers in smooth muscle cells. Arterial VSMCs preincubated under growth factor-free and serum-free conditions (for 24 hours) and treated with TSP1 (2.2 nmol/L) displayed phosphorylation of SHP1 in a temporal fashion similar to that of SIRP-\(\alpha\) (Figure 1F). Treatment of VSMCs with TSP1 did not result in SHP2 phosphorylation (Figure 1G) and did not alter total SHP1 or SHP2 protein levels within the time course of the experiment (Figure 1, F and G, densitometry presented).

Treatment of VSMCs with the NO Donor Sodium Nitroprusside Does Not Stimulate Phosphorylation of SIRP-\(\alpha\) or SHP1

The NO donor S-nitroso-N-acetylpenicillamine was reported to activate SHP1,\textsuperscript{19} and our laboratory reported that TSP1 regulates NO signaling in vascular cells.\textsuperscript{20–22} We were interested in exploring what role exogenous NO could play in our studies. We tested this in VSMCs by determining SIRP-\(\alpha\) phosphorylation in the presence of sodium nitroprusside (SNP), a prodrug that is metabolized by VSMCs to NO.\textsuperscript{23,24} Once again, TSP1 phosphorylated SIRP-\(\alpha\) and SHP1, whereas treatment with SNP had no effect on SIRP-\(\alpha\) or SHP1 phosphorylation (Supplemental Figure 1, A and B).

TSP1-Stimulated Phosphorylation of VSMC SIRP-\(\alpha\) May Not Completely Depend on CD47 Activation

CD47 and SIRP-\(\alpha\) are expressed on vascular cells,\textsuperscript{25} and SIRP-\(\alpha\) activation requires CD47 in phagocytic cells.\textsuperscript{25,26} This conclusion is based, in part, on studies using a TSP1-derived peptide (peptide 4N1K, KRFFYVMMWKK) that was reported to interact specifically with CD47.\textsuperscript{27} However, the validity of this contention is not clear because peptide 4N1K has effects on CD47 null cells.\textsuperscript{28} Nevertheless, to test whether CD47 is required for TSP1 activation of SIRP-\(\alpha\), we first treated cells with a CD47-targeting morpholino oligonucleotide to block mRNA translation.\textsuperscript{29} Western blot confirmed suppression of CD47 protein (by 37%) (Figure 2A). A control nonspecific morpholino (scrambled) did not alter total CD47 expression in treated cells (Figure 2A), and neither the CD47 targeting nor the control morpholinos significantly changed total SIRP-\(\alpha\) expression (Figure 2B, densitometry presented). TSP1 in CD47 gene-suppressed cells still phosphorylated SIRP-\(\alpha\) (Figure 2C). In further experiments, we used a CD47 monoclonal antibody (Ab) that blocks TSP1 binding to CD47,\textsuperscript{15–17, 30} as we reported, and also blocks CD47 interaction with SIRP-\(\alpha\).\textsuperscript{31} Treating VSMCs with CD47-blocking Ab had no effect on basal or TSP1-mediated SIRP-\(\alpha\) phosphorylation (Figure 2D) and did not alter total SIRP-\(\alpha\) protein levels (Figure 2E, densitometry presented). These data, although interesting,
Figure 1. TSP1 binds to and activates nonphagocytic SIRP-α and its downstream signal transducer SHP1. (A) Coimmunoprecipitation in arterial VSMCs of TSP1 and SIRP-α. Immunoprecipitation was with monoclonal Ab to TSP1, SIRP-α, or an isotype-matched control IgG antibody. Results presented are representative of six separate experiments. Plastic wells coated with TSP1 (B) or the recombinant domain of TSP1 containing the C-terminal (E123CaG1, C) at the indicated concentrations were incubated with 125I-SIRP-α at room temperature. Bound radioactivity was quantified and data are presented as the mean ± SEM. of three separate experiments. VSMCs were incubated in basal medium with TSP1 (2.2 nmol/L) for the indicated time points, cell lysate prepared, protein separated by SDS-PAGE electrophoresis, membranes probed with a phospho-tyrosine antibody (D) or lysates immunoprecipitated with a SIRP-α Ab, protein separated via SDS-PAGE electrophoresis, and nitrocellulose membranes probed with Ab to total SIRP-α and to phosphorylated tyrosine residues (E), as well as phosphorylated SHP1 (Y536) (F) and p-SHP2 (G). Densitometry is presented as mean ratios of p-tyr-SIRP-α to total SIRP-α and total SIRP-α to β-actin (± SEM) (D), p-SHP1 to total SHP1, p-SHP1 to β actin, and total SHP1 to β actin (± SEM) (F), and p-SHP2 to total SHP2 and total SHP2 to β actin (± SEM) (G). Representative data from four independent experiments are presented. *Statistically significant difference (P<0.05 compared with untreated).
cannot exclude a possible role for CD47 in TSP1-mediated phosphorylation of SIRP-α in VSMCs. That is, there is the possibility of cross-talk, especially in light of CD47 null data in rTEC (vide infra).

**TSP1 Activation of SIRP-α Does Not Require β Integrins**

TSP1 has several functional domains, and we reported that TSP1, via its C-terminal domain, binds CD47.17 To test whether β integrin could be mediating SIRP-α phosphorylation, we used the TSP1-based peptide 753 (M.W. 685, sequence-acAELDVP) that is derived from the N-terminal domain of the protein and is known to activate integrins.32 Treating VSMCs with peptide 753 (10 μmol/L, 10 minutes) phosphorylated SIRP-α (Supplemental Figure 2A) without changing total SIRP-α protein levels (Supplemental Figure 2C, densitometry presented). The N-terminal domain of TSP1 is known to engage β integrins.32 To explore the role of β integrins in this process, we treated VSMCs with a β integrin–blocking Ab (clone Ha2/5, BD Biosciences) followed by TSP1 (2.2 nmol/L), and determined SIRP-α phosphorylation. Interestingly, TSP1-stimulated phosphorylation of SIRP-α was not altered by β integrin Ab blockade (Supplemental Figure 2B). Under basal conditions, treatment of VSMCs with the β integrin Ab alone did not increase SIRP-α phosphorylation and had no effect on total SIRP-α protein levels (Supplemental Figure 2C, densitometry presented). Peptide 753–mediated activation of SIRP-α was also not inhibited with the β integrin Ab blockade (Supplemental Figure 2B). In contrast, a monoclonal SIRP-α Ab blocked TSP1-mediated phosphorylation of both SIRP-α (data not shown) and the downstream signal transducer SHP-1 (Supplemental Figure 2D).

**TSP1-Stimulated O₂⁻⁻ Production in VSMCs Requires SIRP-α**

We have reported concurrent upregulation of TSP1 and ROS in ischemia33 and IRI.3,15 To determine whether TSP1 is capable of stimulating O₂⁻⁻ production in VSMC via activation of SIRP-α, we used a specific small interfering RNA (siRNA) (oligo 3) to suppress SIRP-α, then treated cells with TSP1 (2.2 nmol/L for 60 minutes).
and determined $O_2^-$ levels via a cytochrome c reduction assay. TSP1 significantly stimulated VSMC $O_2^-$ production (Figure 3A). Gene suppression of SIRP-$\alpha$ using siRNA abrogated TSP1-stimulated $O_2^-$ generation (Figure 3B). Conversely, a control siRNA (scrmb siRNA) had no effect on TSP1-stimulated $O_2^-$ production (Figure 3B). Western blot analysis confirmed a reduction in SIRP-$\alpha$ expression by the targeting siRNA (40%) (Figure 3C). Treatment of VSMCs with the SIRP-$\alpha$ siRNA (oligo 3) did not change CD47 protein expression (Figure 3D). Corroborating these data, TSP1-stimulated $O_2^-$ production was inhibited by more than half in cells treated with a SIRP-$\alpha$ Ab (Figure 3E).

**TSP1, via SIRP-$\alpha$, Phosphorylates the Nox1/2 Organizer Subunit p47phox**

A major NADPH oxidase family member found in rodent large vessel arterial VSMCs is Nox1. Activation of Nox1 (and Nox2) and subsequent enzymatic $O_2^-$ formation occur on assembly of several subunit proteins, including p47phox (reviewed by Lassègue and Griendling). This latter process requires serine phosphorylation of p47phox by Nox1 (Figure 4B), suggesting that SIRP-$\alpha$ is upstream of TSP1-mediated phosphorylation of the Nox subunit.

**TSP1–SIRP-$\alpha$ Signaling Stimulates VSMC $O_2^-$ Production and Inhibits Vasodilation**

TSP1 is a large, 480-kDa protein found in the blood of healthy persons at 100-picomolar concentrations and at low nanomolar concentrations in disease. It is unknown whether TSP1 can cross the subendothelial barrier to gain access to the arterial VSMC compartment. To test this, aortic endothelium-intact arterial rings were incubated with TSP1 (2.2 nmol/L) for 20 minutes and tissue sections cut from the middle of the vessels prepared. TSP1-treated arterial rings showed increased TSP1 on both luminal endothelial cells and throughout the subendothelial VSMC compartment (Figure 5A). Gene suppression of SIRP-$\alpha$ using siRNA abrogated TSP1-stimulated phosphorylation of p47phox (Figure 4B), suggesting that TSP1 inhibits NO-meditated vasodilation.

**TSP1 Stimulates Phosphorylation of SIRP-$\alpha$ and $O_2^-$ Production in Human Renal Tubular Endothelial Cells**

Renal tubular epithelial cells (rTECs) express Noxs, in parenchymal cells, we treated human rTECs with TSP1. Human rTECs expressed SIRP-$\alpha$ and SHP1 (Figure 6A). TSP1 (2.2 nmol/L) treatment promoted rapid phosphorylation of SIRP-$\alpha$ and SHP1 (Figure 6C) concurrent with stimulating increased $O_2^-$ production (Figure 6D). On the other hand, TSP1-stimulated phosphorylation of SIRP-$\alpha$ and SHP1 was blocked in rTECs treated with a SIRP-$\alpha$ Ab (Figure 6C). In these experiments a general phospho-tyrosine antibody was again employed. Therefore, we confirmed these results by immunoprecipitatiing for SIRP-$\alpha$ and probing for changes in tyrosine phosphorylation. Here, too, TSP1 treatment increased p-SIRP-$\alpha$ expression in human rTECs (Figure 6E). Having previously reported rTEC express CD47, we tested whether CD47 is required for TSP1-mediated SIRP-$\alpha$ phosphorylation in rTECs. Wild-type (CD47+/+) and null (CD47−/−) rTECs obtained from kidneys of the respective strains of mice were pre-treated with a SIRP-$\alpha$ antagonist Ab (1 $\mu$g/ml) for 15 minutes followed by exogenous TSP1 (2.2 nmol/L) for 60 minutes. TSP1 treatment stimulated SIRP-$\alpha$ phosphorylation in wild-type rTECs (Figure 6F), which was inhibited by SIRP-$\alpha$ Ab treatment. However, 2.2 nmol/L TSP1 treatment did not increase p-SIRP-$\alpha$ levels in CD47 null rTECs (Figure 6F). Total SIRP-$\alpha$ and SHP1 protein levels did not vary between wild-type and CD47 null rTEC or treatment groups (data not shown). rTEC undergo apoptotic cell death post IRI and a TSP1-derived peptide is known to promote endothelial cell death. We tested whether TSP1, via SIRP-$\alpha$, promoted rTEC apoptosis. Cells were treated with TSP1 (2.2 nmol/L) for 24 hours, and apoptosis and cell viability were assessed. Under these conditions TSP1 did not stimulate apoptosis or decrease cell viability (Supplemental Figure 3).

**TSP1–SIRP-$\alpha$ Signaling Is Upregulated in Renal IRI Concurrent with Increased ROS Production, Decreased Blood Flow, and Increased Tissue Injury**

IRI is a major cause of cellular and organ dysfunction and, in transplantation, of graft failure. Pathologic ROS contribution significantly to this process, and ROS mitigation is a therapeutic goal for limiting tissue injury in several
Figure 3. TSP1-stimulated O$_2^-$ production in VSMCs requires SIRP-$\alpha$. (A) VSMCs were incubated with vehicle or TSP1 (2.2 nmol/L) for 60 minutes. Superoxide production was measured in the 28,000g membrane fraction and calculated from the initial linear rate of superoxide dismutase–inhibitable cytochrome $c$ reduction. Data represent the rate of superoxide production ($\pm$SEM). *Statistically significant difference ($P<0.05$) compared with untreated. (B) VSMCs were transfected with SIRP-$\alpha$ siRNA (oligo 3) or a control scrambled (scrbm) siRNA and incubated with TSP1 (2.2 nmol/L) for 60 minutes. Superoxide production was measured as described above. Data represent the rate of superoxide production ($\pm$SEM). *Statistically significant difference ($P<0.05$) compared with scrbm; $^\#$statistically significant difference ($P<0.05$) compared with scrbm siRNA+TSP1. (C) VSMCs were transfected with several SIRP-$\alpha$ or scrambled (scrbm) siRNA oligos. Western blot analysis was performed for SIRP-$\alpha$. A representative blot from three separate experiments is presented. Densitometry is presented as the mean ratio of total SIRP-$\alpha$ to $\beta$-actin ($\pm$SEM). *Statistically significant difference ($P<0.05$) compared with scrbm control. (D) VSMCs were transfected with SIRP-$\alpha$ (oligo 3) or control scrambled siRNA. Western blot analysis was performed for CD47. Densitometry is presented as the mean ratio of total CD47 to $\beta$-actin ($\pm$SEM) from three experiments. (E) VSMCs were treated with a SIRP-$\alpha$–blocking Ab (1 $\mu$g/ml) for 10 minutes, followed by TSP1 (2.2 nmol/L) for 60 minutes. Superoxide production was measured. Data represent the rate of superoxide production ($\pm$SEM). *Statistically significant difference ($P<0.05$) compared with untreated control; $^\#$statistically significant difference ($P<0.05$) compared with TSP1 treated.
mRNA levels were decreased post IRI in wild-type kidneys compared with sham controls (Figure 7E).

**Disrupting TSP1–SIRP-α Signaling Suppresses Nox1 Transcription and Oxidative Stress, Increases Reperfusion Blood Flow, and Limits Renal IRI Injury**

To further assess the role of the TSP1–SIRP-α signaling cascade in promoting renal IRI, we treated mice with the SIRP-α–blocking Ab that we have shown herein prevents TSP1-stimulated phosphorylation of SIRP-α and inhibits subsequent O$_2^-$ production. Following IRI, SIRP-α Ab treated mice demonstrated restoration of kidney blood flow to near preschismic levels after 24 hours (Figure 8A), concurrent with decreased p-SIRP-α, p-SHP1, total SIRP-α, Nox1 and 3-nitrotyrosine protein expression (protein and/or densitometry shown) and decreased TSP1, SIRP-α and Nox1 mRNA (Figure 8B). Nox2 protein levels were unchanged in both treatment groups. In IRI-challenged animals, treatment with a SIRP-α Ab decreased oxidative stress as quantified by DHE and 4-HNE immunofluorescence (Figure 8C), concordant with less renal tubular injury and neutrophil infiltration (Figure 8D), decreased proinflammatory cytokine and chemokine transcript expression (CCL2, CXCL2, IL1-β, TNF-α) (Figure 8E), and less cell death (as detected by TUNEL staining with results in kidneys from SIRP-α Ab–treated animals [Figure 8F] approaching levels found in sham-operated kidneys [Figure 7E]). From a functional perspective, serum urea and creatinine levels were lower after IRI in animals treated with the SIRP-α Ab compared with control IgG Ab–treated animals (Figure 8G). Previously we reported that CD47 null mice were protected from renal IRI. Therefore, we treated CD47 null mice with the SIRP-α antagonist Ab and challenged them with renal IRI. Interestingly, we found no further improvement in serum urea and creatinine levels in SIRP-α Ab–treated CD47 null mice after IRI compared with untreated CD47 null animals (Supplemental Figure 4).

**DISCUSSION**

In a myeloid leukemia cell line, SIRP-α inhibits expression of the Nox2 subunit and is associated with decreased hydrogen peroxide production. However, nonphagocytic SIRP-α has not previously been linked directly to pathologic ROS production. Our present results implicate a role for SIRP-α in the stimulation of Nox-mediated O$_2^-$ production in multiple nonphagocytic cell types. The data indicate that cells treated with a physiologically relevant concentration of TSP1 demonstrate a SIRP-α–dependent increase in O$_2^-$ production. Either siRNA knockdown or antibody blockade of SIRP-α inhibited both TSP1-mediated phosphorylation of the Nox organizer subunit p47$^{phox}$ and O$_2^-$ production without changing basal ROS production, consistent with SIRP-α playing an active role in stimulating O$_2^-$ production.
TSP1 (2.2 nmol/L, 60 minutes), or TSP1+a SIRP-

murine thoracic aortas. The endothelium was removed and the resulting arterial rings were incubated with vehicle, TSP1 (2.2 nmol/L, 60 minutes), or TSP1+Tempol (30 μM) and constricted with phenylephrine (3×10⁻⁷ M). Endothelium-independent vasodilation was stimulated by SNP (10⁻⁹ to 10⁻⁵ M). Data represent the means±SEM of four experiments. *Statistically significant difference (P<0.05) in relaxation between TSP1 versus TSP1 + Tempol.

Figure 5. TSP1, via SIRP-α, stimulates VSMC O₂⁻⁻ production and inhibits vasodilation. (A) Murine aortic segments were freshly harvested from wild-type male C57BL/6 mice, incubated in standard myograph buffer with TSP1 (2.2 nmol/L, 60 minutes), and tissue sections prepared for immunofluorescence. Representative images of three independent experiments are presented. TSP1 appears red. The red arrows highlight the vessel lumen. Murine (B) and rat (C) aortic rings were prepared from freshly harvested thoracic aortas. The endothelium was removed and the resulting arterial rings were incubated with vehicle, TSP1 (2.2 nmol/L, 60 minutes), or TSP1+Tempol (30 μM) and constricted with phenylephrine (3×10⁻⁷ M). Endothelium-independent vasodilation was stimulated by SNP (10⁻⁹ to 10⁻⁵ M). Data represent the means±SEM of four separate samples. (E) Murine thoracic aortas were harvested and endothelial-free arterial rings were incubated with vehicle, TSP1 (2.2 nmol/L, 60 minutes), or TSP1+a SIRP-α–blocking Ab (1 μg/ml) and constricted with phenylephrine (3×10⁻⁷ M). Endothelium-independent vasodilation was stimulated by SNP (10⁻⁹ to 10⁻⁵ M). Data represent the means±SEM of four experiments. *Statistically significant difference (P<0.05) in relaxation between TSP1 versus TSP1+SIRP-α Ab.

Signaling through SIRP-α is reported to occur via interactions with integrins, growth factors and CD47. In myeloid cells, SIRP-α phosphorylation has been demonstrated in the absence of CD47. Our data indicate that in nonmyeloid cells, TSP1 stimulates the phosphorylation of SIRP-α and its downstream effector SHP1 through a process that may depend on interaction with CD47. Gene suppression of CD47 by 37% and CD47 monoclonal Ab blockade did not prevent TSP1-mediated activation of SIRP-α in VSMCs. It is still possible that a residual amount of CD47 in gene-silenced cells is still sufficient to mediate TSP1-induced SIRP-α phosphorylation. Moreover, the pharmacologic properties of the employed CD47 Ab has to be more fully characterized using protein binding experiments to determine whether the Ab inhibits TSP1–SIRP-α binding or the Ab modifies CD47–SIRP-α interaction. Interestingly, treatment of murine CD47 null rTEC with 2.2 nmol/L TSP1 for 60 minutes did not stimulate SIRP-α phosphorylation. Also, CD47 null mice treated with a SIRP-α antibody did not exhibit further improvement in serum creatinine after renal IRI compared with isotype control-treated animals. Together, these data suggest an interaction between TSP1 and SIRP-α, and they also suggest an important role of CD47. Indeed, our previous studies showed that TSP1 activates Nox1-dependent ROS production in a CD47-dependent manner. In conjunction with the current data, it would appear that TSP1 could mediate its ROS-inducing effect via several mechanisms, including (1) parallel activation of CD47 and SIRP-α with these two pathways exhibiting cross-talk or (2) activation of CD47 followed by downstream activation of SIRP-α. Future studies are required to further delineate the precise molecular mechanisms by which TSP1 stimulates ROS generation.

In human umbilical vein endothelial cells, angiotensin II–stimulated ROS production was associated with increased SHP1 activity. We now find in two distinct nonphagocytic cell types, VSMCs and rTECs, that TSP1 treatment stimulates SIRP-α and downstream SHP1 and increased O₂⁻⁻ production. VSMCs and rTECs, depending on the vascular bed, express both Nox1 and Nox2, and the assembly of both of these enzyme complexes is promoted by the serine phosphorylation of the p47phox subunit, whereas a SIRP-α Ab blocked TSP1-mediated phosphorylation of this essential Nox organizing subunit, and both the SIRP-α–blocking Ab and
SIRP-α siRNA abrogated TSP1-stimulated $O_2^{\cdot-}$ production. These data identify SIRP-α as a new, physiologically important activator of Nox-derived $O_2^{\cdot-}$ production in vascular and epithelial cells. Further work will define the dominant Nox isoform responsible for TSP1–SIRP-α–induced ROS following renal IRI.
We have reported that TSP1 limits arterial vasodilation via inhibition of NO signaling. Results from our present study suggest that TSP1 inhibits arterial vasodilation, in part by stimulating pathologic $\text{O}_2^{-\text{aq}}$ production. The $\text{O}_2^{-\text{aq}}$ scavenger Tempol decreased TSP1-mediated inhibition of NO-driven vasodilation in murine and rat arteries, supporting a role for TSP1 in promoting arterial $\text{O}_2^{-\text{aq}}$ production. A SIRP-α Ab reversed TSP1-mediated inhibition of vasodilation, placing SIRP-α upstream in this process. These data obtained using whole arteries are unlikely to be merely the result of TSP1-mediated inhibition of NO bioavailability because before analysis, vessels were rendered endothelial free and thus lacked an endogenous source of NO. Importantly, these data are, to our knowledge, the first to characterize SIRP-α as an acute regulator of arterial vasodilation.

IRI is characterized by defects in tissue blood flow, increased thrombosis, pathologic ROS production, and cell death. We herein identify SIRP-α as a new receptor for the matricellular protein TSP1. These results are also the first to identify a soluble ligand of SIRP-α. TSP1, on engaging SIRP-α in nonphagocytic cells, stimulates NADPH oxidase–derived $\text{O}_2^{-\text{aq}}$ production. Contrary to previous work (reviewed by Barclay et al and Matozaki et al), we identify SIRP-α as an activating, rather than inhibitory, receptor in two nonphagocytic cell types, specifically VSMCs and rTECs. In vivo, TSP1, SIRP-α, and SHP1 were induced by and promoted IRI. Consistent with this notion, blocking TSP1–SIRP-α signaling with a SIRP-α monoclonal Ab inhibited IRI-mediated expression of these proteins, lowered $\text{O}_2^{-\text{aq}}$ production in cells and tissues, restored NO-mediated vasodilation (in ex vivo vessel preparations), augmented reperfusion blood flow, preserved end-organ integrity, and improved renal function after IRI. Consistent with our previous findings, CD47 is likely to play a parallel and supportive role in this process. Which of the two pathways predominates is still open to question.

CONCISE METHODS

Detailed methods are available are available in the Supplemental Material.

Reagents and Cells

Human rTECs and rat aortic VSMCs were purchased from Lonza (Switzerland) and maintained in recommended medium. Cells were used between passages 3 and 7. TSP1 was purchased from Athens Research & Technology (Athens, GA). CD47 morpholino oligonucleotides and corresponding mismatched control morpholino were purchased from GeneTools, Inc. (Philmonth, Oregon). The TSP1-derived peptide 753 was synthesized by Dr. Henry C. Krutzsch and kindly provided by Dr. David D. Roberts (National Cancer Institute, National Institutes of Health, Bethesda, MD). The SIRP-α monoclonal Ab (clones C20 for treatment applications and A1 for Western blot) and monoclonal CD47 Ab (clone OX101) were purchased from Santa Cruz Biotechnology, and the β integrin–blocking Ab (clone Ha2/5) was purchased from BD Biosciences.

Animals

Male C57BL/6 wild-type mice and Sprague–Dawley rats were obtained from The Jackson Laboratory (Bar Harbor, ME) and Taconic (Hudson, NY), respectively. All studies were performed using protocols approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and in accordance with National Institutes of Health guidelines.

Murine rTEC Cultures

Kidneys from age-matched male wild-type and CD47 null mice were excised and digested, and primary rTEC were harvested and cultured as previously described with several minor modifications.

Protein-Binding Assays

Recombinant SIRP-α was labeled using NaI by the iodogen method as we previously published. Immulon 2HB Removawells (Thermo, Franklin, MA) were coated with 50 μl of TSP1 at several concentrations for 16–20 hours at 4°C. Nonspecific binding was blocked by incubating the wells with DPBS with Ca2+ and Mg2+ and 1% BSA. Radiolabeled SIRP-α was then added and incubation conducted for 2 hours at room temperature. Following extensive washing, bound radioactivity was quantified.

Coimmunoprecipitation

Immunoprecipitation was performed as we have described, with minor modification.

Western protein analysis

Tissue or cells were homogenized in ice-cold lysis buffer. Supernatants were collected and lysates quantified using a Bradford assay (Bio-Rad, Hercules, CA). Thirty micrograms of total protein were resolved by SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad). In blots for CD47, nonreducing Laemmli buffer was used with 8% SDS-PAGE. Blots were probed with primary antibody to the respective proteins and visualized on an Odyssey Imaging System (Licol, Lincoln, NE). The intensity of the bands was quantified using ImageJ.

RNA Extraction and Quantification by Real-Time PCR

Total RNA was extracted using Qiagen RNeasy Mini Kits (Qiagen, Hilden, Germany) as per the manufacturer’s instructions. RNA was quantified using the Take3 Gen5 spectrophotometer (BioTek, Winskoosi, VT). One microgram of RNA was treated with DNase I (amplification grade; Invitrogen, Carlsbad, CA) and then reverse-transcribed using the SuperScript III First Strand Synthesis Supermix (Invitrogen). cDNA was amplified using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) in 10-μl volumes in triplicate with gene-specific primers and probed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions.
Figure 7. TSP1-SIRP-α signaling is upregulated in renal IRI concurrent with increased ROS production, decreased blood flow, and increased tissue injury. Male C57BL/6 mice were challenged with 20 minutes of unilateral renal ischemia and 24 hours of reperfusion (n=8) or sham surgery (n=6) and Western blot for (A) TSP1, SIRP-α, p-SIRP-α, p-SHP1, Nox1, Nox2, 3-nitrotyrosine, and β actin was performed. Data shown are mean±SEM. *Statistically significant (P<0.001) IRI compared with sham-operated kidneys. (B) RT-PCR analysis of renal mRNA expression of the indicated genes in IRI and sham-operated kidneys. Data shown are mean±SEM. *Statistically significant (P<0.05) IRI compared with sham. (C) Representative kidney tissue sections from IRI and sham-operated mice stained for TSP1 and SIRP-α. TSP1 colored green; SIRP-α colored red; nuclei colored blue. Scale bar, 50 μm (magnification ×20). (D) Quantitative analysis and photomicrographs of tubular damage and neutrophil invasion in juxtamedullary sections from IRI and sham-operated mice are shown (magnification, ×200; arrow highlights tubular injury); serum urea and creatinine from the same. Data shown are means±SEM. *Statistically significant (P<0.05) IRI compared with sham. (E) Representative tissue sections and quantitative analysis from IRI and sham-operated mice stained with DHE or 4-HNE and visualized by microscopy (magnification, ×200). RT-PCR analysis of Nox1 and Nox2 mRNA from sham and post-IRI kidneys. Data shown are means±SEM. *Statistically significant (P<0.05) IRI compared with sham. (F) Representative tissue sections and quantitative analysis from IRI and sham-operated mice stained by TUNEL assay and visualized by microscopy (magnification, ×200). *Statistically significant (P<0.05) IRI compared with sham.
Assessment of p47\textsuperscript{phox} Phosphorylation

We determined the phosphorylation of p47\textsuperscript{phox} (critical cytosolic subunit of Nox) in p47\textsuperscript{phox}\textsuperscript{-} immunoprecipitates using an antiphosphoserine Ab as described.\textsuperscript{68} Briefly, cells were lysed and protein from the cytosolic fraction incubated with anti-p47\textsuperscript{phox} Ab. Immune complexes were recovered with Protein G Plus-Agarose. Immunoprecipitates were then subjected to Western blotting with a phospho-serine Ab.

Measurement of O$_2$\textsuperscript{−} via Cytochrome c Reduction

Cells were suspended in 200 μl of ice-cold disruption buffer. The suspension was lysed by five freeze/thaw cycles and passed through a 30-gauge needle five times.\textsuperscript{68} The cell lysate was centrifuged at 1000g for 10 minutes at 4°C to remove intact cells, nuclei, and debris. The supernatant was transferred to another Eppendorf tube and was centrifuged at 28,000g for 15 minutes at 4°C to collect the membrane fraction pellet, which was resuspended in 50 μl disruption buffer. Membrane fractions (2 μg/well) were added to cytochrome c–containing oxidase assay buffer. After a 5-minute baseline measurement, NADPH (180 μM) was added to initiate the reaction. O$_2$\textsuperscript{−} was measured as the initial linear rate of superoxide dismutase–inhibitable cytochrome c reduction.\textsuperscript{69}

siRNA Transfection

Cells were plated the day before transfection to achieve 30%–50% confluence in DMEM medium containing 10% fetal calf serum without antibiotics. The siRNA transfections were performed by using Lipofectamine 2000 (Invitrogen) in Opti-MEM according to the manufacturer’s instructions.

Morpholino Oligonucleotide Protein Suppression of CD47

Cells were seeded onto 60-mm$^2$ plates in DMEM medium containing 10% fetal calf serum. CD47 morpholino was transfected with a final concentration of 10 μM using the delivery agent Endoporter (6 μM/ml; Genetools, Philomath, OR) according to protocol instructions.

Tissue Histology and Microscopy

Kidneys embedded in paraffin were sectioned at 3 μm and stained with hematoxylin and eosin by standard methods. Markers of tubular damage (tubular dilatation, cell necrosis, infarction, and cast formation) were scored in “blinded” fashion on randomly selected cortical-medullary fields (magnification, ×200). Light microscopy images were acquired under identical settings using a Zeiss Axiosvert 40CFL microscope and Axiovision software, version 4.8 (Carl Zeiss, Oberkochen, Germany).

Immunofluorescence Staining of Arterial Rings, Human rTECs, and Murine Kidneys

Cryostat sections of vessels were washed with PBS, followed by 0.5% BSA in PBS. Sections were blocked with 2% BSA solution. The slides were incubated at room temperature with primary anti-TSP1 Ab. Slides were washed with BSA solution and incubated with a CY3 goat anti-rabbit secondary Ab in combination with the F-actin dye rhodamine phalloidin. For rTEC, cytospins were generated and fixed with ethanol. Slides were blocked with 5% goat serum and then incubated overnight at 4°C with SIRP-α Ab. Nuclei were stained with Hoechst dye. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope (software version 1.7a). Cryostat sections (5 μm) of mouse kidneys were fixed for 20 minutes with 2% paraformaldehyde. They were then washed, blocked with 2% donkey serum in BSA, incubated with combined primary antibodies for TSP1 and SIRP-α, washed and incubated with secondary antibody. Nuclei were stained with Hoescht dye. After rinses with PBS, sections were coveredslipped with Gelvatol mounting media. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope (software version 2.01).

Cell Apoptosis and Viability Assays

Human rTECs were grown to 80% confluence, treated with TSP1 (2.2 nmol/L) with or without SIRP-α antibody (1 μg/ml) for 24 hours, lysates prepared, proteins then separated by SDS-PAGE, transferred to nitrocellulose membrane and probed for caspase-3. Cell viability was assessed in a 96-well plate using the LIVE/DEAD Viability/Cytotoxicity kit (Molecular Probes). Calcein (0.5 μM) and ethidium homodimer-1 (2 μM) were added to the cells for 30 minutes and fluorescence read using a microplate reader as per manufacturer instructions.

Arterial Myography

Myography of arterial segments was performed as previously published\textsuperscript{5,70} with minor modifications. Animals were anesthetized with pentobarbital (50 mg/kg intraperitoneally). Thoracic aortas of mice and rats were cleared of adherent adipose tissue and excised. The endothelial layer was removed by gently rubbing the luminal side of the vessel along the rough surface of a blunt needle. Arterial segments were mounted on myograph pins (Danish Myo Technology, Atlanta, GA) in 5-ml incubation buffer maintained at 37°C, pH 7.4, gassed with 95% O$_2$ and 5% CO$_2$, and brought to an optimal resting tension. Viability of the vessels was ascertained by a contractile response to potassium chloride. Phenylephrine (Sigma-Aldrich) concentration-response curves (10$^{-9}$ to 10$^{-5}$ M) were generated by measuring contraction plateaus at each concentration. In precontracted vessels, endothelium-independent vasodilation to SNP (Sigma-Aldrich; 10$^{-10}$ to 10$^{-5}$ M) with or without the indicated treatments was then tested.

Detection of ROS in Renal Tissue Sections

ROS in renal tissue sections was assessed using several techniques. The cell-permeable agent dihydroethidium was applied to unfixed frozen sections that were incubated in a light-protected humidified chamber at 37°C for 30 minutes, washed with PBS, and mounted with fluorescence mounting medium. Cryosections of kidneys were washed with PBS, followed by 0.5% BSA in PBS. Sections were blocked with 2% BSA solution. They were incubated at room temperature with primary antibody for 4-HNE. Slides were incubated with CY3 goat anti-rabbit secondary antibody (Jackson ImmunoResearch) in combination with F-actin dye rhodamine phalloidin. Nuclei were stained with Hoescht dye. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope (software version 1.7a).
Disrupting TSP1–SIRP-α signaling suppresses Nox1 transcription and oxidative stress, increases reperfusion blood flow, and limits renal IRI injury. Wild-type mice were treated with a SIRP-α–blocking Ab or isotype control (CTRL) Ab and challenged with renal ischemia and 24-hour reperfusion (n=8 per group). (A) Laser Doppler images of renal blood flow. Red indicates blood flow. Results represent the means±SEM of four measurements from five mice in each group. *Statistically significant difference (P<0.001) for SIRP-α compared with isotype control at 30 minutes; **statistically significant difference (P<0.001) for SIRP-α compared with isotype control at 24 hours of reperfusion. (B) Western and RT-PCR analysis was performed for the indicated proteins and genes. Data shown are means±SEM, with representative Western blots.*Statistically significant difference (P<0.001) for SIRP-α compared with isotype control Ab. (C) Quantification and representative tissue sections from SIRP-α or control Ab treated mice after renal IRI stained with DHE or 4-HNE and visualized by microscopy (magnification, ×200) (n=5 per group). (D) Representative tissue sections and analysis of renal tubular damage and neutrophilic invasion from SIRP-α and control Ab–treated mice (magnification, ×200; inset ×400). Data shown are means±SEM, *statistically significant difference (P<0.001) for SIRP-α compared with control Ab. Arrows highlight rTEC death and cast formation. (E) RT-PCR analysis of inflammatory cytokines and chemokines. Data shown are means±SEM. *Statistically significant difference (P<0.001) for SIRP-α compared with control Ab. (F) Quantiﬁcation and tissue sections from control or SIRP-α Ab–treated mice after renal IRI mice stained by TUNEL assay (magnification, ×200). Data shown are means±SEM (n=6 per group). *Statistically significant difference (P<0.001) for SIRP-α compared with control Ab. (G) Serum urea and creatinine from mice. Data shown are means±SEM (n=6 per group). *Statistically signiﬁcant difference (P<0.05) for SIRP-α compared with control Ab.
IRI
Mice were anesthetized using isoflurane and oxygen titrated to effect, and body temperature was maintained at 36°C with the aid of a rectal temperature probe, warming pad, and warming lamp. A microaneurysm clip was placed to occlude the left, or both, renal pedicles for 20 minutes, after which the clip was removed. The abdomen was closed with 5/0 monofilament suture. Mice were used for laser Doppler experiments 30 minutes or 24 hours after reperfusion, and then euthanized. Blood was collected and kidney tissue snap frozen, placed in RNA later, embedded in optimal cutting temperature compound, or fixed in 10% neutral buffered formalin.

Laser Doppler Blood Flow Analysis
Real-time kidney perfusion and blood flow was measured using laser Doppler imaging (MoorLDI-2α; Moor Instruments, Devon, UK). Briefly, animals were anesthetized and core temperature maintained at 36°C. Organ blood flow was assessed at baseline, in response to ischemia and reperfusion at 30 minutes and 24 hours. Results are expressed as the percentage change from baseline control of the region of interest.

In Vivo SIRP-α Ab Treatment
Age-matched male wild-type C57BL/6 or CD47 null mice were randomly assigned to receive a SIRP-α monoclonal Ab (clone C20; Santa Cruz Biotechnology; 0.4 μg/g body weight intraperitoneally in 100 μl sterile PBS) or an IgG isotype-matched control Ab (Santa Cruz Biotechnology) 90 minutes before surgery.

Statistical Analyses
Statistical analyses were performed using GraphPad Prism software. Data were analyzed by one-way ANOVA followed by the Tukey test for multiple comparisons. For grouped analysis, data were analyzed by two-way ANOVA followed by Bonferroni post hoc test. A P value of <0.05 was assumed to indicate a statistically significant difference.

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DISCLOSURES
J.S.I. is chair of the Scientific Advisory Boards of Vasculox, Inc. (St. Louis, MO), and Radiation Control Technologies, Inc. (Rockville, MD), and holds equity interests in the same.

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CORRECTION


Please note the following should have been included in the Acknowledgments section for the above published article in the June 2014 issue of JASN. “This work was also supported by P30-DK079307 (J.S.I.).”
Materials and Methods

Animals. Male C57BL/6 wild type mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Male Sprague Dawley rats were obtained from Taconic (Hudson, NY). Animals were housed for one week prior to use and had access to water and standard rodent chow ad libitum. Studies were performed using protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh and in accordance with NIH guidelines. Euthanasia was achieved by isoflurane inhalational anesthesia (1.5%) and concurrent cervical dislocation (mice) and or CO₂ inhalation from a pressurized tank followed by cervical dislocation (rats).

Reagents and cells. Human renal tubular epithelial cells (rTEC) and rat aortic VSMC were purchased from Lonza (Switzerland) and maintained in recommended medium. Cells were used between passages 3-7. TSP1 was purchased from Athens Research & Technology (Athens, GA). SIRP-α and CD47 morpholino oligonucleotides complementary to a 5′-UTR sequence rat mRNAs and corresponding 5 base mismatched control morpholino were purchased from GeneTools, Inc. (Philmonth, Oregon). The TSP1-derived peptide 753 was synthesized by Dr. Henry C. Krutzsch and kindly provided by Dr. David D. Roberts (NCI, NIH, Bethesda, MD). The SIRP-α monoclonal Ab (clones C20 for treatment applications and A1 for Western immunoblot) and monoclonal CD47 Ab (clones OX101) were purchased from Santa Cruz Biotechnology and the β integrin blocking Ab (clone Ha2/5) was purchased from BD Biosciences.

Murine rTEC cultures. Primary murine wild type and CD47 null cells were harvested as previously published with modifications⁵. Briefly, mice were euthanized, flushed with PBS, and kidneys removed. The renal cortex was manually resected into pieces of approximately 1 mm³
and digested at 37°C for 25 minutes in DMEM/F12 medium (Invitrogen, Carlsbad, CA) with 1mg/ml type II collagenase (Worthington Chemicals), BSA (Sigma Aldrich, St Louis, MO) and DNase (Sigma Aldrich). The kidney digest was washed through a 40 μm sieve and spun down at 300 g for 5 minutes. The cell pellet was resuspended in defined K1 medium: DMEM/F12 medium supplemented with 25 ng/ml epidermal growth factor (Sigma Aldrich), 1 ng/ml PGE₁ (Cayman Chemicals, Ann Arbor, MI), 5 × 10⁻¹¹ M triiodothyronine (Sigma Aldrich), 5 × 10⁻⁸ M hydrocortisone (Sigma-Aldrich), insulin–transferrin–sodium selenite media supplement (Sigma Aldrich), 1% penicillin/ streptomycin (Cellgro, Manassas, VA), 25 mM HEPES (Invitrogen), and 5% FCS (Invitrogen). The cell suspension was then cultured on collagen-coated Petri dishes (BD Biosciences) in K1 medium until epithelial colonies were established. Experiments were commenced after the cells had reached 90% confluence, which was usually between 3-4 days after the isolation procedure.

**Protein binding assays.** The recombinant human extracellular domain of human SIRP-α fused to a modified human Fc domain was a gift of Dr. William Frazier (Washington University, St. Louis, MO). Recombinant SIRP-α was labeled using Na₁²⁵I by the iodogen method as we previously published²⁴. Immulon 2HB Removawells (Thermo, Franklin, MA) were coated with 50 μl of TSP1 at several concentrations for 16-20 h at 4°C. Nonspecific binding was blocked by incubating the wells with DPBS with Ca²⁺ and Mg²⁺ and 1% BSA for 2 h at room temperature. Radio-labeled SIRP-α was then added and incubation conducted for 2 h at room temperature. Following extensive washing, bound radioactivity was quantified.

**Co-immunoprecipitation.** Immunoprecipitation was performed as we have published with minor modification.² Arterial vascular smooth muscle cells (passages 2-8) were grown in full media and then collected in NP-40 lysis buffer with protease inhibitor cocktail (Sigma Aldrich).
Protein levels were quantitated using a DC™ assay (Bio-Rad, Hercules, CA). Two hundred micrograms of protein was precipitated using either a TSP1 antibody (clone 6.1, Abcam, Cambridge, UK) or mouse IgG1 control antibody (Santa Cruz Biotechnology, Dallas, TX), or a SIRP-α (clone C20) or goat IgG control antibody (both Santa Cruz Biotechnology). For the phosphotyrosine immunoprecipitation, vascular smooth muscle cells or human rTEC (passages 2-6) were serum starved over 24 h, and then treated with TSP1 (2.2 nmol/L) for 1 h, lysate prepared and measured, and then precipitated with a SIRP-α antibody (clone C20, Santa Cruz Biotechnology). Immune complexes were recovered with Protein G Plus-Agarose (Thermo Scientific Pierce, Rockford, IL), washed four times and centrifuged at 600 g at 4°C for 5 min. Pellets were suspended in sample buffer and boiled at 95°C for 5 min. Protein was then separated via gel electrophoresis and transferred to membranes and blotted against TSP1, SIRP-α (clones C20, Santa Cruz Biotechnology), or phosphotyrosine (Millipore, Billerica, MA).

**Western protein analysis.** Following 24 h of serum starvation, the indicated treatments of cells were carried out at 37°C in fetal calf serum- and growth factor-free medium with 0.1% BSA. Following treatment, the cells were rinsed with ice-cold PBS and lysed at 4°C in lysis buffer that contained: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1X protease inhibitors cocktail (Sigma) and 1X phosphatase inhibitors cocktail (Roche Applied Science, Hercules, CA). Cell lysates were centrifuged at 17,000 g for 20 min. A DC™ assay (BioRad, Life Sciences Research, Hercules, CA) was used to quantify total protein. Cell lysates mixed with SDS sample buffer were boiled at 95°C for 5 min, electrophoretically separated on 7.5% PAGE gels for approximately 1 h at 150 V, and transferred to nitrocellulose membrane (BioRad) for 2 h at 400 mA. Membranes were blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE).
and incubated overnight at 4°C with primary Ab. The following Ab were employed – anti-SIRP-α, anti-SHP1, anti-SHP2, anti-CD47 and anti-Nox1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine and phosphoserine Abs were obtained from Millipore (Billerica, MA). Anti-phospho-S591 SHP1, phopho-Y536 SHP1, phosphor-Y542 SHP2, phosphor-Y580 SHP2 and anti-Nox2 Ab were obtained from Abcam (Cambridge, MA). The β-actin Ab was obtained from Cell Signaling Technology (Danvers, MA). The intensity of the bands was quantified using the Odyssey software or Image J (rsbweb.nih.gov/ij/).

**RNA extraction and quantification by real-time PCR.** Total RNA was extracted using Qiagen RNeasy® Mini Kits (Qiagen, Hilden, Germany) as per the manufacturer’s instructions. RNA was quantified using the Take3 Gen5 spectrophotometer (BioTek, Winooski, VT). One microgram (1 µg) of RNA was treated with DNase I (amplification grade, Invitrogen) and then reverse-transcribed using the Superscript III First Strand Synthesis Supermix (Invitrogen). cDNA was amplified using Platinum® Quantitative PCR SuperMix-UDG (Invitrogen) in 10 µl volumes in triplicate with gene-specific primers and probed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. Thermal cycling conditions were 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data were analyzed using the ΔΔCt method with expression normalized to the housekeeping gene and WT-sham-operated animals used as the referent control. The following primer sequences were employed: TSP1 (Mm01335418_m1), CD47 (Mm00495005_m1), SIRP-α (Mm00455928_m1), SHP1 (Mm00442278_m1), SHP2 (00448434_m1), Nox1 (Mm00549170_m1), and Nox2 (Mm01287743_m1), TNF-α (Mm00441889_m1), IL-6 (Mm00446190_m1), CCL2 (Mm00441242_m1), CXCL2 (Mm00436450_m1), IL-1β (Mm00434228_m1) and HPRT1 (Mm00446968_m1) (Taqman,
Assessment of p47<sub>phox</sub> phosphorylation. Phosphorylation of p47<sup>phox</sup> is consistent with increased Nox2 or 1 activity in cells. The phosphorylation of p47<sup>phox</sup> (critical cytosolic subunit of Nox) in p47<sup>phox</sup> immunoprecipitates was determined using an anti-phosphoserine Ab as we published. Briefly, cells were lysed and protein extracted. Equal amounts of protein (300 µg) from the cytosolic fraction as determined by the Bio-Rad protein assay (Life Science Research, Hercules, CA) were incubated overnight at 4°C with anti-p47<sup>phox</sup> Ab (6 µg, Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were recovered with Protein G Plus-Agarose (Thermo Scientific Pierce), washed four times and centrifuged at 600g at 4°C for 5 min. Pellets were suspended in sample buffer and boiled at 95°C for 5 min. Immunoprecipitates were then subjected to Western blotting with a phospho-serine Ab (1:1000, clone 4A4, Millipore).

Measurement of O<sub>2</sub><sup>−</sup> via cytochrome c reduction. Cells were suspended in 200 µl of ice-cold disruption buffer (8 mM potassium, sodium phosphate buffer, pH 7.0, 131 mM NaCl, 340 mM sucrose, 2 mM NaN<sub>3</sub>, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail). The suspension was lysed by five freeze/thaw cycles and passed through a 30-gauge needle five times. Cell disruption was confirmed by phase-contrast microscopy. The cell lysate was centrifuged at 1000g for 10 min at 4°C to remove unbroken cells, nuclei, and debris. The supernatant was transferred to another eppendorf tube and was centrifuged at 28,000g for 15 min at 4°C to collect the membrane fraction pellet, which was resuspended in 50 µl disruption buffer. Throughout all these procedures, extreme care was taken to maintain the lysate at a temperature of 4°C. Membrane fractions (2 µg/well) were added to cytochrome c-containing oxidase assay buffer (65 mM sodium phosphate buffer, pH 7.0, 1 mM EGTA, 10 µM FAD, 1 mM MgCl<sub>2</sub>, 2 mM NaN<sub>3</sub>, and 0.2 mM cytochrome c). After a 5 min baseline
measurement, NADPH (180 µM) was added to initiate the reaction. O$_2^{-}$ was measured as the initial linear rate of SOD-inhibitable cytochrome c reduction quantified at 550 nm using the extinction coefficient 21.1 mM$^{-1}$ cm$^{-1}$.

**siRNA transfection.** Cells were plated the day before transfection to achieve 30–50% confluence in DMEM medium containing 10% fetal calf serum without antibiotics. The small interfering RNA (siRNA) transfections were performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in serum free Opti-MEM according to the manufacturer’s instructions and were carried out in 10-cm$^2$ plates with a final siRNA concentration of 46 nM. DMEM containing 5% FBS was added to culture plates 10 h post-transfection. At 48 h post transfection, the cells were serum and growth factor starved with 0.1% BSA medium for 24 h followed by treatments as indicated in the figure legends. Confirmation of target suppression was provided by Western blot analysis.

**Morpholino oligonucleotide protein suppression of CD47.** Cells were seeded onto 60-mm$^2$ plates in DMEM medium containing 10% fetal calf serum. CD47 morpholino was transfected with a final concentration of 10 µM using the delivery agent Endoporter (6 µl/ml, Genetools, Philomath, OR) according to protocol instructions. At 48 h post transfection, the cells were serum and growth factor starved with 0.1% BSA medium for 24 h followed by treatments as indicated in the figure legends. Gene silencing was monitored at the protein level by Western blotting of cell lysates collected 72 h following transfection.

**Tissue histology and microscopy.** Kidneys embedded in paraffin were sectioned at 3µm and stained with haematoxylin and eosin by standard methods. Markers of tubular damage (tubular dilatation, cell necrosis, infarction and cast formation) were scored by calculation of the percentage of tubules in the corticomedullary junction that displayed such features: 0, none; 1, 1-
Histological examination was performed in “blinded” fashion on 6 randomly selected corticomedullary fields (magnification x200). Light microscopy images were acquired under identical settings using a Zeiss Axiovert 40CFL microscope and Axiovision v4.8 software (Zeiss, Oberkochen, Germany). Displayed tissue section images are at 200x magnification, and insets, are included, at 400x magnification.

Immunofluorescence of arterial rings, human rTEC and murine kidneys. Cryostat sections (5 µm) of vessels were washed three times with phosphate buffered saline (PBS), followed by 3 washes of 0.5% BSA in PBS. Sections were blocked with 2% BSA solution for 30 min. The slides were incubated for 1 h at room temperature (RT) with primary anti-TSP1 Ab (AMSBio, Abingdon, UK; 1:200 in 0.5% BSA solution). Slides were washed three times with BSA solution and incubated for 1 h at RT with a CY3 goat anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories; 1:500 in 0.5% BSA solution) in combination with 1:250 dilution of F-actin dye Rhodamine Phalloidin (Invitrogen). For rTEC, cytospins were generated and fixed with ethanol. Slides were blocked with 5% goat serum and then incubated overnight at 4°C with SIRP-α Ab (clone A-1), 1:50. Slides were washed with PBS and incubated with anti-mouse PE (Immunotec Research Inc, Swanton, VT) 1:500 for 1 h at room temperature. Nuclei were stained with Hoechst dye (bisbenzamide 1mg/100ml water) for 30 sec. After three rinses with PBS, sections were coverslipped with Gelvatol mounting media. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope (software version 1.7a). For whole organs cryostat sections (5 µm) of mouse kidneys were fixed for 20 minutes with 2% paraformaldehyde. Sections were then washed three times with phosphate buffered saline (PBS), followed by 3x washes with solution of 0.5% BSA in PBS. Sections were blocked with 2% donkey serum in BSA solution for 30 minutes. The slides were incubated for 2 hours at room temperature (RT)
with combined primary antibodies for TSP1 (PA1-29196, Thermo Scientific) at 1:100 and SIRP-α (clone C-20, Santa Cruz) at 1:200 in 0.5% BSA solution. Slides were washed three times with BSA solution and incubated for 1 hour at RT with CY3 donkey anti goat secondary antibody (705165147, Jackson Immuno) diluted 1:500, combined with donkey anti rabbit Alexa 488 (A-21206, Invitrogen) diluted 1:1000 in BSA solution. Nuclei were stained with Hoeschts dye (bisbenzamide 1mg/100ml water) for 30 seconds. After three rinses with PBS, sections were coverslipped with Gelvatol mounting media. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope (software version 2.01).

**Arterial myography.** Myography of arterial segments was performed as previously published with minor modifications. Animals were anesthetized with pentobarbital 50 mg/kg i.p. To exsanguinate the abdominal vena cava was transected and the vasculature was gently flushed with chilled 4ºC PBS buffer via puncture of the left ventricle. Thoracic aortas of mice and rats were cleared of adherent adipose tissue and excised. The endothelial layer was removed by gently rubbing the luminal side of the vessel along the rough surface of a blunt needle. Arterial segments approximately 2 mm in length were mounted on myograph pins (Danish Myo Technology, Atlanta, GA) in 5ml incubation buffer maintained at 37°C, pH 7.4, gassed with 95% O₂ and 5% CO₂, and brought to an optimal resting tension by increasing tone every 10 sec. Rings were allowed to stabilize for 1 h, replacing the PSS solution every 20 min. Viability of the vessels was ascertained by a contractile response to potassium chloride (100 mM KCl in PSS solution) for 30 min. Rings were then washed 3 times with PSS and allowed to stabilize to baseline. Phenylephrine (PE; Sigma-Aldrich) concentration–response curves (10⁻⁹ to 10⁻⁵ M) were generated by measuring contraction plateaus at each concentration. After vessel segments reached a stable plateau phase induced by a PE concentration producing 80% maximum
contraction (EC\textsubscript{80}), a single concentration of acetylcholine (ACh; Sigma-Aldrich, 10\textsuperscript{-6} M) was added to assess endothelium function. Aortic rings were then washed and incubated in the presence or absence of a SIRP-\(\alpha\) monoclonal blocking Ab (1 \(\mu\)g/ml, 15 min) ± TSP1 (2.2 nmol/L) ± Tempol (30 \(\mu\)M). Vessels contracted with an EC\textsubscript{80} of PE were allowed to reach a stable plateau and endothelium-independent vasodilation to sodium nitroprusside (SNP, Sigma-Aldrich, 10\textsuperscript{-10} to 10\textsuperscript{-5} M) was then tested.

**Detection of ROS in renal tissue sections.** Assessment of ROS in renal tissue sections was done using the several techniques. The cell permeable agent dihydroethidium (DHE, 10\(\mu\)M, Molecular Probes) was applied to unfixed frozen sections, incubated in a light-protected humidified chamber at 37\(^{\circ}\)C for 30 minutes, washed with PBS and mounted with Gelvital mounting medium. In other experiments cryostat sections (5 micron) of kidneys were washed three times with PBS, followed by 3x washes with solution of 0.5% BSA in PBS. Sections were blocked with 2% BSA solution for 30 minutes. The slides were incubated for 1 h at room RT with primary antibody for 4-HNE (Alpha Diagnostics), 1:100 in 0.5% BSA solution. Slides were washed three times with BSA solution and incubated for 1 h at RT with CY3 goat anti-rabbit secondary antibody (Jackson Immuno), diluted 1:500 in BSA solution, in combination with 1:250 dilution of F-actin dye Rhodamine Phalloidin (Invitrogen). Nuclei were stained with Hoeschts dye (bisbenzamide 1mg/100ml water) for 30 sec. After three rinses with PBS, sections were coverslipped with Gelvatol mounting media. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope (software version 1.7a).

**Cell apoptosis and viability analysis.** Human rTEC were grown to 80% confluence, serum starved in 0.1% FBS overnight, and treated with TSP1 (2.2nM) and/or SIRP-\(\alpha\) antibody (clone C-20) for 24 h. Cells in 60mm plates were washed with cold PBS, and collected in RIPA buffer
for protein quantification. Proteins were then separated by SDS-PAGE, transferred to nitrocellulose membrane and probed for caspase-3 (Cell Signaling Technology). Additionally, cell viability was assessed in a 96-well plate using the LIVE/DEAD Viability/Cytotoxicity kit (Molecular Probes). Calcein (0.5µM) and ethidium homodimer-1 (2µM) were added to the cells for 30 minutes and fluorescence read using a microplate reader as per manufacturer instructions.

**Ischemia reperfusion injury.** Mice were anaesthetized using isoflurane and oxygen titrated to effect, and body temperature maintained at 36°C with the aid of a rectal temperature probe, warming pad and lamp. A microaneurysm clip was placed to occlude the renal pedicles for 20 min after which the clip was removed. The abdomen was closed with 5/0 monofilament suture. Mice were used for Laser Doppler experiments 30 min or 24 h after reperfusion, and euthanized. Blood was collected and kidney tissue snap frozen, placed in RNAlater, embedded in OCT compound, or fixed in 10% neutral buffered formalin.

**Laser Doppler blood flow analysis.** Real time kidney perfusion and blood flow was measured using laser Doppler imaging (MoorLDI-2%, Moor Instruments, Devon, UK) as we have published 3, 4. Briefly, animals were anesthetized and core temperature maintained at 36°C. Organ blood flow was assessed at baseline, in response to ischemia and reperfusion at 30 min and 24 h. Results are expressed as the percent change from baseline control of the region of interest.

**In vivo SIRP-α Ab treatment.** Wild type C57BL/6 mice were randomized to receive either a SIRP-α monoclonal Ab (clone C20, Santa Cruz Biotechnology, 0.4µg/gram body weight i.p. in 100 µl sterile PBS) or an IgG isotype-matched control Ab (Santa Cruz Biotechnology) 90 min before surgery.
**Statistical analysis.** Statistical analyses were performed using GraphPad Prism software. Data were analyzed by 1-way ANOVA followed by Tukey test for multiple comparisons. For grouped analysis, data were analyzed by 2-way ANOVA followed by Bonferroni post hoc test. A *p* value of $< 0.05$ was assumed to be significant.

**References**


Supplemental Figure 1. Treatment of VSMC with the NO pro-drug sodium nitroprusside does not stimulate phosphorylation of SIRP-α or SHP1. VSMC in minimal medium were treated with TSP1 (2.2 nmol/L) or the NO pro-drug sodium nitroprusside (SNP, 10 μM) for 10 min, cells lysed and proteins separated and blots probed for p-SIRP-α (A, C) and p-SHP1 (B) or β-actin. Representative data from 4 independent experiments are presented. Densitometry is presented as mean ratio of p-tyr-SIRP-α to β-actin (± S.E.M.) and as the mean ratio p-SHP1 to β-actin (± S.E.M.). * = statistically significant difference (p < 0.05) TSP1 compared to SNP or control (untreated).
Supplemental Figure 2. TSP1 activation of SIRP-α does not require β integrins. (A) VSMC in basal medium were treated with peptide 753 (10 µM), or a β integrin blocking Ab (1 µg/ml) ± TSP1 (2.2 nmol/L) for 10 min (B), cell lysate prepared and Western blot analysis performed. Densitometry is presented as the mean ratio of p-SIRP-α to β-actin (± S.E.M.) (A, B) or total SIRP-α to β-actin (± S.E.M.) (B). (C) VSMC in basal medium were treated with peptide 753 (10 µM) ± a β integrin blocking Ab (1 µg/ml) for 10 min, cell lysate prepared and Western blot analysis performed. Densitometry is presented as the mean ratio of p-SIRP-α to β-actin (± S.E.M.) or total SIRP-α to β-actin (± S.E.M.). (D) VSMC in basal medium were treated with TSP1 (2.2 nmol/L) ± a SIRP-α blocking Ab (1 µg/ml) for 10 min, cell lysate prepared and Western blot analysis performed. Results are representative of 4 separate experiments. Densitometry is presented as the mean ratio of p-SHP1 to β-actin (± S.E.M.). All demonstrated results are representative of 4 separate experiments; * = statistically significant difference (p < 0.05) compared to untreated and Ab treated and # = statistically significant difference (p < 0.05) compared to TSP1 treated.
Supplemental Figure 3. TSP1 treatment dose not stimulate cell apoptosis or cell death in human rTEC. (A) Human rTEC were treated in minimal medium with 0.1% BSA with TSP1 (2.2 nmol/L) ± a SIRP-α Ab (1 μg/ml) for 24 h and protein expression of caspace 3 determined via Western immunoblot. A representative blot from 3 separate experiments is shown. Data are presented as the means ratio of target protein to β-actin (± S.E.M.). (B) Human rTEC were cultured in minimal medium with 0.1% BSA and treated with TSP1 (2.2 nmol/L) for 24 h and cell viability determined using the LiveDead Cell Viability kit (Molecular Probes) as per the manufacture's instructions. Data are presented as the means (± S.E.M.) of the % live and % dead cells.
Supplemental Figure 4. SIRP-α Ab treatment does not improve serum urea or creatinine in IRI-challenged CD47 -/- mice. CD47 null age matched male mice were treated with a SIRP-α blocking Ab or isotype IgG control (CTRL) Ab (0.4 μg/g body weight i.p.) and challenged with 20 min of renal ischemia and 24 h of reperfusion, n=8 per group. Serum urea and creatinine were measured 24 h after reperfusion. Results are presented as the means (± S.E.M.).