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Blocking Thrombospondin-1/CD47 Signaling Alleviates Deleterious Effects of Aging on Tissue Responses to Ischemia

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Objective—Decreased blood flow secondary to peripheral vascular disease underlies a significant number of chronic diseases that account for the majority of morbidity and mortality among the elderly. Blood vessel diameter and blood flow are limited by the matricellular protein thrombospondin-1 (TSP1) through its ability to block responses to the endogenous vasodilator nitric oxide (NO). In this study we investigate the role TSP1 plays in regulating blood flow in the presence of advanced age and atherosclerotic vascular disease.

Methods and Results—Mice lacking TSP1 or CD47 show minimal loss of their resistance to ischemic injury with age and increased preservation of tissue perfusion immediately after injury. Treatment of WT and apolipoprotein E–null mice using therapeutic agents that decrease CD47 or enhance NO levels reverses the deleterious effects of age- and diet-induced vasculopathy and results in significantly increased tissue survival in models of ischemia.

Conclusion—With increasing age and diet-induced atherosclerotic vascular disease, TSP1 and its receptor CD47 become more limiting for blood flow and tissue survival after ischemic injury. Drugs that limit TSP1/CD47 regulation of blood flow could improve outcomes from surgical interventions in the elderly and ameliorate vascular complications attendant to aging. (*Arterioscler Thromb Vasc Biol.* 2007;27:000-000.)

Key Words: nitric oxide ■ thrombospondin-1 ■ perfusion ■ ischemia ■ wound healing

Complications from peripheral vascular disease, including coronary artery disease and myocardial infarction, stroke, and ischemic vascular disease affect some 80% of people over the age of 65. The elderly have significant alterations in vascular anatomy including a loss of vascular networks¹ and alterations in vascular response to injury.² Age is a recognized risk factor for complications after surgery including delayed and incomplete wound healing and tissue loss and accounts for significant morbidity and mortality in this group.^{3–6} Animal studies have confirmed the impact of aging on wound healing.^{7–9}

Tissue perfusion is regulated through the control of blood vessel diameter, which itself is controlled by the contractile state of vascular smooth muscle cells (VSMCs). Nitric oxide (NO) is a primary and ubiquitous dilator of blood vessels.¹⁰ NO is constitutively produced in blood vessels by endothelial nitric oxide synthase (eNOS). NO activates soluble guanylate cyclase (sGC) leading to cGMP production and vasodilation. In aged vascular cells,¹¹ animals, and people, both eNOS expression and NO production¹² are decreased.

We recently reported that thrombospondin-1 (TSP1) blocks NO-driven VSMC relaxation in a CD47-dependent

manner.^{13–15} NO-driven alterations in blood flow are substantially greater in the absence of TSP1 or CD47. Given the deleterious effects of aging on the cardiovascular system, we wanted to determine whether blocking of TSP1 inhibition of NO signaling would provide significant tissue protection in senescent animals. Aged WT and apolipoprotein E (apoE)-null mice (with diet-driven vasculopathy) demonstrated increased tissue necrosis in response to a fixed ischemic challenge compared with young animals. In contrast, senescent TSP1-null and CD47-null animals subjected to the same ischemic challenge demonstrated tissue preservation comparable to that in young animals. Suppression of CD47 in senescent or apoE-null animals also resulted in increased tissue survival after ischemic injury.

Materials and Methods

Reagents

Isosorbide dinitrate (ISDN) and L-nitro-N-methyl arginine (L-NAME) were purchased from Sigma. A CD47 morpholino antisense oligonucleotide (CGTCACAGGCAGGACCCACTGCCCA) and a mismatched control morpholino were purchased from GeneTools.

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Animals

C57BL/6 WT, TSP1-null,¹⁶ and CD47-null¹⁷ mice were maintained in a pathogen-free environment with ad libitum access to standard rat chow and water. ApoE-null mice (B6.129P2-Apoe<tmlUnc>/J) were purchased from Jackson Labs (Bar Harbor, Me) and maintained on either a standard rat chow diet or a 40% fat diet (Harlan Teklan, Madison, Wis).¹⁸ Animals used were 12 to 18 months of age, except as indicated when young animals (aged 2 to 4 months) were used for control purposes. Care and handling of animals was in accordance with the Animal Care and Use Committees of the National Cancer Institute and of Washington University School of Medicine.

Ischemic Soft Tissue Flap Model

Animals underwent creation of a random myocutaneous (McFarlane) flap as previously described.¹⁴ Some animals received either ISDN (1 mg/mL) or L-NAME (0.5 mg/mL) ad libitum in the drinking water during the postoperative interval.

Estimation of Survival Area in Flaps

The necrotic areas of dorsal myocutaneous McFarlane flaps were determined as previously described.¹⁴

Laser Doppler Analysis of Tissue Perfusion

Core temperature was monitored via rectal probe and maintained at 37°C by a heated stage. Anesthesia was obtained with 1.5% isoflurane. A MoorLD1-2λ scanner (Moor Instruments) was used with the following parameters: scan area, 1.6×2.5 cm; scan speed, 4 ms per pixel; scan time, 1 minute 54 sec; override distance, 25 cm. The override distance was 20 cm.

Hindlimb Ischemia

Wild-type, TSP1-null, CD47-null underwent ligation of the femoral artery at the level of the inguinal ligament as previously described¹⁵ with a 5-0 nylon suture placed around the femoral artery and tied, resulting in complete vessel occlusion.

Blood Oxygen Level–Dependent MRI Imaging

WT and TSP1 null mice 14 to 18 months of age underwent in vivo analysis of tissue perfusion and blood flow using blood oxygen level–dependent (BOLD) MRI. MRI images were acquired using a Bruker Biospin 4.7 T scanner and isoflurane anesthesia as previously described.¹⁴ T2 mapping spin echo images were obtained using a multi-slice multi-echo (MSME) sequence with a 10-echo train and an echo time of 15 ms. The scan time for a T2 mapping image set (NEX=1) by the MSME sequence was 10 minutes.

Hind Paw Perfusion Assay

Fourteen-month-old mice underwent treatment of the hind paw with 5% mustard oil (Chem Services Inc) in sesame oil (50 μL to dorsal paw), which is known to induce NO-dependent vasoactive responses.^{19,20}

Morpholino Suppression of CD47

Flap soft tissue units and underlying wound beds were injected with control vehicle (normal saline), a CD47 morpholino, or a control morpholino (10 μmol/L) in sterile saline. Flap survival was determined as described.

Mitochondrial Viability Assay

Mitochondrial viability of hindlimb muscle biopsies was assessed by the reduction of a tetrazolium salt to water insoluble formazan through mitochondrial oxidation as described.²¹ Results were expressed as absorbance normalized to dry tissue weight.

Determination of Tissue cGMP

Skeletal muscle biopsies of equal wet weight were excised, frozen in liquid nitrogen (LN₂) and pulverized, and then washed with 6% (wt/vol) trichloroacetic acid (TCA) at 4°C. Homogenates were centrifuged and supernatants washed 4 times with 5 volumes of

water saturated diethyl ether. The extracts were lyophilized and resuspended in assay buffer for analysis via immunoassay (Amersham, GE Healthcare).

Histology

Tissue units were excised, fixed in 10% buffered formaldehyde, paraffin embedded, and sectioned at a thickness of 5 μm. Sections were then stained with hematoxylin and eosin (H&E). Review of each slide was performed by an independent pathologist blinded to the origin of each tissue slide.

Statistics

Results are presented as the mean±SD of a total of 164 aged animals (12 to 18 months old) of the following genetic type: WT=67, TSP1-null=56, CD47-null=31, apo E-null=10. Where indicated studies were performed in comparable numbers of young WT, TSP1- and CD47-null mice aged 2 to 4 months. Significance was calculated with Student *t* test and 1-way ANOVA using a software package (Origin) with *P*<0.05.

Results

Thrombospondin-1 Limits Tissue Necrosis in Aged Animals

Aged (14- to 18-month-old) sex-matched WT and TSP1-null mice underwent McFarlane flap surgery. Wild-type flaps demonstrated near total necrosis with only 20±6% survival obtained at 7 days (Figure 1A and 1B). This is significantly less than the survival seen in young mice of the same background¹⁴ (*P*<0.05). Flaps in senescent TSP1-null mice demonstrated near total tissue survival with minimal necrosis (6±3%). Survival did not differ significantly from that of young TSP1-null mice¹⁴ (*P*>0.05). Histological review of WT tissue flaps from senescent mice demonstrated loss of the epidermis and ulceration, coagulative necrosis of subcutaneous collagen, and absence of hair follicles (supplemental Figure 1a and 1c, available online at <http://atvb.ahajournals.org>). TSP1-null flaps demonstrated normal histology with minimal inflammatory cell infiltration (supplemental Figure 1b and 1d).

Nitric Oxide Increases Aged Tissue Survival After Ischemia

WT aged mice that underwent random myocutaneous flaps and received ISDN ad libitum in the drinking water demonstrated a moderate but significant increase in tissue survival 41±5% (*P*<0.05; Figure 1C). Treatment of WT aged mice with L-NAME further decreased flap survival (8±4% versus 20±6%, *P*<0.05) compared with untreated (Figure 1A and 1C), whereas L-NAME failed to diminish flap survival in TSP1-null animals. Given the near complete survival of random flaps in untreated aged TSP1-null animals treatment with ISDN did not dramatically alter flap survival (Figure 1C).

Tissue cGMP Is Limited by TSP1 and Age

Skeletal muscle from WT and TSP1-null young (12 weeks old) and aged (18 months old; Figure 1D) or old mice alone (Figure 1E) mice was analyzed for cGMP. Consistent with previous reports,^{22,23} cGMP levels decreased significantly in aged WT animals. In contrast, cGMP was slightly elevated in muscle samples from young TSP1-null mice and did not fall with age. After 24 hours of ischemia, old TSP1-null animals

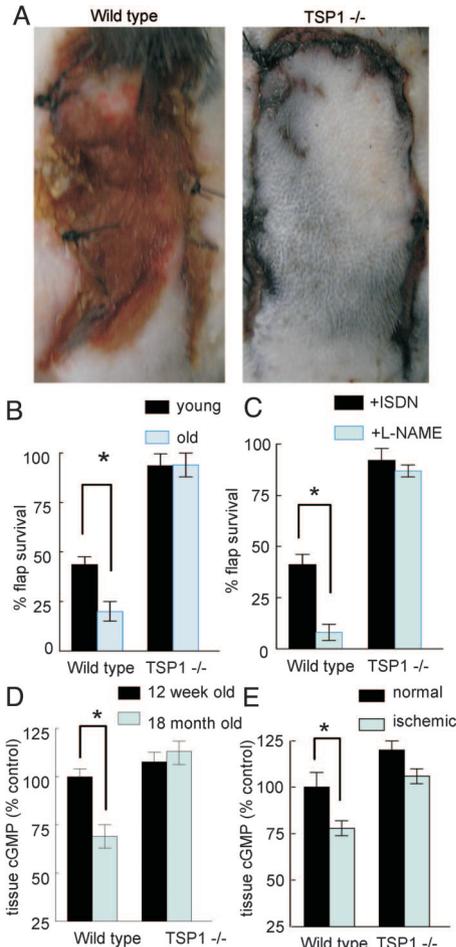


Figure 1. TSP1 impairs ischemic tissue survival in aged animals. WT and TSP1-null (A, B) mice 2 to 4 and 12 to 16 months of age underwent random dorsal myocutaneous flaps, and tissue survival was determined. Results represent the mean \pm SD of 16 animals. Aged animals (C) underwent flap surgery and received ISDN or L-NAME in the drinking water postoperatively. Results represent the mean \pm SD of 16 animals. Skeletal muscle from young (12 weeks) and old (18 month) animals (D) or old animals \pm 24 hours of ischemia (E) was processed for cGMP analysis. Results represent the mean \pm SD of 4 pairs of animals.

demonstrated significantly less decrease in tissue cGMP compared with wild-type (Figure 1E).

TSP1 Limits Immediate Responses to Ischemia in Senescent Animals

Mice (14 to 18 months old) underwent random myocutaneous flaps and laser Doppler analysis of tissue perfusion (Figure 2A). TSP1-null and CD47-null flaps demonstrated greater flap perfusion than WT flaps (Figure 2B; supplemental Figure II and movie). Null flaps exhibited less overall loss of tissue perfusion immediately after flap elevation and a progressive increase in tissue perfusion during the postoperative interval. WT flaps demonstrated progressive decreases in flap perfusion and showed no tendency to recovery.

Thrombospondin-1 Limits Hindlimb Survival of Ischemia in Aged Animals

Mice (age 14 to 18 months) underwent ligation of the femoral artery. Aged wild-type animals demonstrated minimal remodel-

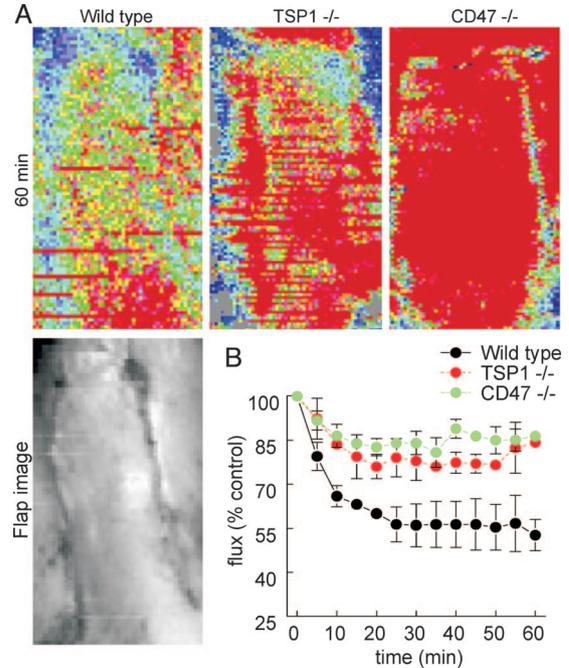


Figure 2. Thrombospondin-1 limits immediate responses to ischemia in senescent animals. Mice 14 to 18 months of age underwent dorsal McFarlane flap surgery and perfusion was determined via laser Doppler (A, B). Animals were maintained at 37°C on a heated stage. Images and data are representative of 18 mice, 6 of each strain.

ing of existing collateral vessels in the ischemic limb and significantly less than young mice (Figure 3A and 3D). In contrast TSP1-null limbs demonstrated extensive vascular remodeling of existing collateral vessels with both young and old animals performing equally well (Figure 3B and 3D). Laser Doppler analysis of hindlimb perfusion 72 hours after ligation demonstrated less restoration of perfusion in wild-type (Figure 3A and 3C) than in TSP1-null limbs (Figure 3B and 3C). Mitochondrial viability of muscle biopsies from hindlimbs was only minimally decreased in limbs undergoing vascular ligation compared with contralateral control limbs in TSP1-null specimens (Figure 3E) Aged WT samples showed a pronounced decrease in MTT signal between ischemic limb muscle compared with normal limb muscle, and between young and old tissue samples (Figure 3E). H&E sections of hindlimbs demonstrated marked sterile necrosis of muscle fibers and loss of cell nuclei in ischemic WT tissue (supplemental Figure IIIA and IIIB). Ischemic TSP1-null muscle demonstrated minimal sterile necrosis of muscle fibers and increased cell nuclei.

Loss of TSP1 Immediately Enhances Tissue Perfusion After Hindlimb Ligation in Older Animals

Aged WT and TSP1-null animals showed a profound decrease in perfusion immediately after vascular ligation of the hindlimb (Figure 4A and 4B). However, the TSP1-null animals showed a progressive increase in hindlimb perfusion compared with WT limbs and had significantly more restoration of flow within the first postoperative hour.

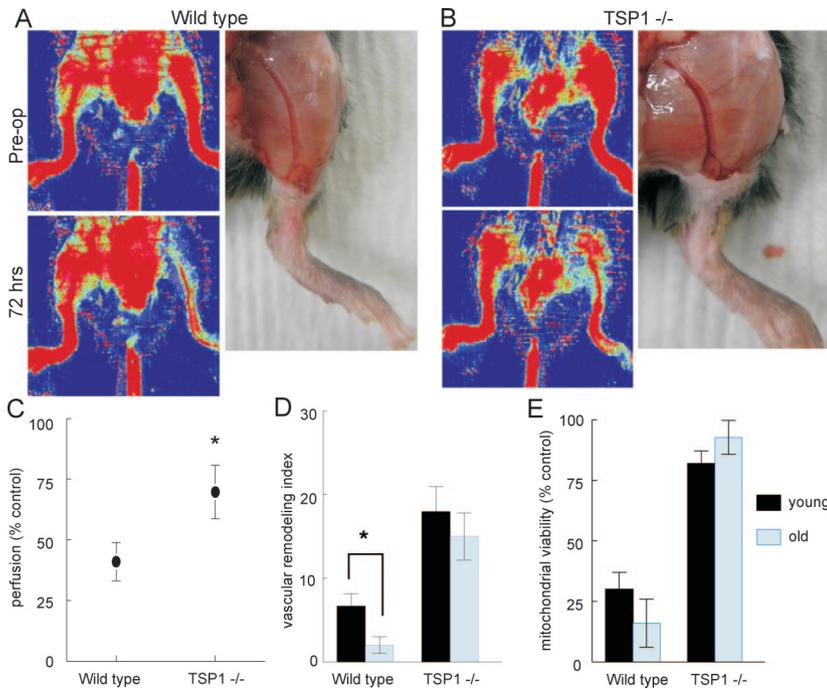


Figure 3. Thrombospondin-1 limits hindlimb survival to ischemia in aged animals. WT (A) and TSP1-null (B) mice 2 to 4 and 14 to 18 months in age underwent femoral artery ligation. Doppler analysis of limb perfusion was performed at 72 hours postoperatively (A to C; $P > 0.05$). Images pictured are from representative aged animals. Under $5\times$ magnification, visible vessels on the surface of the vastus medialis was quantified in both young and old animals (D). Mitochondrial viability of muscle from the tibialis anterior was determined and quantified (E). Results represent the mean \pm SD of 12 animals.

Aged TSP1-Null Animals Demonstrate Enhanced Responses to Exogenous NO in Ischemic Tissue

To further define the role of TSP1/NO signaling in acute responses to tissue ischemia, WT and TSP1-null mice (14 to 18 months of age) underwent ligation of the femoral artery. Three days after the procedure, animals underwent BOLD MRI in the presence of an NO challenge (10 $\mu\text{mol/L}$ DEA/NO). In the absence of TSP1, NO-driven blood flow changes showed a progressive increase to levels slightly above those found in the control limb (Figure 4D). WT animals demonstrated a significant deficit in tissue blood flow responses to exogenous NO challenge after proximal ligation compared with the nonoperated limb (Figure 4C). T_2 maps showed dramatic differences in WT animals between control and ischemic hindlimbs (Figure 4c). In contrast, blood flow was the same in ischemic and control hindlimbs in TSP1-null mice (Figure 4d).

CD47 Limits Tissue Survival to Ischemia in Aged Animals

We have reported that the effects of TSP1 on tissue ischemia are mediated by CD47.¹⁵ After random myocutaneous flap surgery, tissue survival in senescent CD47-null mice ($93 \pm 4\%$) resembled levels obtained in aged TSP1-null animals (Figure 5A). In contrast to the decrease with age in WT animals, flap tissue survival in CD47-null animals did not decrease with age (Figure 5A).

TSP1 and CD47 Limit Vasodilatory Responses in Aged Animals

WT and CD47-null mice aged 2 to 4 months underwent mustard oil application to the right hind paw and perfusion measured by Doppler. Young WT and CD47-null animals demonstrated equivalent levels of hind paw perfusion under basal conditions (Figure 5B and 5C). However, on applica-

tion of mustard oil, CD47-null animals experienced a marked increase of the baseline perfusion as compared with WT. Likewise, young TSP1-null mice demonstrated enhanced perfusion in response to a mustard oil challenge compared with WT (Figure 5D). Senescent CD47- and TSP1-null animals had 20% to 25% greater perfusion compared with WT animals after mustard oil treatment, with persistence of perfusion gains over time (Figure 5E).

Morpholino Suppression of CD47 Increases Aged Tissue Survival After Ischemia

WT mice 14 to 18 months in age underwent random myocutaneous flaps and were treated with control vehicle, a CD47 morpholino (supplemental Figure IVA and IVB) or control morpholino (data not shown) injected directly to the flap and wound bed at the time of surgery. Postoperative tissue survival was increased in animals treated with a CD47 morpholino as compared with control treated animals ($91 \pm 4\%$ versus $31 \pm 6\%$, respectively; Figure 6A). Control morpholino treated flaps displayed degrees of tissue necrosis comparable to untreated aged WT animals (data not shown). Interestingly, CD47 morpholino-treated WT flaps demonstrated substantial remodeling of existing collateral flap vessels (supplemental Figure IVC). Mitochondrial viability was markedly increased in flaps that received the CD47 morpholino as compared with vehicle, missense morpholino, or untreated flaps (Figure 6B).

Ischemic Tissue Necrosis in Aged Animals With Atherosclerotic Peripheral Vascular Disease Is Minimized by CD47 Suppression

ApoE-null mice 12 to 16 months of age fed a high-fat diet for a minimum of 8 months underwent flap elevation. On postoperative day 7 apoE-null animals demonstrated significantly decreased tissue survival comparable to or even

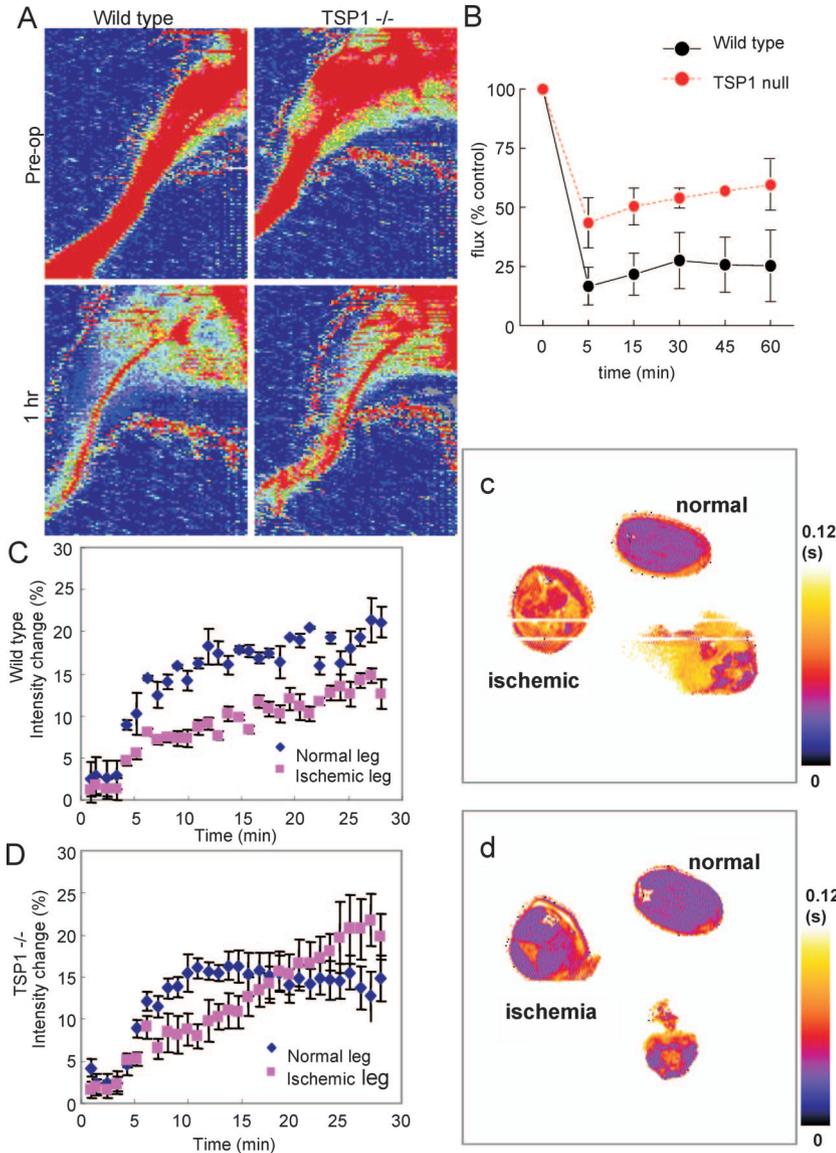


Figure 4. Loss of TSP1 minimizes tissue loss after acute vascular interruption in aged animals. Mice aged 14 to 18 months underwent laser Doppler analysis of hindlimb perfusion followed by ligation of the femoral artery and immediate analysis (A and B). Results represent the mean \pm SD of 6 pairs of animals. Aged WT (C) and TSP1-null (D) mice underwent femoral artery ligation, and BOLD MRI images were obtained from T₂* weighted gradient echo sequences. DEA/NO (100 nmol/g bodyweight) was administered 5 minutes after starting the scan. Values are presented as mean \pm SE of 4 and 5 experiments in wild-type and TSP1-null mice respectively. T₂ maps of normal and ischemic hindlimbs of aged WT (c) and TSP1-null (d) animals.

slightly worse than changes seen in WT animals of comparable age (supplemental Figure IVD and IVE). Despite diet-induced atherosclerosis, these animals showed increased tissue survival after CD47 morpholino treatment ($84 \pm 7\%$ versus $5 \pm 5\%$, respectively; Figure 6C). Routine histological staining of an artery from an aged apoE-null animal on a high-fat diet 3 weeks after temporary ligation demonstrated significant neointimal enlargement and plaque formation (supplemental Figure IVF).

Discussion

Altered tissue perfusion secondary to atherosclerotic peripheral vascular disease is a common cause of numerous diseases of the elderly.^{24,25} Such vasculopathy is endemic in Western societies.²⁶ The consequences of atherosclerotic vascular disease in the elderly are altered blood flow²⁷ and inadequate delivery of nutrients and oxygen to tissues with attendant tissue ischemia, necrosis, and loss. Even in the absence of atherosclerotic changes, age-related decreases in nitric oxide synthase activity^{28,29} and cellular and tissue cGMP^{22,23} can

impair the ability of senescent vasculature to dilate, thereby limiting the delivery of blood to tissues.

Recently, we demonstrated that NO-driven relaxation of VSMCs is regulated by TSP1.³⁰ In both endothelial and VSMCs,^{30,31} TSP1 via CD47 blocks the ability of endogenous or exogenous NO to elevate cGMP levels. As a physiological consequence, tissue perfusion in response to NO is significantly greater in the absence of TSP1 or CD47.¹⁴ Although the absence of TSP1 or CD47 conferred a survival advantage on ischemic tissues in young animals (10 to 16 weeks of age), it was not clear that ischemia in the elderly or in the presence of age-associated vasculopathy would be ameliorated by blocking the TSP1-CD47 pathway.

In the present study we found that TSP1, in a CD47-dependent manner, limits ischemic tissue survival under conditions of advanced age and atherosclerotic vasculopathy. In the absence of TSP1 or CD47, senescent mice were able to maintain perfusion after an ischemic insult. The acute effects of TSP1/CD47 signaling on perfusion of aged ischemic tissues is consistent with the immediate enhancement of

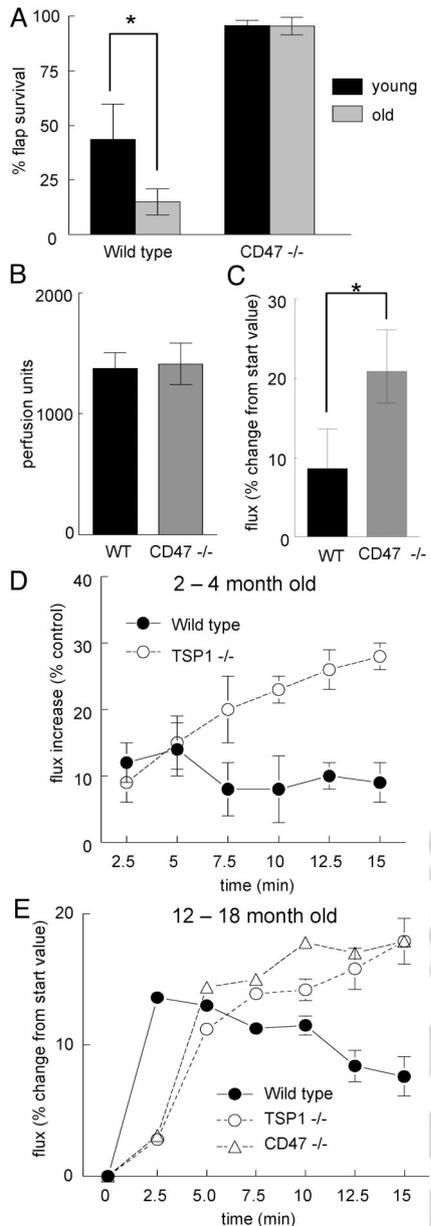


Figure 5. TSP1 through CD47 limits ischemic tissue survival and NO-driven tissue perfusion in elderly animals. Mice aged 2 to 4 and 12 to 16 months underwent random dorsal flaps and tissue survival determined (A). Results represent the mean \pm SD of 18 animals (12 CD47-null and 6 WT). Mice 8 to 16 weeks old (B, C, D) and 14- to 16-month-old animals (E) underwent laser Doppler analysis of paw perfusion. Data were acquired pretreatment (B) and at 5 minutes after application of 5% mustard oil (C) to the hind paw, or in young (D) or aged (E) WT, TSP1-, and CD47-null mice. Results represent the mean \pm SD of 5 pair of WT, CD47-null, and TSP1-null animals from each age group (B and C) or the mean \pm SD of 24 mice, 8 of each strain (D and E).

tissue perfusion in muscle units of TSP1-null animals exposed to exogenous NO.¹⁴ Even more remarkable, senescent TSP1-null animals demonstrate a greater perfusion increase in response to exogenous NO than young WT animals (see Figure 4D and¹⁴). Superior perfusion and tissue survival responses of aged TSP1- and CD47-null animals were observed consistently for random cutaneous flaps, hindlimb vascular ligation, and a noninvasive mustard oil/hind paw

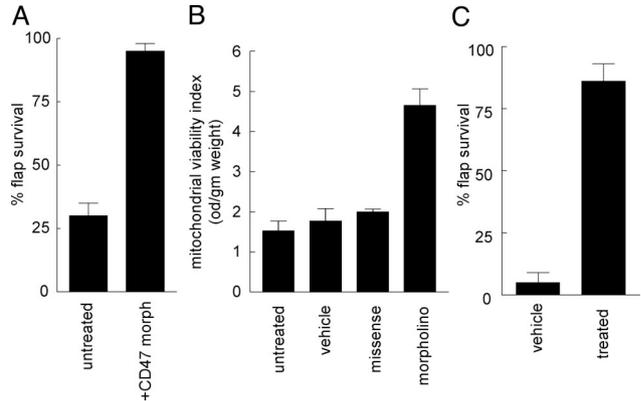


Figure 6. Morpholino suppression of CD47 increases aged tissue survival to ischemia. WT mice 14 to 18 months of age underwent random dorsal flaps. Animals were treated with control vehicle, missense control morpholino (data not shown), or a CD47 morpholino (A; 10 μ mol/L in PBS injected in the flap and wound bed) and flap survival determined. Results presented represent the mean \pm SD from 12 animals (6 treated with control vehicle and 6 treated with a CD47 morpholino). Mitochondrial viability of flaps was determined and quantified (B). ApoE-null mice on a high-fat diet and of at least 12 months of age underwent random flaps. Animals were treated with control vehicle or a CD47 morpholino and flap survival determined on postoperative day 7 (C). Results presented represent the mean \pm SD from 10 animals (5 treated with control vehicle and 5 treated with a CD47 morpholino).

assay despite the comparable soft tissue vascular densities in wild-type and null animals.³² The NO dependence for tissue survival of ischemic injury in WT animals is supported by positive effects of NO supplementation with ISDN and negative effects of NOS inhibition with L-NAME. In the absence of TSP1, tissue survival is less sensitive to NO modulation. This is not surprising because TSP1 limits cGMP accumulation in both vascular cells^{30,31} and tissue (Figure 1D and 1E), and prior studies have documented increased tissue TSP1 levels with age.³³ Thus, we propose that the NO insufficiency of aging may be attributable to increased TSP1 antagonism of NO/cGMP signaling as well as loss of NOS activity.

ApoE-null mice on a high-fat diet develop a hyperlipidemic state analogous to that in humans. Blood vessels of these mice show luminal narrowing and hypertrophy of the medial layers with atherosclerotic plaque.³⁴ Comparable atherosclerotic changes in people have been associated with decreased local NO production by vascular endothelium.^{35,36} Interestingly, TSP1 expression has been found to increase with age in atherosclerotic blood vessels^{33,37} and in several end organs.³⁸⁻⁴⁰ Morpholino suppression of CD47 improved ischemic tissue survival in apoE-null animals in the presence of diet-induced atherosclerotic vasculopathy, and also in aged wild-type animals. These results suggest that targeting of TSP1 or CD47 in regional vascular beds could improve tissue perfusion under conditions of both advanced age and peripheral vascular disease.

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Disclosures

None.

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