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RESEARCH PAPER

Thrombospondin-1 is an inhibitor of pharmacological activation of soluble guanylate cyclase

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Background and purpose: Soluble guanylate cyclase (sGC) is the signal transduction enzyme most responsible for mediating the effects of nitric oxide (NO). Recently, NO-independent small molecule activators of sGC have been developed that have promising clinical activities. We have shown that the secreted matrix protein thrombospondin-1 (TSP-1) binds to CD47 and potently inhibits NO stimulation of sGC in endothelial and vascular smooth muscle cells (VSMCs) and platelets. Here we show that TSP-1 signalling via CD47 inhibits sGC activation by NO-independent sGC activating small molecules.
Experimental approach: Vascular smooth muscle cells and washed human platelets were pretreated with TSP-1 (2.2 nM) in the presence of haeme-dependent sGC activators (YC-1, BAY 41-2272), and a haeme-independent activator (meso-porphyrin IX), and cGMP levels were measured. The effect of sGC activators on platelet aggregation and contraction of VSMC embedded in collagen gels was also assayed in the presence and absence of TSP-1.
Key results: Thrombospondin-1 inhibited sGC activator-dependent increase in cGMP in VSMC and platelets. TSP-1 pretreatment also inhibited the ability of these agents to delay thrombin-induced platelet aggregation. TSP-1 pretreatment reduced the ability of sGC activating agents to abrogate VSMC contraction *in vitro*.
Conclusions and implications: This work demonstrates that TSP-1 is a universal inhibitor of sGC, blocking both haeme-dependent and haeme-independent activation. These data coupled with the reported increases in TSP-1 with age, diabetes, ischaemia/reperfusion, and atherosclerosis implies that the therapeutic potential of all drugs that activate sGC could be compromised in disease states where TSP-1/CD47 signalling is elevated.
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Abbreviations: ACD, acid citrate dextrose; DEANO, diethylamino NONOate; PPIX, meso-porphyrin IX; PRP, platelet rich plasma; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside; TSP-1, thrombospondin-1; VSMC, vascular smooth muscle cell

Introduction

Soluble guanylate cyclase (sGC) is a heterodimeric haeme protein with an approximate mass of 150 kDa (~70 kDa α and ~80 kDa β) (Kamisaki *et al.*, 1986). sGC catalyses the production of the second messenger molecule cGMP from GTP (Arnold *et al.*, 1977). sGC is the primary receptor for nitric oxide (NO), and NO-cGMP signalling controls diverse aspects of mammalian physiology including vascular smooth muscle relaxation (Ignarro and Kadowitz, 1985), platelet function (Buechler *et al.*, 1994), neurotransmission (Garthwaite *et al.*, 1988; Snyder and Bredt, 1991), and gastric motility (Vanneste *et al.*, 2007). NO binds to the haeme of guanylate cyclase releasing an axial histidine ligand to stimulate its production of cGMP over 200-fold (Gerzer *et al.*, 1981; Ignarro *et al.*, 1986; Burstyn *et al.*, 1995; Stone and Marletta, 1994; Zhao *et al.*, 1999). Cardiovascular diseases are often attributed to an insufficiency of endogenous NO and associated sGC signalling (Challah *et al.*, 1997; Freedman *et al.*, 1998); however, the clinical application of NO is limited to indirect NO donors (SNP, nitroglycerin) (Feelisch, 1998) that cannot be used chronically due to tolerance (Munzel *et al.*, 2005) and toxicity issues (Rindone and Sloane, 1992). This has led to the development of NO independent small molecule activators of sGC that directly stimulate sGC as well as lower the EC_{50} for NO

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activation of sGC. These are termed haeme-dependent activators. Another class activate sGC to a similar extent as NO but are independent of the presence of haeme or NO. These are termed haeme-independent activators (Evgenov *et al.*, 2006). The precise binding sites for the haeme-dependent activators including YC-1, BAY 41-2272, BAY 41-8543, CFM-1571 and A-350619 are still a matter of controversy (Kharitonov *et al.*, 1999; Stasch *et al.*, 2001; Lamothe *et al.*, 2004), but they do not bind the haeme like NO and probably bind to an allosteric site in the N-terminal portion of the protein (Hu *et al.*, 2008). The haeme-independent activators including BAY 58-2667 and HMR-1766 bind instead of the haeme and induce the fully activated conformation of the enzyme (Evgenov *et al.*, 2006). Both BAY 58-2667 (Frey *et al.*, 2008) (ClinicalTrials.gov Identifier: NCT00559650) and HMR-1766 are currently in phase II clinical trials as drugs for treating cardiovascular disease.

We have previously shown that the activation of sGC by NO is controlled by the ubiquitous transmembrane receptor CD47 and its ligand thrombospondin 1 (TSP-1) (Isenberg *et al.*, 2006a, 2008d). TSP-1 binding to CD47 inhibits NO activation of sGC with an EC_{50} in the picomolar range (Isenberg *et al.*, 2005; 2009a). Interruption of this process by an antisense morpholino directed at CD47 or by some antibodies that bind to TSP-1 or CD47 restores sGC sensitivity to NO. Additional studies have shown that antisense suppression of CD47 or antibody targeting of TSP-1 or CD47 can improve acute tissue survival of fixed ischaemic injuries, ischaemia-reperfusion injuries associated with organ transplantation, and soft tissue injuries caused by ionizing radiation (Isenberg *et al.*, 2007c; 2008a,c). Finally, TSP-1 limits blood pressure responses to exogenous vasoactive agents (Isenberg *et al.*, 2009b). Increased TSP-1 expression is associated with several major chronic diseases including atherosclerosis, diabetes, and aging, and this is beginning to be recognized as a cause of deficient NO/cGMP signalling, suggesting that drugs targeting CD47 could provide benefit to these patients (Riessen *et al.*, 1998; Roth *et al.*, 1998; Favier *et al.*, 2005; Isenberg *et al.*, 2007b; 2008b).

The work presented herein details the inhibitory effect of TSP-1/CD47 signalling on sGC activating compounds for endpoints related to cardiovascular function. The implications of this work are discussed regarding the diminished efficacy of these drugs in the presence of physiological and pathophysiological amounts of TSP-1.

Methods

Reagents

Meso-porphyrin IX (PPIX) was purchased from Frontier Scientific (Logan, UT). 5-[1-(phenylmethyl)-1H-indazol-3-yl]-2-furanmethanol (YC-1) and isobutylmethylxanthine (IBMX) were purchased from EMD chemicals (Darmstadt, GER). 3-(4-Amino-5-cyclopropylpyrimidine-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridine (BAY 41-2272) was purchased from Alexis (Lausen, Switzerland). Thrombin was obtained from Sigma (St Louis, MO, USA); M199 medium, glutamine and penicillin/streptomycin from Gibco (Grand Island, NY, USA); SmBm was from Lonza (Walkersville, MD, USA). TSP-1 was prepared, as previously described, from fresh platelets obtained under an approved protocol from the transfusion service of the National Institutes of Health (Roberts *et al.*, 1994). The nitric oxide donor diethylamine/NONOate (DEA/NO) was kindly provided by Dr Larry Keefer (NCI-Frederick, MD, USA). PPIX, YC-1 and BAY 41-2272 stock solutions were made up in DMSO (Sigma). IBMX stock solutions were made up in ethanol.

Preparation of human platelets

Platelets, obtained from healthy volunteers through the NIH blood bank, were pelleted from platelet-rich plasma (PRP) by centrifugation for 10 min at 200 g. They were then resuspended in acid citrate dextrose (ACD; 85 mM citric acid, 65 mM sodium citrate, 100 mM glucose, pH 5.1) at a ratio of 1:10 at room temperature. Platelets were pelleted again and resuspended in 10 mL Tyrode buffer (137 mM NaCl, 3 mM KCl, 12 mM $NaHCO_3$, 0.3 mM $NaHPO_4$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, 5 mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), 3.5 mg·mL^{-1} BSA, pH 7.4). The final platelet number was adjusted to 6.5×10^5 μL^{-1} in a cuvette containing 500 μL of Tyrode buffer.

Platelet aggregation assay

Aggregation of human platelets under high shear conditions was assessed using a standard optical aggregometer (Lumi-Dual Aggregometer; Chrono-Log, Havertown, PA, USA) at 37°C and 1200 rpm in a volume of 500 μL buffer with a final platelet concentration of 6.5×10^5 platelets·μL^{-1} over a 5 min interval. Preincubation with TSP-1 was for 15 min prior to addition of sGC activators or vehicle control, which were incubated 5 min prior to the initiation of aggregation with thrombin.

Collagen gel matrix contraction assay

Analysis of *in vitro* porcine VSMC (white hairless Yucatan minipig, as described in Isenberg *et al.*, 2008c) contraction was performed as described previously (Isenberg *et al.*, 2007c). Briefly, a 4 mL volume of type I collagen (3 mg·mL^{-1}) was mixed with 0.4 mL of 10× M199 medium, 37 μL of 100× glutamine, 37 μL of penicillin streptomycin and 0.5 mL of 11.8 mg·mL^{-1} Na bicarbonate, and the pH of the mixture was neutralized with 0.1 M NaOH. The mixture was seeded with porcine VSMCs (50 000 cells per well), and 75 μL aliquots (per well) were placed into a 96-well plate (Nunc, Denmark). Plates were incubated for 4 h at 37°C and 5% CO_2 to allow gel formation and spreading of cells and then further incubated for 10 h in smooth muscle basal medium (SmBm) containing 0.1% BSA and the specified treatment agent. Following this, gels were gently detached from the wells with a 2 μL pipette tip and 10% FCS was added to initiate contraction. Contraction was assessed as changes in the area of the gel measured from digital images of the plate using Image Pro software (Media Cybernetics, Inc., Bethesda, MD, USA).

Intracellular cGMP assay

Fresh human platelets, 5×10^5 platelets·mL^{-1} in Tyrode buffer, were preincubated with TSP-1 for 15 min and then challenged

with sGC activators or vehicle control along with IBMX (30 μM) for 2 min at 37°C, and total cGMP was determined via immunoassay according to the manufacturer's instructions (RPN226, GE Healthcare, Amersham, UK).

Porcine VSMC were seeded at 100 000 cells per well in each well of 12 well plates (Nunc) in smooth muscle growth medium for 24 h at 37°C and 5% CO_2. Cells were deprived of serum overnight with SmBm containing 0.1% BSA. Before the start of the assay, cells were washed once with PBS and placed in SmBm without BSA to aid later protein measurement. Cells were treated for 15 min at 37°C with TSP-1 prior to the addition of IBMX (30 μM) and specified sGC activators or vehicle control. The assay was terminated by rapidly decanting the media from the wells and adding 1× lysis buffer (immunoassay kit, RPN226) on a bed of dry ice. Cells were subjected to two freezing and thawing cycles on dry ice to maximize the extraction of cGMP. cGMP was determined via immunoassay according to the manufacturer's instructions (as before).

Statistics

Data are presented as the mean ± SD of at least $n = 3$. Significance was calculated using Student's *t*-test with $P < 0.05$ taken as being statistically significant.

Results

TSP-1 inhibited the delay in platelet aggregation induced by the sGC activators

Elevated intracellular cGMP levels inhibit platelet aggregation (Mellion *et al.*, 1981). Washed human platelets aggregated briskly when exposed to 1 $U \cdot mL^{-1}$ of thrombin, and this was significantly delayed by treatment of the platelets with either 16 μM BAY 41-2272, 100 μM YC-1 or 100 μM PPIX (Figure 1A–C). Concentrations were chosen based on EC_{50} values for each drug determined by preliminary titration experiments (data not shown). BAY 41-2272 was the most potent followed by YC-1 and PPIX (15%, 74% and 93% aggregated respectively) (Figure 1D). Consistent with the ability of physiological concentrations of TSP-1 to reverse an NO-mediated delay in platelet aggregation (Isenberg *et al.*, 2008d), pretreatment of the platelets with 2.2 nM TSP-1 almost completely abolished the delay in thrombin-induced aggregation caused by the sGC activating compounds (Figure 1D).

TSP-1 inhibited the sGC activator-stimulated relaxation of smooth muscle cells

The collagen gel contraction assay is an established surrogate assay that can be used to measure the vasorelaxant properties of compounds *in lieu* of more complex organ bath apparatus. Here we used the assay to show the effect of TSP-1 on the vasorelaxant properties of sGC activating compounds. Porcine VSMC embedded in collagen gels contracted in response to the addition of 10% foetal calf serum; TSP-1 had no significant effect on this contraction (Figure 2). However, 100 μM YC-1, 10 μM BAY 41-2272 and 10 μM PPIX all effectively inhibited this contraction to FCS (the concentrations used were based on initial dose-response titrations; data not shown), and preincubation of the gels with TSP-1 limited the ability of these compounds to inhibit the contraction to FCS (Figure 2).

TSP-1 inhibited the accumulation of cGMP stimulated by sGC activators

Although the above results in platelets and VSMC are consistent with the ability of TSP-1 to inhibit the pharmacological activation of sGC, to prove this hypothesis we needed to exclude the possibility that TSP-1 reverses these functional responses by acting on a target downstream of sGC. Our previous studies have excluded the possibility that TSP-1 inhibits NO-mediated signalling through sGC by modulating cGMP phosphodiesterase activities in endothelial or VSMC (Isenberg *et al.*, 2005; 2006b). However, TSP-1 has been shown to inhibit functional responses stimulated by a cell permeable cGMP analogue, and the target for this inhibition in platelets was identified as cGMP-dependent protein kinase (Isenberg *et al.*, 2008d). The results shown in Figures 1 and 2 could result from this downstream inhibition rather than direct inhibition of sGC. To clearly establish whether TSP-1 can inhibit sGC activation by agents other than NO, we examined cGMP levels following the same treatments. Primary porcine VSMC and washed human platelets both exhibited increases in cGMP accumulation as a result of treatment with sGC activators (Figure 3A,B) at similar concentrations to those effective in the functional assays above. The experiments were carried out in the presence of the pan-phosphodiesterase inhibitor (PDE) IBMX to confine the effect of these activators to sGC activation. This is necessary because YC-1 is also a PDE inhibitor with an IC_{50} of 10 μM (Evgenov *et al.*, 2006). Pretreatment of the porcine VSMCs with 2.2 nM TSP-1 significantly inhibited the ability of the sGC activators to increase cGMP production (Figure 3A). Also, in the washed human platelets, pretreatment with 2.2 nM TSP-1 significantly inhibited the ability of the sGC activators to increase cGMP production (Figure 3B).

Discussion

Previously we have shown that TSP-1, through CD47 ligation, is a potent inhibitor of endogenous NO cardiovascular signalling via sGC inactivation (Isenberg *et al.*, 2005; 2008d). Here we demonstrated that TSP-1 universally inhibits sGC activation by several NO-independent activators. The actions of these pharmacological activators of sGC in both functional (platelet aggregation and gel contraction) and biochemical assays of cardiovascular function were effectively limited in the presence of 2.2 nM TSP-1. Although present in the plasma at lower concentrations (0.04–0.25 $\mu g \cdot mL^{-1}$) (Mosher, 1990), TSP-1 levels are increased acutely as part of a physiological response by platelets in a local wound environment; where platelets act as a vasoconstrictor to stem bleeding (Isenberg *et al.*, 2007a). TSP-1 levels are also increased chronically in the plasma of patients with pathological conditions such as acute myocardial infarction, acute liver failure, chronic liver disease, chronic renal disease (Booth and Berndt, 1987), diabetes (Varma *et al.*, 2008), ischaemic heart disease (Sezaki

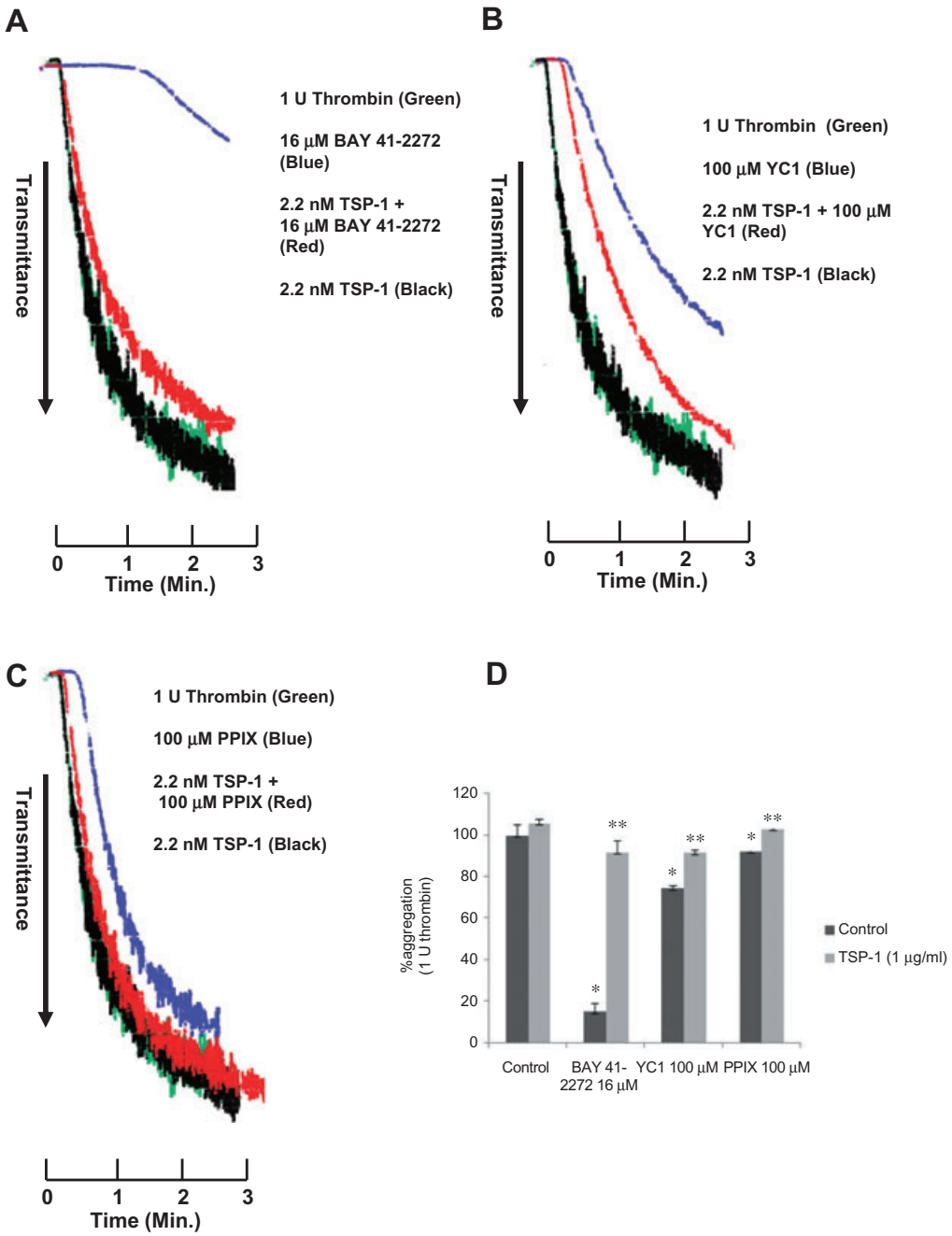

Figure 1 Washed human platelets (325×10^6 cells per condition) were treated with vehicle or with BAY 41-2272 (16 μM, A), YC-1 (100 μM, B), and PPIX (100 μM, C) for 1 min or pretreated for 15 min with TSP-1 followed by 1 min for the respective drug or TSP-1 alone. Platelet aggregation was induced with thrombin (1 U) and assessed under high shear conditions. (D) Represents the replicate platelet aggregation data expressed as mean ± SD ($n = 3$). *$P < 0.05$ versus control, **$P < 0.05$ versus non-TSP-1 pretreated. TSP-1, thrombospondin-1.

et al., 2005) and atherosclerosis (Roth *et al.*, 1998), and possibly contribute to the pathology of these and associated diseases. Interestingly, a review by Chirkov and Horowitz (2007) describes a condition of platelet hyperaggregability and impaired vascular tissue responses to nitric oxide associated with potential increases in TSP-1. Also, platelets taken from patients with unstable angina pectoris have been found to be significantly less responsive to the anti-agreggatory effect of sodium nitroprusside (Chirkov *et al.*, 1996). When considered in the context of our present work, these findings suggest that TSP-1 may be one of the factors contributing to cardiovascular NO resistance and the lack of NO bioavailability found with aging.

While the mechanism of TSP-1/CD47-induced inhibition of sGC is not yet known, these experiments do imply that the inhibition is not simply due to oxidation of the sGC haeme. If this were the case, TSP-1 treatment would result in a potentiation of PPIX stimulated cGMP production due to the higher affinity of ferric sGC or apo-sGC for PPIX (Ignarro *et al.*, 1984), not the inhibitory effect seen here. Interestingly, while PPIX provided the highest level of inhibition of the collagen gel contraction, it was the weakest at increasing cGMP flux. There

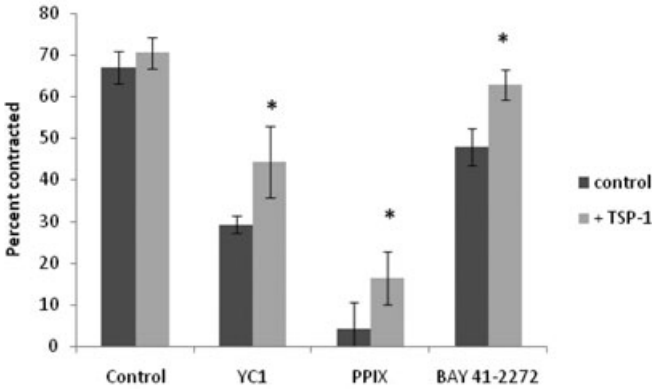

Figure 2 Porcine VSMCs were seeded into collagen gels at 50 000 cells per well. Wells were treated with TSP-1 (2.2 nM) or left untreated for 12 h in basal media. The sGC activator drugs (100 μM YC-1, 10 μM BAY 41-2272, and 10 μM PPIX) or vehicle were added at the same time as contraction was initiated with 10% FCS. Contraction was measured as a function of change in gel area after 12 h at 37°C and 5% CO_2. Data are expressed as mean ± SD (n = 4). *P < 0.05 versus non-TSP-1 pretreated. PPIX, meso-porphyrin IX; TSP-1, thrombospondin-1; VSMC, vascular smooth muscle cell.

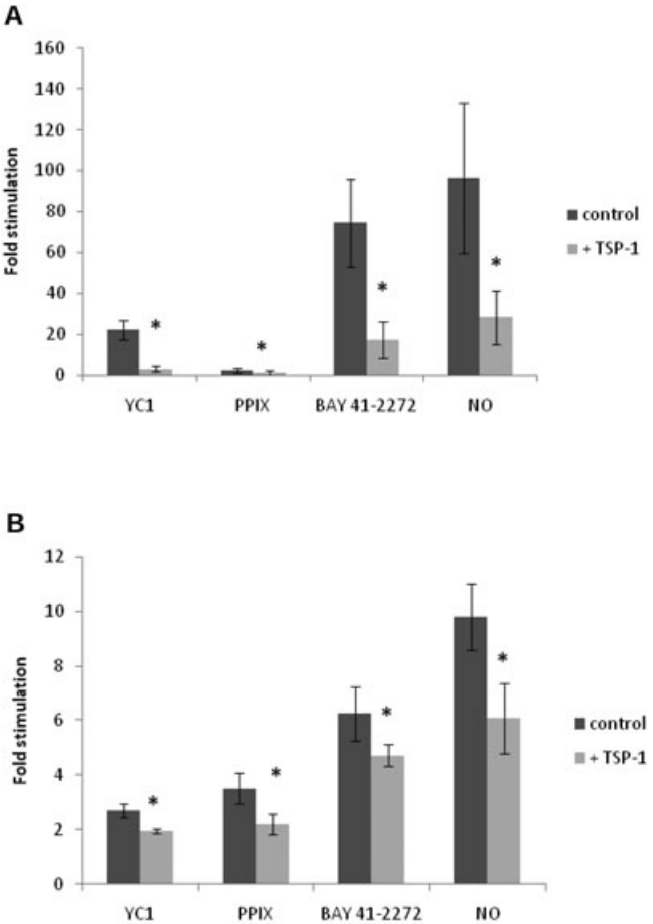

Figure 3 Porcine VSMCs (A) or washed human platelets (B) were pretreated for 15 min with TSP-1 followed by 5 min for the respective drug or vehicle control (YC-1 10 μM, PPIX 100 μM, BAY 41-2272 10 μM, DEANO 1 μM). Cells were lysed and cGMP measured by ELISA. VSMCs were deprived of serum overnight prior to the assay. The data are expressed as mean ± SD (n = 4 VSMC, n = 3 platelets) in units of fold increase over control. *P < 0.05 versus non-TSP-1 pretreated. PPIX, meso-porphyrin IX; TSP-1, thrombospondin-1; VSMC, vascular smooth muscle cell.

are probably additional pathways perturbed in the smooth muscle contraction model that is affected by PPIX.

The desired effect of any sGC activating drug is to increase the amount of cGMP produced in the system and increase the downstream effects. However, as we showed here, TSP-1 potently blocks several types of primary sGC activators. Furthermore, we have previously shown that TSP-1 potently inhibits the canonical NO-sGC-cGMP pathway downstream of sGC at the cGMP-dependent kinase (Isenberg *et al.*, 2008d). Finally, TSP-1 blocks the activity of cell permeable cGMP analogues (8-Br-cGMP) to drive down stream signalling in vascular cells (Isenberg *et al.*, 2005), further limiting the effect of any drug meant to directly increase the levels of cGMP.

In summary, our data show that TSP-1/CD47 potently inhibits not only the physiological actions of sGC induced by NO, but also the activity of different types of pharmacological activators of sGC. TSP-1 is increased in many cardiovascular conditions that would be a target for treatment with therapeutic sGC activators and may limit their effectiveness. Hence, treatment of cardiovascular diseases with sGC activating agents would be more effective in conjunction with therapeutics targeting TSP-1/CD47.

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Conflicts of interest

There are no conflicts of interest.

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