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Thrombospondin-1 and CD47 regulate blood pressure and cardiac responses to vasoactive stress

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ABSTRACT

Nitric oxide (NO) locally regulates vascular resistance and blood pressure by modulating blood vessel tone. Thrombospondin-1 signaling via its receptor CD47 locally limits the ability of NO to relax vascular smooth muscle cells and increase regional blood flow in ischemic tissues. To determine whether thrombospondin-1 plays a broader role in central cardiovascular physiology, we examined vasoactive stress responses in mice lacking thrombospondin-1 or CD47. Mice lacking thrombospondin-1 exhibit activity-associated increases in heart rate, central diastolic and mean arterial blood pressure and a constant decrease in pulse pressure. CD47-deficient mice have normal central pulse pressure but elevated resting peripheral blood pressure. Both null mice show exaggerated decreases in peripheral blood pressure and increased cardiac output and ejection fraction in response to NO. Autonomic blockade also induces exaggerated hypotensive responses in awake thrombospondin-1 null and CD47 null mice. Both null mice exhibit a greater hypotensive response to isoflurane, and autonomic blockage under isoflurane anesthesia leads to premature death of thrombospondin-1 null mice. Conversely, the hypertensive response to epinephrine is attenuated in thrombospondin-1 null mice. Thus, the matricellular protein thrombospondin-1 and its receptor CD47 serve as acute physiological regulators of blood pressure and exert a vasopressor activity to maintain global hemodynamics under stress.

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1. Introduction

Cardiovascular homeostasis requires constant regulation of tissue perfusion and blood flow through coordinated interactions of the autonomic nervous system, heart, lungs and blood vessels. Meeting regional metabolic demands requires rapid and efficient redistribution of blood flow. Nitric oxide (NO) is a major physiological regulator of blood vessel diameter and blood flow (Arnal et al., 1999; Ignarro, 2002). In response to specific stressors, arterial endothelium increases its production of NO, which diffuses into the adjacent vascular smooth muscle cells (VSMC) and causes cGMP-mediated relaxation by activating soluble guanylate cyclase (sGC). This results in vessel dilation and increased blood flow. Direct cGMP-dependent and indirect activation of cGMP phosphodiesterases (PDE) provides negative feedback to limit NO/cGMP signaling (Mullershausen et al., 2003). The matricellular protein thrombospondin-1 (TSP1), which is produced by vascular cells and circulates at 100–200 pM levels in plasma (Bergseth et al., 2000), controls a second pathway that limits NO signaling by preventing activation of sGC (Isenberg et al., 2005b, 2006b). TSP1 also inhibits signaling downstream of cGMP, and in platelets cGMP-dependent protein kinase (cGK) is a second target of TSP1 (Isenberg et al., 2008b). Physiological levels of TSP1 potently inhibit NO-driven relaxation of contracting VSMC and thereby limit the ability of NO to increase tissue blood flow at rest and under stress (Isenberg et al., 2007a). The ability of TSP1 to block NO/cGMP signaling in vascular cells requires its cell surface receptor CD47 (Isenberg et al., 2006a). Targeting of either TSP1 or CD47 relieves the inhibition of sGC and significantly enhances tissue survival and blood flow after local ischemic challenges (Isenberg et al., 2007b,c).

NO also has systemic cardiovascular activities. Some NOS knockout mice exhibit hypertensive phenotypes (Ortiz and Garvin, 2003). Systemic administration of NO donors or nitrovasodilators alters blood pressure and cardiac function, leading to extensive use of these agents for treating chronic and acute cardiovascular diseases (Hermann et al., 2006). Based on their ability to limit NO signaling, we hypothesized that TSP1 and CD47 could also regulate systemic cardiovascular responses. We show here that TSP1 and CD47-null mice exhibit alterations in resting blood pressure and

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hyperdynamic responses to vasoactive challenges. These results demonstrate for the first time that a matricellular protein can acutely regulate blood pressure and cardiovascular responses to stress.

2. Results

2.1. TSP1-null mice are hypertensive with activity

Telemetry implants in WT and TSP1 null mice (Fig. 1) or WT and CD47 null mice (Fig. 2) allowed continuous monitoring of central arterial blood pressure during 12 h light/12 h dark cycles. TSP1 null mice demonstrated significant decreases in pulse pressure relative to WT mice during both active and inactive periods (Fig. 1, $P<0.045$). All other blood pressure parameters were comparable in WT and TSP1 null mice during the inactive light period of the day (Fig. 1A). During the active dark cycles, however, TSP1 null mice demonstrated significant increases in diastolic and MAP and a significant decrease in hear rate relative to WT mice (Fig. 1, Table 1). Systolic blood pressure was elevated to a lesser extent in TSP1 null mice and did not achieve significance (Table 1). Given that TSP1 null mice have higher cGMP levels in their VSMC, which decreases contraction, the increases in MAP and DBP are not a direct consequence of losing the vasopressor activity of TSP1 but could reflect an increase in autonomic tone in the null mice to compensate for the loss of TSP1 signaling (see below) or other activities of TSP1 that affect vascular function independent of CD47/cGMP signaling.

In contrast to TSP1 null mice, CD47 null mice demonstrated normal pulse pressure but increased changes in diastolic pressure and heart rate between active and inactive cycles compared to WT (Fig. 2, Table 1). The latter differences could reflect the higher activity level of the CD47 null cohort (23.7 ± 2.72 counts/min versus 11.7 ± 3.8 counts/min in wild type animals).

2.2. CD47 modulates resting peripheral blood pressure

Consistent with the telemetry data, awake TSP1 null mice showed slightly elevated peripheral MAP via tail cuff measurement, but this

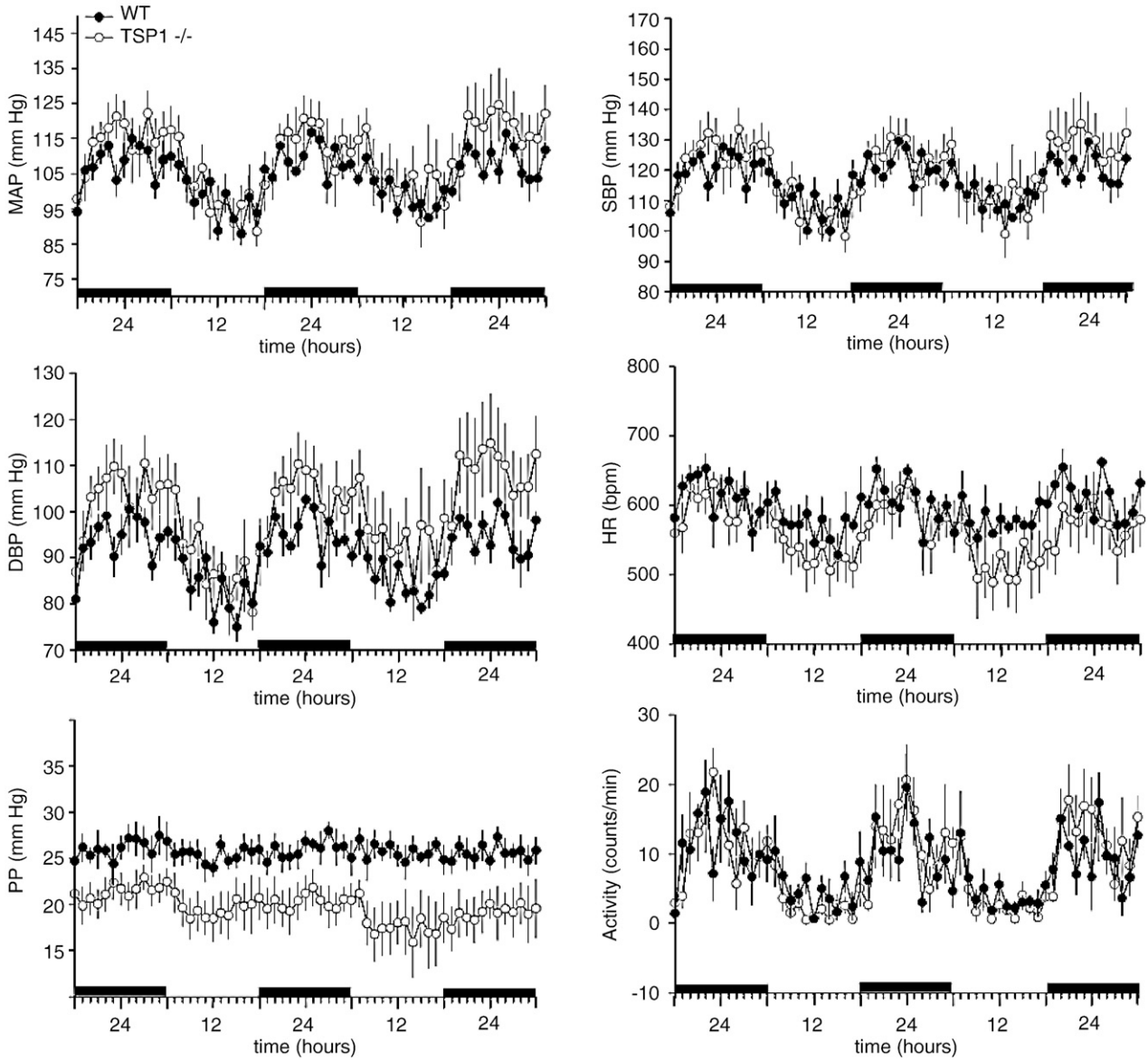


Fig. 1. Telemetry blood pressure and pulse recordings in conscious WT and TSP1 null mice. Following transmitter placement in age and sex matched mice, the mice were permitted to equilibrate for 7 days. Recordings of physiologic data were obtained the second post-operative week. Mean arterial pressure (MAP), diastolic blood pressure (DBP), and pulse pressure (PP), heart rate (HR), systolic blood pressure (SBP), and activity level data are presented ± SE for three consecutive wake (18:00 to 6:00 h)–sleep (6:00 to 1800 h) cycles over 72 h. Results are from 4 mice of each strain.

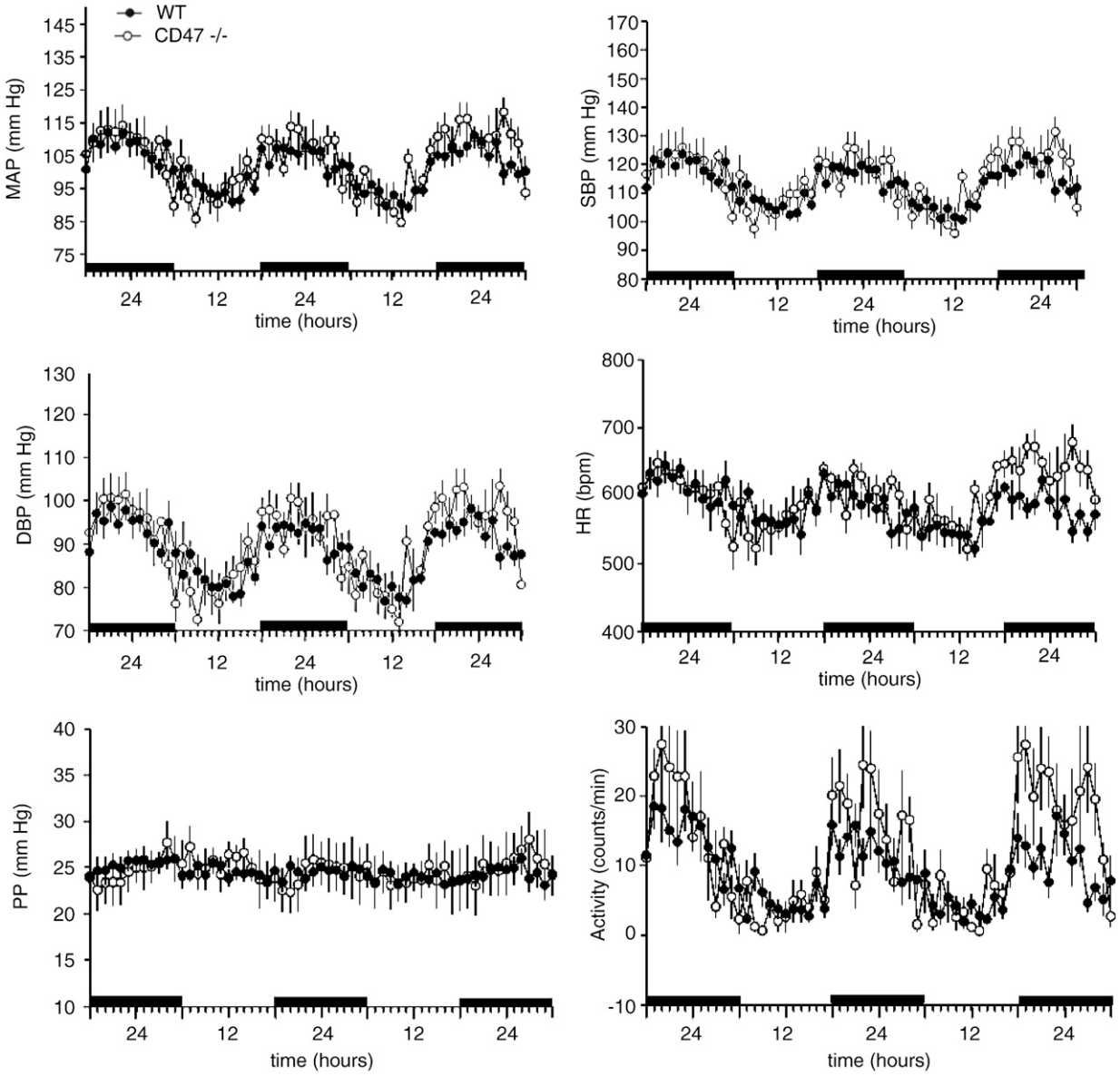


Fig. 2. Telemetry blood pressure and pulse recordings in conscious WT and CD47 null mice. Following transmitter placement in age and sex matched mice, mice were permitted to equilibrate for 7 days. Recordings of physiologic data were obtained the second post-operative week. Mean arterial pressure (MAP), diastolic blood pressure (DBP), and pulse pressure (PP), heart rate (HR), systolic blood pressure (SBP), and activity level data are presented ± SE for three consecutive wake (18:00 to 6:00 h)–sleep (6:00 to 1800 h) cycles over 72 h. Results are from 4 mice of each strain.

did not achieve significance (Fig. 3A), and their pulse rate did not significantly differ from that of WT (Fig. 3B). In contrast, CD47 null mice demonstrated significant increases in peripheral systolic, diastolic and MAP by tail cuff measurements (Fig. 3A). Comparison of MAP in WT and CD47 nulls ($n=4$) using a carotid catheter method under isoflurane anesthesia also detected approximately 15% higher MAP in CD47 null mice (Fig. 3C). No differences in ECG were found between the three strains (data not shown).

Table 1
Light/dark differences in central blood pressure in wild type and TSP1 null mice

Variable	WT dark	TSP1 null dark	WT light	TSP1 null light	ΔWT ($n=4$)	ΔTSP1 null ($n=4$)	*P* value[a]	CD47 null dark	CD47 null light	ΔCD47 null ($n=4$)	*P* value[b]
MAP (mmHg)	108.73 ± 0.41	115.79 ± 7.22	98.80 ± 0.74	100.31 ± 6.41	9.92 ± 0.83	15.48 ± 1.54	0.03	110.46 ± 4.06	95.08 ± 2.24	15.38 ± 1.93	0.018
SBP (mmHg)	121.11 ± 0.61	125.95 ± 7.48	110.91 ± 1.12	109.7 ± 6.44	10.20 ± 0.76	16.21 ± 2.00	0.05	122.24 ± 4.52	106.65 ± 2.16	15.59 ± 2.36	0.039
DBP (mmHg)	95.10 ± 0.94	105.60 ± 7.09	85.31 ± 0.66	90.48 ± 6.38	9.80 ± 0.98	15.12 ± 1.2	0.03	97.30 ± 3.68	81.83 ± 2.67	15.47 ± 1.50	0.005
HR (bpm)	610.1 ± 15.6	587.0 ± 28.5	581.9 ± 14.9	529.9 ± 33.3	28.2 ± 6.0	57.1 ± 5.2	0.01	632.4 ± 1.6	575.1 ± 17.4	57.3 ± 4.6	0.001
PP (mmHg)	25.94 ± 1.37	20.25 ± 1.96	25.55 ± 1.08	19.17 ± 2.14[c]	0.39 ± 0.32	1.07 ± 0.91	0.51	24.91 ± 2.06	24.74 ± 2.05	0.16 ± 0.96	0.729
Activity (counts/min)	10.69 ± 1.85	12.09 ± 3.10	4.82 ± 1.02	2.98 ± 0.41	5.86 ± 0.91	9.11 ± 2.79	0.34	19.29 ± 4.03[c]	4.63 ± 1.27	14.66 ± 2.86	0.036

[a] *P*-values for ΔWT versus ΔTSP1 null.
[b] *P*-values for ΔWT versus ΔCD47 null.
[c] Significantly differs from WT ($P=0.04$).

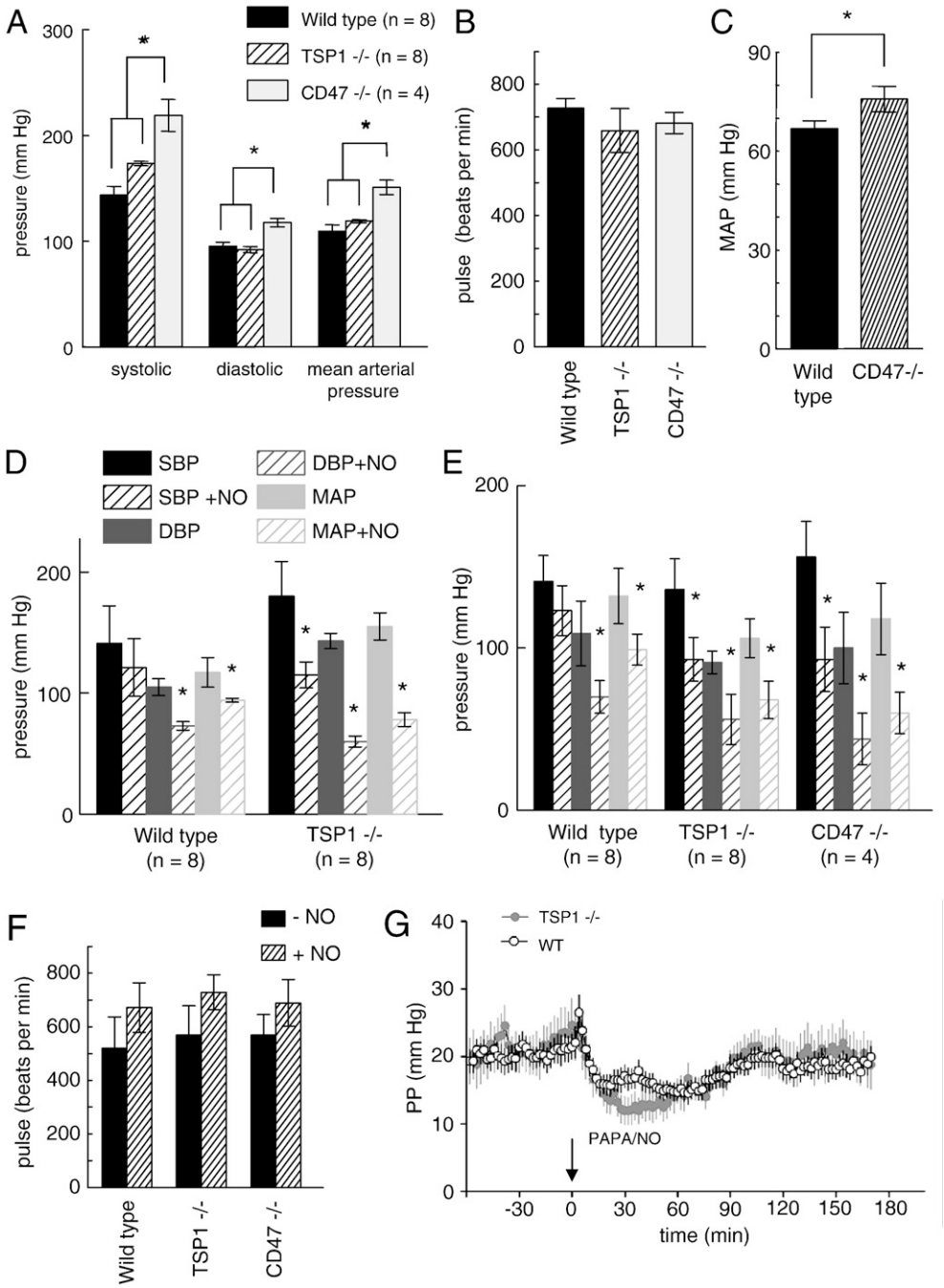


Fig. 3. TSP1 and CD47 limit blood pressure changes in response to NO. Age and sex matched awake WT, TSP1 and CD47 null mice underwent analysis of blood pressure (A) and pulse (B) via tail cuff. Blood pressure was assessed via a carotid arterial catheter in 5 WT and 4 CD47 −/− mice under anesthesia (C). Awake WT and TSP1 null were treated with a rapid releasing NO-donor (1 μl/g i.p. of 100 mM DEA/NO) and blood pressure measured by tail cuff (D). Awake WT, TSP1 and CD47 null mice underwent treatment with an intermediate releasing NO-donor (1 μl/g i.p of 100 mM PAPA/NO) and blood pressure (E) and pulse (F) measurements obtained via tail cuff. Results are of the mean ± SD of 8 mice each of WT and TSP1 null and 4 CD47-null. Awake WT and TSP1 null mice underwent telemetric analysis of physiologic data before and after treatment with PAPA/NO. Pulse pressure (PP ± SE, G) is presented from 4 mice of each strain. Experiments were repeated a minimum of 3 times. Asterisk (*) indicates pressure values following treatment that significantly differ from baseline pressure values ($P<0.05$).

2.3. TSP1 and CD47 limit blood pressure responses to NO

The above moderate differences in basal blood pressure parameters suggest that endogenous TSP1 and CD47 play subtle roles in resting blood pressure regulation. In addition to their common regulation of NO/cGMP signaling, the differences between TSP1- and CD47-null mice could reflect anatomical effects of these gene deletions or the ability of TSP1 to act through additional TSP1 receptors. Furthermore, several homeostatic pathways could compensate for the expected hypertensive activity of TSP1/CD47 signaling. Because our primary goal was define the roles of TSP1 and CD47 in acute cardiovascular regulation, we next examined acute responses of the mice to specific vasoactive challenges. Because TSP1/CD47 signaling limits NO responses in VSMC in vitro by preventing sGC activation (Isenberg et al., 2006a,b) and tissue blood flow in response to exogenous NO in vivo (Isenberg et al., 2007a), we proposed that the null mice should exhibit a greater acute hypotensive response following a systemic NO challenge. Age and sex matched WT and TSP1-null mice were challenged via i.p. injection using 1 μl/g of 100 mM DEA/NO, and awake resting peripheral blood pressure and

pulse were determined by tail cuff. NO treatment significantly decreased systolic, diastolic, and MAP in WT mice, but significantly greater decreases in blood pressure measurements were noted in mice lacking TSP1 (Fig. 3D). Mean pulse values tended to increase modestly in all mice following DEA/NO challenge (data not shown). Time course data demonstrated delayed recovery of baseline pressure levels in TSP1 null versus WT mice (data not shown).

Similar differences were found when mice were challenged using an NO donor with slower release kinetics (PAPA/NO, $t^{1/2} = 15$ min, Thomas et al., 2002, Fig. 3E), with the drop in peripheral MAP always greater in the absence of TSP1. Interestingly, CD47 null mice treated with PAPA/NO showed the greatest drop in peripheral MAP (Fig. 3E). Mean pulse was only modestly increased above resting values in mice treated with PAPA/NO (Fig. 3F). Central pulse pressure measurements using telemetry confirmed an enhanced hypotensive response to PAPA/NO challenge in the TSP1 nulls (Fig. 3G). These results are consistent with the increased responsiveness of sGC to exogenous NO in VSMC from both null mice in vitro (Isenberg et al., 2006a).

2.4. TSP1 and CD47 limit cardiac responses following NO challenge

In addition to its effects on arterial tone, NO increases cardiac function in both normal (Prendergast et al., 1997) and failing hearts. (Inglessis et al., 2004) To investigate the potential role of its antagonist TSP1 in systemic cardiac physiology, age and sex matched WT, TSP1 and CD47 null mice underwent cardiac Doppler analysis (Fig. 4). Under 1.5% isoflurane anesthesia and at constant core temperature (35.5 °C), TSP1 and CD47 null mice demonstrated elevated heart rates (Fig. 4A). These differences were maintained following i.p. injection with a rapidly releasing NO donor (0.5 µl/g body weight of 100 mM DEA/NO, $t^{1/2} = 2$–4 min, Thomas et al., 2002), which increased heart rate in all mice. Both null strains demonstrated differences in ejection fractions compared to WT mice after induction of isoflurane general anesthesia (data not shown).

More profound alterations in cardiac ejection fraction and cardiac output were observed after exogenous NO challenge. WT mice demonstrated mild increases in ejection fraction and cardiac output (12.2 ± 5.3 and 13.2 ± 15.3% increases respectively) which rapidly returned to pre-treatment baseline (Fig. 4B, C). In contrast, TSP1 null mice demonstrated dramatic increases in ejection fraction and cardiac output (44 ± 2.6 and 48 ± 1.4% respectively). CD47 null mice showed similarly enhanced cardiac output and ejection fraction when treated with the NO donor (43.3 ± 10.6 and 54.5 ± 8.6 respectively). In both TSP1 and CD47 null mice, the increase in cardiac function always persisted longer than in WT mice.

The above cardiac responses may be secondary to direct effects of TSP1/CD47 on arterial tone. However, TSP1/CD47 signaling is known to limit tissue cGMP in skeletal muscle (Isenberg et al., 2007b),

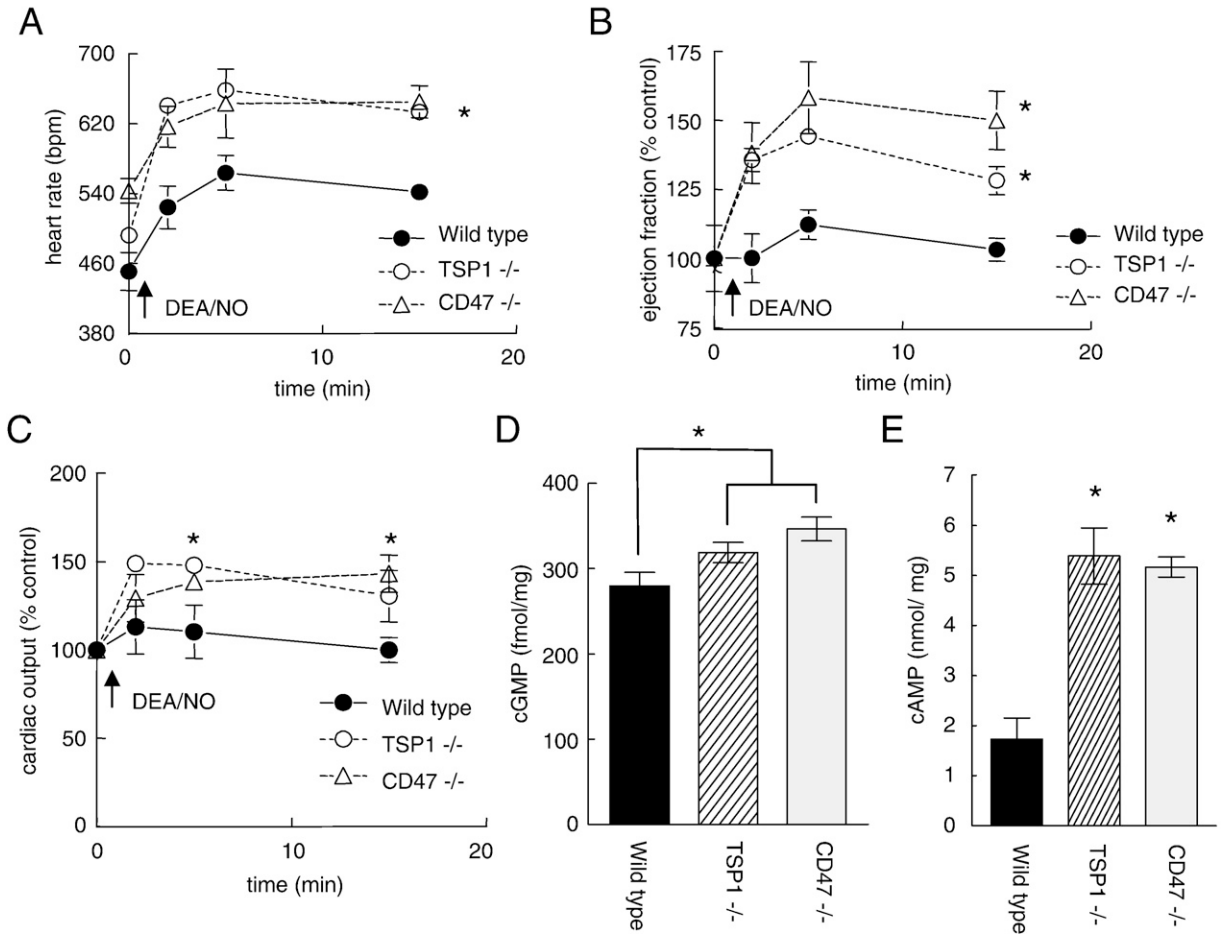


Fig. 4. TSP1 and CD47 modulate cardiac responses to vasoactive challenge. Age and sex matched WT ($n = 4$), TSP1 null ($n = 4$), and CD47 null ($n = 4$) underwent echocardiography. After baseline recordings, mice were challenged with NO (0.5 µl/g body weight of 100 mM DEA/NO i.p.) and data gathered at 2, 5 and 15 min. Heart rate (A) is presented as the mean values ± SD, whereas ejection fraction (B) and cardiac output (C) as presented as percent control. Asterisk (*) indicates curves are of statistically significant difference when compared with corresponding WT mice (A, B) or individual points of curves are statistically significant compared to WT (C) ($P < 0.05$). Age and sex matched WT, TSP1 and CD47 null mice underwent euthanasia via cervical dislocation. Hearts were excised, pulverized in liquid nitrogen and tissue cGMP (D) or cAMP (E) levels determined. Results are expressed as the mean ± SD for 6 of each strain (cGMP) and 4 of each strain (cAMP). Asterisk (*) indicates statistically significant difference between TSP1 and CD47 null when compared with corresponding WT samples (D, E) ($P < 0.05$).

suggesting that some cardiac changes in the nulls may represent local effects of TSP1 in the heart. Consistent with this hypothesis, analysis of tissue cGMP levels in left ventricular samples demonstrated a significant elevation of cardiac muscle cGMP in TSP1 and CD47 null mice compared to WT (Fig. 4D). Cardiac function also depends on cAMP levels, which in turn are regulated by several PDEs that are modulated by cGMP (Zaccolo and Movsesian, 2007). Whole tissue cAMP levels were markedly increased in hearts from TSP1 and CD47 null mice (Fig. 4E).

2.5. TSP1 limits cardiovascular collapse following autonomic blockade

Input from the autonomic nervous system is a critical homeostatic mechanism to maintain MAP and to minimize alterations in blood pressure due to vascular stress. Conversely, autonomic blockade using a centrally active agent such as hexamethonium chloride removes sympathetic tone and enhances cardiovascular responses to alterations in NO levels (Scrogin et al., 1998; Shibao et al., 2007), Because autonomic tone and TSP1 signaling limit the vasodilation response to NO via different mechanisms, we proposed that autonomic blockade might further enhance responses of TSP1 null mice to endogenous NO. Telemetric blood pressure analysis demonstrated dramatically greater decreases in central MAP ($P<0.05$) after central autonomic blockade using a limited dose of hexamethonium chloride in awake TSP1 and CD47 null mice compared to WT (Fig. 5A). The hypotensive response in TSP1 and CD47 null mice following autonomic blockade was also significantly prolonged compared to WT mice.

We further compared responses to autonomic blockade in mice maintained under general anesthesia using 1.5% isoflurane by challenging with a higher dose of hexamethonium than used in Fig. 5A. Cutaneous perfusion was measured every 2.5 min via Doppler (Fig. 5B). Both WT and TSP1 null mice demonstrated decreased cutaneous perfusion and eventual cardiovascular collapse and death. However, loss of perfusion and death was significantly faster in the absence of TSP1 and diverged from that of WT mice after 5 min. WT mice sustained cutaneous perfusion for an additional 10 min, indicating an important role for endogenous TSP1 in maintaining perfusion under this combined stress.

2.6. TSP1 augments acute blood pressure responses to epinephrine

Autonomic stimulation of sympathetic nerves leads to norepinephrine-stimulated vasoconstriction of arteries (Lee et al., 2003). This can be mimicked by treatment with epinephrine (0.05 μg/animal via i.p. injection), which produced the expected increase in peripheral MAP in WT mice (Fig. 5C). However, TSP1 null mice did not show a significant increase in MAP, presumably because of the greater opposing NO signaling in these mice. Mean pulse values were moderately increased in both WT and TSP null mice (Fig. 5D). Remarkably, the same dose of epinephrine proved fatal to CD47 null mice, precluding further measurements (data not shown).

2.7. TSP1 limits blood pressure response to isoflurane

Central anesthetic agents are known to have strong effects upon blood pressure and cardiac performance (Becker and Haas, 2007; Reich et al., 2005; Torri et al., 2000). To assess whether differential responses to anesthesia contributed to the greater sensitivity of TSP1 null mice in Fig. 5B, we compared peripheral blood pressure responses to isoflurane. Induction of isoflurane inhalation anesthesia resulted in a greater decrease in tail cuff blood pressure in TSP1 null mice than in WT (Fig. 5E).

To determine whether the differential response to isoflurane was associated with altered circulating TSP1 levels following exposure to isoflurane we determined plasma TSP1 levels in groups of 5 WT mice before and after anesthesia. Plasma TSP1 levels tended to decrease following 30 min under inhalation isoflurane (34 ± 18 versus 13 ± 10 ng/ml), but the difference did not achieve significance ($P=0.066$ by two-tailed t-test). Although this could suggest that isoflurane lowers blood pressure in WT mice by decreasing TSP1, we caution that circulating TSP1 should not acutely increase blood pressure because it cannot cross the subendothelial basement membrane to engage VSMC CD47 and inhibit their relaxation by NO.

3. Discussion

Previous studies have shown that disrupting some components of elastic matrix in blood vessels can affect blood pressure. Partial loss of vascular elastin in mice results in elevated pulmonary circulatory pressures, altered vascular compliance, and cardiac hypertrophy (Shifren et al., 2008; Wagenseil et al., 2007, 2005). Loss of the associated protein fibulin-5 also causes a decrease in vascular compliance (Kelleher et al., 2004). Finally, polymorphisms in fibrillin-1 in humans are associated with elevated carotid pulse pressure (Medley et al., 2002). These phenotypes can all be attributed to the effects of these structural matrix proteins on the elastic properties of resistance vessels. In contrast, TSP1 is not a structural component of blood vessels. However, its presence in the ECM at very low levels is sufficient to limit VSMC responses to the potent physiological vasodilator NO (Isenberg et al., 2007a, 2006b).

The present results demonstrate a broader role for TSP1 in controlling peripheral and central blood pressure and cardiovascular responses to several vasoactive challenges. TSP1 null mice have a lower central pulse pressure than WT mice and exhibit several activity-dependent alterations in central and peripheral blood pressure parameters. These differences are not consistent with a simple hypotensive response due to elevated vascular cGMP levels in the TSP1 null but may reflect homeostatic efforts to compensate for loss of the hypertensive activity of TSP1. However, the enhanced hypotensive responses of TSP and CD47 null mice to NO challenge are consistent with and extend our previous finding that TSP1 null mice at rest demonstrate a greater regional increase in skeletal muscle blood flow in response to NO (Isenberg et al., 2007a). Hydrolysis of cGMP by PDEs has been considered the major negative regulator of NO signaling in vascular physiology (Mullershausen et al., 2003; Rybalkin et al., 2003). However, TSP1 also regulates NO signaling by limiting sGC and cGK activation (Isenberg et al., 2005b). Elevated basal cGMP levels in tissue and vascular cells from TSP1 or CD47 null mice show that cGMP signaling is continuously modulated by physiological concentrations of TSP1 independent of PDE activity (Isenberg et al., 2006a). Thus, TSP1 in the ECM surrounding VSMC constantly signals via CD47 to enhance blood pressure and temper both regional and systemic vasodilator activities of endogenous NO.

The null mice also exhibit exaggerated responses to anesthesia or loss of autonomic regulation and a more limited response, in the case of TSP1 null mice, to epinephrine. These results indicate an interdependence of TSP1/CD47 and autonomic regulation. Because autonomic tone also differs between asleep and awake animals, a slight sympathetic overcompensation may account for the increased MAP in TSP1 null mice during their active cycles. This could be a compensation for the decreased pulse pressure in the TSP1 nulls, which is consistent with TSP1 being a vasopressor. However, additional studies will be required to identify why CD47 null mice lack a decreased pulse pressure. These differences between the CD47 null and TSP1 null mice may reflect some compensation for loss of TSP1 by other thrombospondins expressed in vascular cells such as TSP2 and TSP4 (Lamy et al., 2007; Lopes et al., 2003; Stenina et al., 2003a). Our recent data indicates that TSP4 has some activity to inhibit NO/cGMP signaling in endothelial cells via CD47, but TSP2 is much less active, and both are weaker than TSP1 (Isenberg et al., 2009). Furthermore, the absence of TSP2 in vascular cells does not

result in elevated cGMP levels. Alternatively, other CD47 ligands such as SIRPα (Lee et al., 2007) may influence cardiovascular signaling through CD47 in a TSP1-independent manner.

Cardiac function is regulated by cGMP and cAMP. As previously shown for cGMP in skeletal muscle (Isenberg et al., 2007b), cGMP levels are increased in cardiac muscle of TSP1 and CD47 nulls. Although cGMP can influence cAMP levels by modulating PDEs, this feedback is unlikely to explain the greater increase in cAMP in null heart tissue. CD47 ligation in VSMC and T cells decreases cAMP levels by inhibiting adenylate cyclase in a heterotrimeric G_i-dependent manner (Manna and Frazier, 2003; Wang et al., 1999), suggesting that TSP1/CD47 signaling may regulate cardiac cAMP levels via similar mechanism. However, the enhanced cardiac responses to NO in TSP1 and CD47 null mice probably also reflect compensation for differences in peripheral vasodilation in these mice.

Because homeostatic compensation mechanisms limit changes in resting cardiovascular parameters, the roles of TSP1 and CD47 in maintaining systemic cardiovascular physiology become more evident following vasoactive challenge. In WT mice, moderate doses of NO enhance cardiac function and heart rate (Kojda and Kottenberg, 1999; Mohan et al., 1996; Shah and MacCarthy, 2000), but these responses are modest and rapidly dampened by autonomic compensation. In contrast, a similar NO challenge in TSP1 or CD47 null mice induces greater increases in heart rate, cardiac output and ejection fraction with delayed return to baseline.

Lack of TSP1 is also associated with less elevation of blood pressure to epinephrine. Conversely, chemical sympathectomy with hexamethonium, which effectively ablates the vasoconstrictor effects of peripheral epinephrine on vascular tone, enhances hypotension in TSP1 and CD47 null mice. Absent sympathetic tone, TSP1 is required for maintenance of blood pressure and cardiac output. Under such conditions, lack of TSP1 leads to accelerated loss of blood pressure, tissue perfusion and death compared to WT.

Because loss of TSP1/CD47 signaling can alter blood pressure and hemodynamic responses to stress, the potential for altered vascular matrix TSP1 levels to increase blood pressure or decrease compliance in response to physiological NO signaling should be considered. TSP1 expression is elevated in the blood vessel wall or surrounding matrix in atherosclerosis, restenosis, tissue ischemia, and diabetes (Chen et al., 1999; Riessen et al., 1998; Roth et al., 1998; Stenina et al., 2003b). In these diseases, accumulation of TSP1 in the matrix surrounding VSMC could chronically limit NO-mediated vasodilation. Some cancers are associated with elevated circulating plasma TSP1 levels (Yamashita et al., 1998). However, it remains unclear whether elevated circulating TSP1 could be hypertensive since it would not have access to CD47 on VSMC.

Given the association between a coding polymorphism in TSP1 and early coronary artery disease (Topol et al., 2001; Zwicker et al., 2006), our results raise the intriguing possibility that altering the antagonism between the TSP/CD47 and NO/cGMP pathways could lead to cardiovascular pathology in people. This polymorphism alters the conformation of signature domain of TSP1, which contains the CD47 binding site (Isenberg et al., 2009), but it is not known whether the polymorphism directly affects CD47 binding. Furthermore, these results suggest that inhibitors of TSP1/CD47 interactions could have therapeutic application to regulate blood pressure, cardiac dynamics, and regional blood flow.

4. Experimental procedures

4.1. Animals

Wild type (WT), TSP1 and CD47 null mouse colonies in a pure C57BL/6 background were housed under pathogen-free conditions and had ad libitum access to filtered water and standard chow. Both null strains were rederived with back crossing before use, and the CD47 null strain was back crossed against the wild type C57BL/6 colony within one year of initiating this study. Handling and care of animals were in compliance with guidelines of the Animal Care and Use Committee of the National Cancer Institute.

4.2. Reagents

The NO donor diethylamine NONOate (DEA/NO) was provided by Dr. Larry Keefer (NCI, Frederick, Maryland). The NO donor (Z)-1-[N-(3-ammoniopropyl)-N-(n-propyl)amino]-NONOate (PAPA/NO) was from Cayman Chemical Company (Ann Arbor, MI). Isoflurane (Forane®, USP) was from Baxter Healthcare Corp. (Deerfield, IL). Epinephrine hydrochloride and hexamethonium chloride were from Sigma-Aldrich (St. Louis, MO).

4.3. Tail cuff blood pressure and pulse measurements

Peripheral systolic, diastolic and mean arterial pressure (MAP) and pulse were measured using a computerized tail cuff system (Hatteras Systems, MC4000 Blood Pressure Analysis System, Cary, NC). Each animal underwent a cycle of 10 preliminary and 50 experimental pressures measurements for data acquisition and calculation. Mice were acclimated to the measuring system for 4 days with studies performed on the 5th day. Recordings were obtained at the same time each day.

4.4. Internal blood pressure measurement via telemetry

Mice were anesthetized with ketamine/xylazine (90 and 10 mg/kg, respectively). The telemeter catheter was inserted into the left carotid artery at the aortic arch, and the telemeter body (model TA11PA-C20, Data Sciences International, St. Paul, MN) placed in a subcutaneous pocket. The signal was processed using a RPC-1 receiver, a 20-channel data-exchange matrix, APR-1 ambient pressure monitor, and a Dataquest ART 2.3 acquisition system (Data Sciences International). Data was acquired for 10 s every 2 min and 100-min averages calculated.

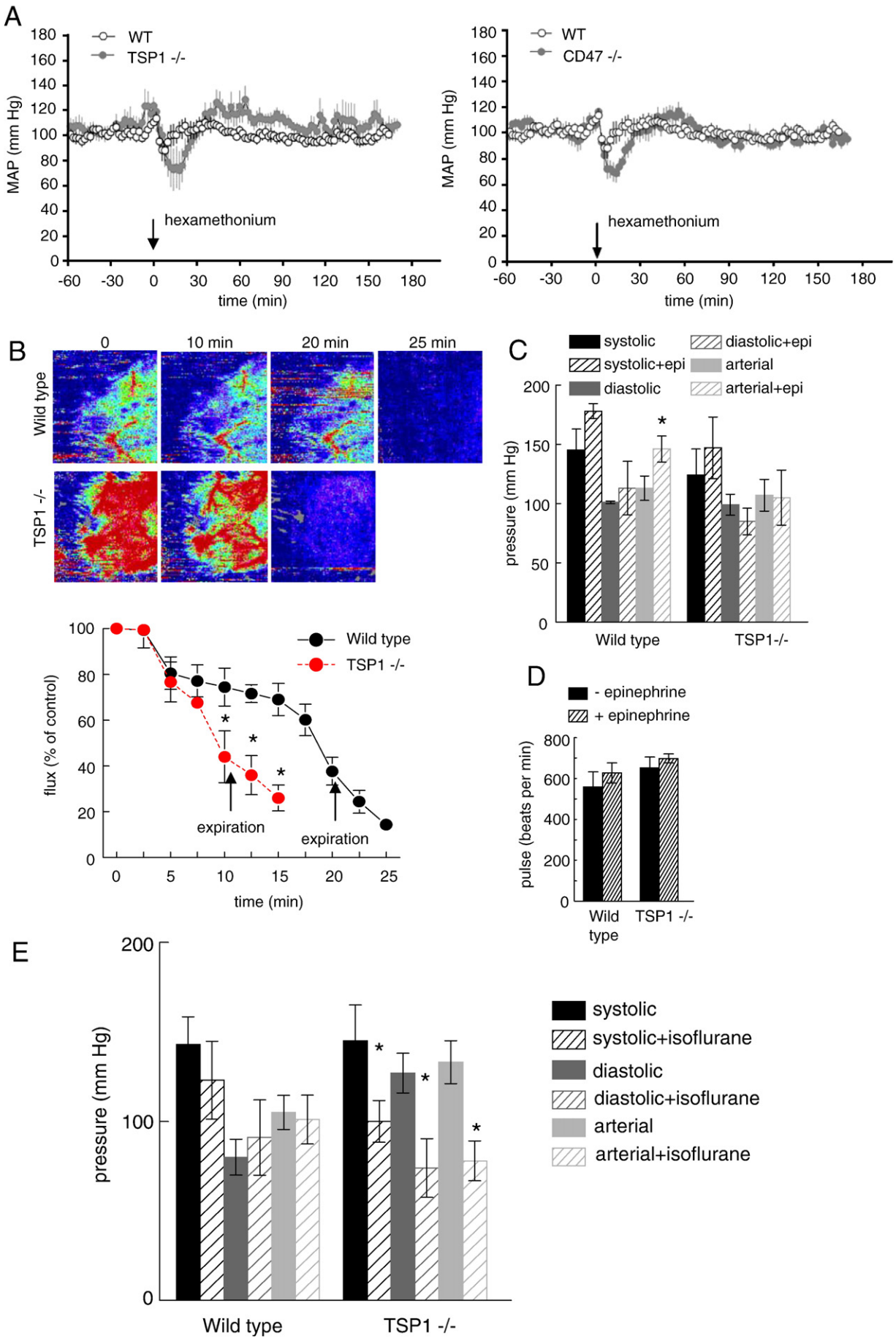
4.5. ECG measurements

ECG measurements were obtained using the ECGenie^{Tm} ECG Screening System (Mouse Specifics, Inc., Boston, MA). Mice were placed on the recording stage and allowed to acclimate to the system, and measurements were obtained passively.

4.6. In vivo echocardiography

Analysis of murine hearts in WT, TSP1 and CD47 null age and sex matched mice was performed using a Vevo 770 High-Resolution In

Fig. 5. TSP1 protects against cardiovascular collapse following autonomic blockade and regulates response to epinephrine and general anesthesia. Telemetric transmitters were placed in age and sex matched WT, TSP1 and CD47 null mice (A). On post-operative day 7 mice were treated with hexamethonium chloride (25 μg/g in 100 μl of sterile PBS i.p.) and MAP ± SE determined. Experiments were repeated a minimum of three times in 4 mice of each strain. (B) WT and TSP1 null mice underwent laser Doppler analysis of cutaneous perfusion at 2.5 min intervals ± hexamethonium (30 μg/g) i.p. Results are the mean ± SD for 4 mice of each strain. Asterisk indicates values are of statistically significant difference when compared with corresponding WT mice ($P<0.05$). Age and sex matched mice underwent blood pressure (C) and pulse (D) analysis via tail cuff before and after treatment with epinephrine (0.05 μg/animal i.p.). Asterisk (*) indicates arterial pressure following treatment significantly differs compared with baseline (C) ($P<0.05$). Age and sex matched mice at a core temperature of 35.5 °C underwent blood pressure analysis before and after receiving 1.5% inhalation isoflurane (E). Asterisk (*) indicates significant difference in change in pressure between TSP1 compared to wild type following treatment ($P<0.05$).


A
WT
TSP1 -/-
MAP (mm Hg)
hexamethonium
time (min)
WT
CD47 -/-
MAP (mm Hg)
hexamethonium
time (min)
B
0
10 min
20 min
25 min
Wild type
TSP1 -/-
Wild type
TSP1 -/-
flux (% of control)
expiration
expiration
time (min)
C
systolic
systolic+epi
diastolic
diastolic+epi
arterial
arterial+epi
pressure (mm Hg)
Wild type
TSP1-/-
D
- epinephrine
+ epinephrine
pulse (beats per min)
Wild type
TSP1 -/-
E
pressure (mm Hg)
Wild type
TSP1 -/-
systolic
systolic+isoflurane
diastolic
diastolic+isoflurane
arterial
arterial+isoflurane

Vivo Imaging System (VisualSonics, Toronto, ON, Canada) with a RMV 707 "high frame" scan head for high frame rate and real-time small animal imaging with a center frequency of 30 MHz and a frequency band 15–45 MHz. Mice were maintained on 1.5% isoflurane and core temperature of 35.5 °C. Long-axis imaging was taken to visualize left ventricle, right ventricle, ascending aorta, and right ventricular outflow tract. The short-axis imaging was taken to view the LV and RV movement during diastole and systole stages. Anatomical M-mode (AM-Mode) provided the ability to obtain anatomically correct LV measurements. All data and images were analyzed by Advanced Cardiovascular Package Software (VS-11560, VisualSonics, Toronto, ON, Canada). Measurements from three continuous cardiac cycles were averaged to obtain a value for each data point.

4.7. Skin blood flow

Skin blood flow was measured on the dorsum of age and sex matched mice using laser Doppler imaging (MoorLD1-2λ, Moor Instruments, Devon, England) (Isenberg et al., 2007b). Anesthesia was provided by 1.5% inhalation isoflurane. Core temperature was monitored by rectal probe. After equilibration to the experimental set-up, analysis of blood flow was performed. The following instrument settings were used: override distance 21 cm; scan time 4 ms/pixel. Results are expressed as a percent of the control baseline for the ROI.

4.8. Plasma TSP1 analysis

Plasma samples were collected using EDTA as anti-coagulant from groups of 5 WT mice with or without prior anesthesia for 30 min using 1.5% inhalation isoflurane. TSP1 levels were quantified using a heparin-BSA capture enzyme-linked immunoassay with detection using a polyclonal rabbit anti-TSP1 antibody as previously described (Isenberg et al., 2005a).

4.9. Cardiac cGMP and cAMP

cGMP and cAMP from cardiac muscle tissue were determined by immunoassay as previously described (Isenberg et al., 2007b).

4.10. Statistics

Results are presented as the mean ± SD or mean ± SE with significance calculated by the Student's *t* test or one-way ANOVA with Tukey post hoc test and significance taken at P values < 0.05. Data represents results of studies performed in a total of 117 mice distributed as follows: WT, $n = 46$; TSP1 null, $n = 46$; CD47 null, $n = 25$.

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