Stainless Steel Ions Stimulate Increased Thrombospondin-1-Dependent TGF-Beta Activation by Vascular Smooth Muscle Cells: Implications for In-Stent Restenosis

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Abstract

Background/Aims: Despite advances in stent design, in-stent restenosis (ISR) remains a significant clinical problem. All implant metals exhibit corrosion, which results in release of metal ions. Stainless steel (SS), a metal alloy widely used in stents, releases ions to the vessel wall and induces reactive oxygen species, inflammation and fibroproliferative responses. The molecular mechanisms are unknown. TGF-β is known to be involved in the fibroproliferative responses of vascular smooth muscle cells (VSMCs) in restenosis, and TGF-β antagonists attenuate ISR. We hypothesized that SS ions induce the latent TGF-β activator, thrombospondin-1 (TSP1), through altered oxidative signaling to stimulate increased TGF-β activation and VSMC phenotype change. ***Methods:*** VSMCs were treated with SS metal ion cocktails, and morphology, TSP1, extracellular matrix production, desmin and TGF-β activity were assessed by immunoblotting. ***Results:*** SS ions stimulate the synthetic phenotype, increased TGF-β activity, TSP1, increased extracellular matrix and downregulation of desmin in VSMCs. Furthermore, SS ions increase hydrogen peroxide and decrease cGMP-dependent protein kinase (PKG) signaling, a known repressor of TSP1 transcription. Catalase blocks SS ion attenuation of PKG signaling and increased TSP1 expression. ***Conclusions:*** These data suggest that ions from stent alloy corrosion contribute to ISR through stimulation of TSP1-dependent TGF-β activation.

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Introduction

In-stent restenosis (ISR) is a fibroproliferative response of the vessel wall to injury, characterized by initial inflammatory responses, VSMC proliferation, late loss of cells and accumulation of extracellular matrix in the neo-intimal layer, resulting in loss of luminal area [1]. ISR remains a significant clinical problem despite advances in stent design and the use of drug-eluting stents [2]. Metal alloys commonly used for stents are subject to stress/strain fracture related to crevice and pitting corrosion due to both mechanical and environmental factors. Reports of stent fractures are increased, particularly after drug-eluting stent deployment [3–5]. Furthermore, the polymer and diamond-like carbon coatings applied to stent surfaces are known to crack and degrade with time,

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particularly when subject to mechanical strain [6, 7]. This degradative process releases metallic ions that localize to tissue surrounding implants [8]. This process is significant, because increased stent corrosion is associated with increased rates of neointimal proliferation [9, 10]. Yet, the mechanisms underlying this response are not understood. High concentrations of metal ions are toxic to VSMCs in vitro and sublethal concentrations stimulate both inflammatory and fibrotic reactions [11, 12]. Stainless steel (SS) is particularly conducive to stimulation of fibrous tissue formation and inflammatory (allergic) responses, which are associated with increased rates of restenosis [11]. Stent implantation, SS surfaces and iron and manganese, components of SS, are all known to increase reactive oxygen species (ROS) [13–15]. Despite the fact that restenosis involves both inflammatory and fibroproliferative responses, there have been no studies addressing the role of metal ions on the behavior of vascular smooth muscle cells (VSMCs) in ISR.

TGF-β is a pluripotent growth factor which drives fibroproliferative responses critical to restenosis through the canonical Smad signaling pathway as well as other associated pathways [16, 17]. TGF-β action is highly regulated: latent TGF-β must be converted to its active form in order to elicit biological activity. Thrombospondin-1 (TSP1) is an activator of latent TGF-β and an extracellular matrix protein that is induced by growth factors and injury [18]. TSP1 binding to the latent complex alters the conformation of the complex which renders TGF-β biologically active [19]. We have mapped the critical sites of interaction between TSP1 and the latent TGF-β complex [20] and we have developed a peptide (LSKL) which functions as a competitive antagonist of TSP1 binding to and activation of the latent complex. The LSKL peptide is an inhibitor of TSP1-dependent TGF-β activation both in vitro and in several different rodent disease models in vivo, including diabetes and hypertension [21, 22]. TSP1 is upregulated following stent deployment and evidence suggests a role for TSP1 in restenosis [23, 24]. TSP1 expression is increased by ROS, partly through attenuation of cGMP-dependent protein kinase (PKG) transcriptional repression [25]. The role of TSP1-dependent TGF-β activation in ISR is not known.

Phenotypic switching of VSMCs is typical of restenotic remodeling in ISR [1, 26]. These synthetic VSMCs acquire myofibroblastic-like characteristics with loss of desmin expression and produce increased extracellular matrix components, including TSP1 and ED-A fibronectin (ED-A FN) [27–30]. Interestingly, α-smooth muscle actin (SMA)-positive cells predominate in the neointima,

suggesting a role for myofibroblast-like cells of either smooth muscle or other origin in restenotic remodeling [1, 26]. Because TGF-β stimulates myofibroblast differentiation and SMC differentiation of mesenchymal progenitors, TGF-β is likely important for these phenotypic changes in ISR [31].

Based on the known role of TGF-β in ISR and the observations of stent metal ion release to the vascular wall, we hypothesized that ions from SS stimulate increased TGF-β activity in VSMCS through upregulation of TSP1. The increased TGF-β activity potentially contributes to VSMC phenotype switching to induce ISR through fibroproliferative responses. We now report that SS ions stimulate increased TSP1 expression which results in enhanced TGF-β activation, extracellular matrix production and loss of desmin expression. Furthermore, we show that SS ions stimulate hydrogen peroxide release and attenuate PKG activity through a catalase-dependent mechanism to upregulate TSP1 expression. These studies identify a novel mechanism by which stent corrosion contributes to ISR.

Materials and Methods

Materials

TSP1 Purification. TSP1 stripped of associated TGF-β was purified from thrombin-stimulated, pooled, outdated human platelet packs purchased from the American Red Cross [32].

Chemicals. Catalase (C9322) and 8-(chlorophenylthio)guanosine 3′:5′-cyclic monophosphate sodium salt (8pCPT-cGMP) (C5438) were purchased from Sigma-Aldrich (St. Louis, Mo., USA). Recombinant human TGF-β1 (240B) was purchased from R&D Systems (Minneapolis, Minn., USA).

Peptides. LSKL, SLLK, GGWSHW were synthesized and purified to >95% purity (Anaspec Inc., San Jose, Calif., USA). LSKL and GGWSHW are peptides from the latency-associated peptide region of latent TGF-β and from the type 1 repeats of TSP1, respectively, which act as competitive antagonists of TSP1-dependent TGF-β activation, and SLLK is an inactive control peptide [19].

Antibodies. The following antibodies were purchased: mouse anti ED-A FN, clone IST-9 and rabbit anti- type I collagen (ab292) (ABCAM); nonimmune mouse IgG, mouse anti-α-SMA, clone 1A4, mouse anti-vimentin (V6389) (Sigma-Aldrich); mouse anti-desmin, clone RD301, rabbit anti-PKG (PA128083), mouse anti-calponin (MA1-37219) (Affinity BioReagents); rabbit anti-phospho Smad 2 (ser465/467) (3101) and rabbit anti-phospho VASP (ser239) (3114) (Cell Signaling Technology); mouse anti-Smad 2/3 (610842) (BD Transduction Laboratories); rabbit anti-β-tubulin, clone H235 (SC9104) (Santa Cruz Biotechnology); rat anti-F4/80 Antigen, cloneA3-1 (MCA497GA) (AbD Serotec); mouse anti-CD68 (MAB1435), mouse anti-CD11b (CBL1512Z) (Chemicon International). Secondary horseradish peroxidase (HRP)-tagged antibodies were purchased from Jackson Immunoresearch Labs.
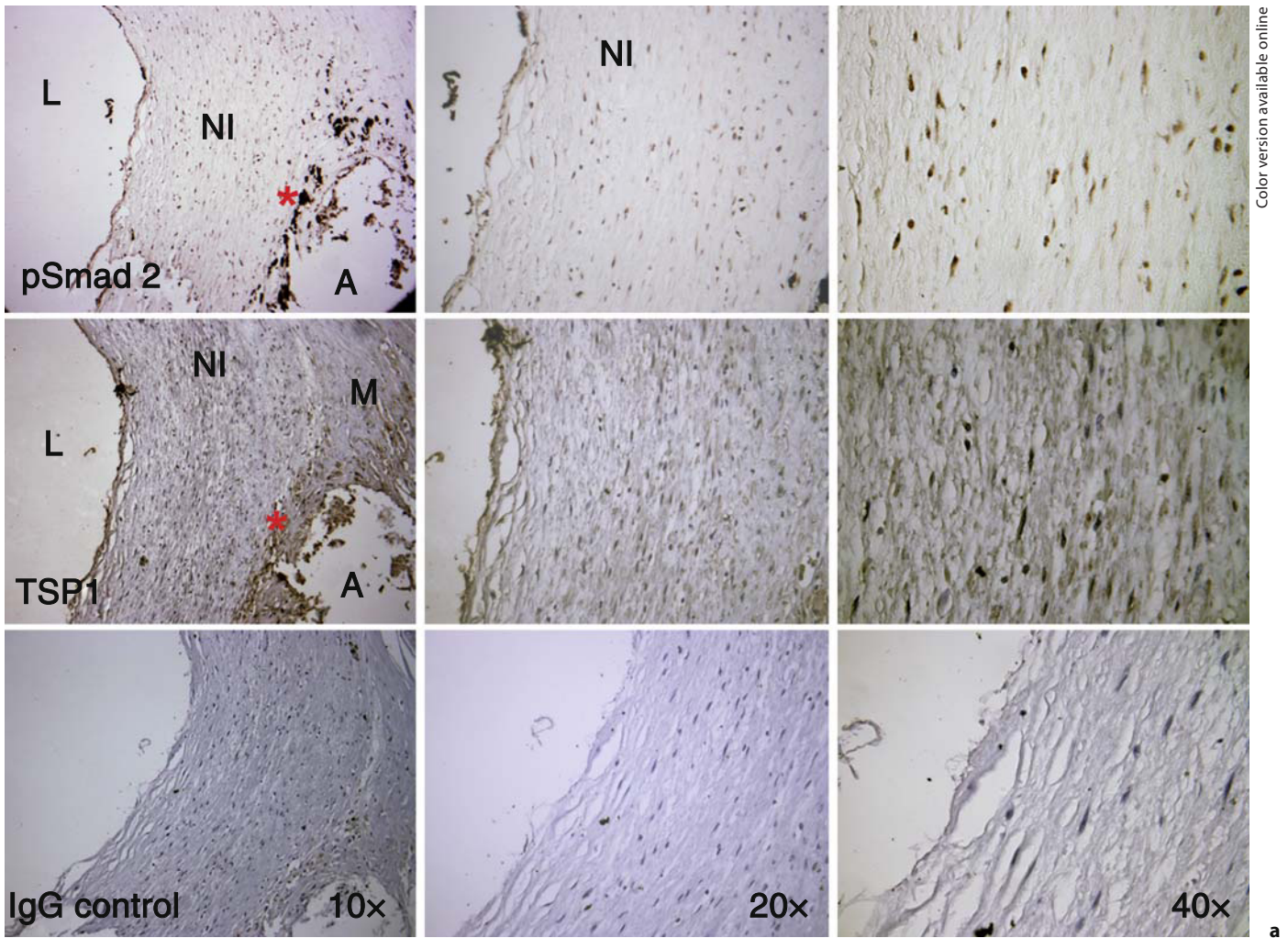
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Fig. 1. TSP1 and active TGF-β (pSmad 2) are expressed in arteries with ISR. **a** Left circumflex artery showing restenotic remodeling from a patient who received a SS drug-eluting stent (Taxus) at least 1 year prior to harvesting of vessels with ISR at the time of cardiac transplant. There is staining for both TSP1 and nuclear pSmad 2 in the endothelium and in the restenotic neointimal VSMCs (NI). There are also macrophages (*) and VSMCs in a region of atheroma (A) adjacent to the restenotic remodeling that also stain for pSmad 2 and TSP1, respectively. L = Lumen; M = media.

Goat anti-rabbit IgG-Biotin (BA1000), horse anti-mouse IgG-Biotin (BA2001) and goat anti-rat IgG-Biotin (BA9400) were purchased from Vector Laboratories and secondary antibody goat anti-mouse IgG Alexa Fluor 488 (A11001) was purchased from Molecular Probes. Mouse monoclonal antibody to TSP1, Clone 133, was developed in our lab [33, 34].

Immunohistochemistry

Sections of human coronary arteries were obtained from existing paraffin-embedded sections under IRB protocol approval X060928009 to B. Brott. The vessel shown in figure 1a is from the left circumflex artery of a patient undergoing a heart transplant who had been implanted with a Taxus stent within 12 months. Figure 1b panels are from a patient who received 4 Cypher stents 12 months prior to autopsy. Results are representative of sections of coronary arteries stained from 3 separate patients with ISR receiving drug-eluting stents. Antigen retrieval was performed by microwaving sections in 10 mM citrate buffer, pH 6.0, for 3 min at full power and for 7 min at 40% power. Sections were incubated in 1% H_2O_2 for 10 min, blocked with 2.5% ovalbumin for 1 h at room temperature, and then incubated with primary antibodies overnight at 4°C. Sections were washed and then incubated with the appropriate biotin-tagged secondary antibodies (1/500 dilution) for 1 h at room temperature. Following washing, streptavidin/HRP (ABC kit PK6100) was added to sections for 30 min at room temperature. Color was developed with the DAB developer (Vector Laboratories SK4100). Some sections were counterstained with hematoxylin. Sections were dehydrated and then mounted with Vectamount media (Vector Labs H5000). Primary antibodies were used at the following concentrations: rabbit anti-phospho Smad 2
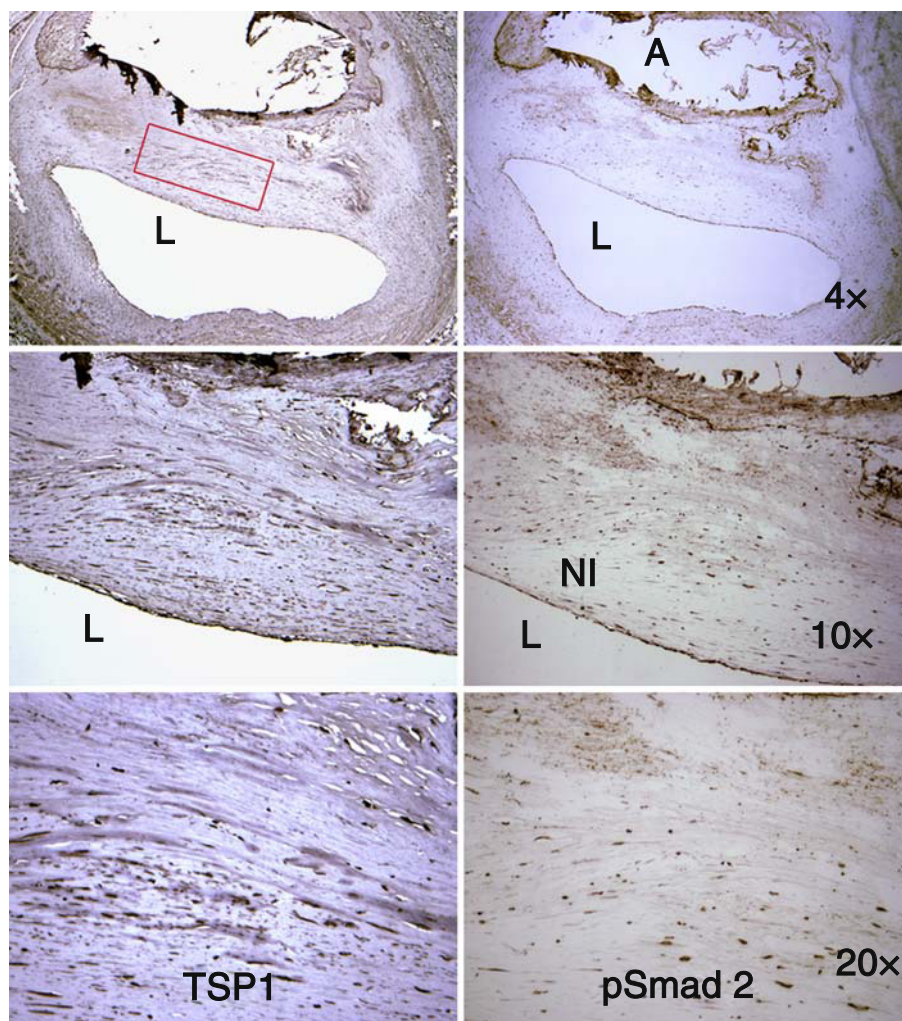
Fig. 1. TSP1 and active TGF-β (pSmad 2) are expressed in arteries with ISR. **b** Section from a proximal left anterior descending artery implanted with 4 Cypher stents 12 months prior to autopsy. The VSMCs in the mature, fibrous neointima (NI) are positive for both TSP1 and nuclear pSmad 2. The rectangle denotes the area shown at 20×. Control sections incubated with nonimmune IgG show no staining. L = Lumen.

(800 ng/ml); mouse anti-TSP1 (10 μg/ml); rat anti-F4/80 (10 μg/ml); mouse anti-rat CD68 (10 μg/ml); mouse anti-CD11b (10 μg/ml). Nonimmune rabbit, rat and mouse IgG were diluted to the final concentration of the relevant primary antibody.

VSMC Isolation and Culture

Primary cultures of rat aortic smooth muscle cells (VSMCs) were derived from 10-week-old female Sprague-Dawley rats (Charles River) using the explant method [35]. Cells were routinely cultured in DMEM with 10% FBS, 2 mmol/l L-glutamine and used between passages 2 and 5.

Immunocytochemistry

Cells were grown on glass coverslips in DMEM with 10% FBS until approximately 60% confluent. Cells were treated for 24 h with 0.1 ppm SS ions in dilute acid or dilute acid alone as a control. Cells were fixed in cold 3% paraformaldehyde for 10 min, washed 3 times in DMEM and permeabilized with 0.1% Triton X-100 for 3 min. Cells were washed and nonspecific binding sites were blocked with 0.1% ovalbumin for 30 min and then incubated with primary antibodies at a final concentration of 4 μg/ml overnight

at 4°C. Cells were washed and incubated with secondary antibody goat anti-mouse IgG Alexa Fluor 488 at a 1/500 dilution and mounted on glass slides using Vectashield. Immunofluorescence was detected using a Nikon Eclipse microscope equipped for epifluorescence at the University of Alabama at Birmingham BERM Center FRET microscopy core facility. All images were obtained at 40× magnification and were processed identically.

SS Ion Solutions

To simulate the SS corrosion products, high-purity atomic absorption standards (Acros Organics USA, Morris Plains, N.J., USA) were used. Simulated ion cocktails representing SS corrosion ions were made by addition of Fe^{3+}, Ni^{2+}, Cr^{3+}, Mn^{2+} and Mo^{6+} at 66, 14, 16, 2 and 2%, respectively. Stock solutions at 10 ppm were prepared from 1 mg/ml stock solutions in 2% HNO_3, except for Cr which is in a stock of 2% HCl. Metals were diluted to their final concentration in serum-free DMEM and incubated in a CO_2 incubator to equilibrate pH prior to adding to cells. DMEM with a volume of acid (DMEM/acid) equal to that used for the ion dilution was used as a control for any effects of the acid diluent.

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Treatment of Cells with SS Ions

VSMCs were grown to 50–60% confluence in 12-well plates in DMEM with 10% FBS. Cultures were rinsed in serum-free DMEM and treated with diluted cocktails of metal ions (0.1–1.0 ppm) dissolved in acid. Dilute DMEM/acid controls were run in each assay. The concentrations of metal ions used in these studies did not cause cell death (data not shown). Furthermore, at 0.1 ppm, the iron and nickel concentrations are approximately 2- and 30-fold, respectively, over levels detected by mass spectrometry normalized per milligram of explanted vascular tissue harvested from autopsy specimens of patients with SS stents [D. Halwani and J. Lemons unpubl. data, UAB Master's thesis]. Cells were treated with metal ions for times indicated. Forty-eight-hour treatments were refreshed at 24 h. Assays were performed in triplicate and extracts pooled in a total volume of 60 μl (20 μl/well) of 2× Laemmli Buffer with protease inhibitors (Sigma-Aldrich P2714) and phosphatase inhibitors (Sigma-Aldrich P2850). Extracts were sonicated for 6 s to shear DNA and boiled at 100°C for 5 min, followed by centrifugation at 10,000 rpm for 2 min. Each assay was repeated a minimum of 3 separate times.

Immunoblotting

Equal volumes of cell extracts were electrophoretically separated and transferred to nitrocellulose membranes per standard protocols. Multiple films were exposed to ensure linearity of the response. In each experiment, membranes were stripped and reprobed with antibody to β-tubulin as a loading control for normalization of cell number.

Hydrogen Peroxide Measurement

The Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (A22188; Invitrogen-Molecular Probes) was used according to manufacturer's instructions. Cell suspensions of 15,000 cells/well in 20 μl of serum-free DMEM in the presence or absence of treatments were then added to wells with pre-incubated (37°C, 30 min) Ampex Red-HRP reagent and incubated at 37°C with 5% CO_2 for 1–4 h. Hydrogen peroxide produced by cells was measured at 1, 2, 3 and 4 h using a Cytofluor 4000 microplate reader (excitation 530 nm, emission 580 nm; PerSeptive Biosystems). Samples were assayed in triplicate for each condition. Hydrogen peroxide (10 μmol/l) was used as a positive control. Wells containing reagents without cells were set as assay background that was subtracted from sample readings.

Statistics

Results of the hydrogen peroxide assay were analyzed by one-way ANOVA with post hoc Tukey analysis using Sigma Stat 3.1. Results with a p value <0.05 are considered significant.

Results

Localization of TSP1 and Active TGF-β in Peri-Stent Coronary Artery

To determine whether TSP1 and active TGF-β are localized to vascular tissues surrounding stent materials, human autopsy specimens obtained from stented coronary arteries were stained for TSP1 and phosphorylated Smad 2 (pSmad 2) as an indicator of active TGF-β signaling. These studies showed localization of both TSP1 and active TGF-β in VSMCs of the restenotic neointima of peri-stent regions (fig. 1a, b). The co-expression of TSP1 and pSmad 2 in VSMCs of the restenotic fibrous neointima suggest that TSP1 might be responsible for locally activating latent TGF-β in these lesions. Serial sections stained for macrophage markers (CD68, CD11b) showed that vessels with long-term (approx. 12 months) stent placement had few macrophages in the actual restenotic lesions (data not shown), although macrophages expressing active TGF-β were prominent in atheromatous regions, distinct from the neointimal areas of restenotic remodeling.

SS Ions Increase TGF-β Activity, TSP1 and Extracellular Matrix Production by VSMCs

Rat VSMCs (p3) were treated with SS ions to determine whether metal ions induce increased TGF-β activity (pSmad 2; fig. 2a). SS ion treatment stimulated increased Smad 2 phosphorylation in VSMCs. Interestingly, acid diluent used for preparing the SS ions increased total Smad 2, although it had no effect on Smad 2 phosphorylation. It is known that synthetic VSMCs express increased TSP1, a known activator of latent TGF-β [36]. Therefore, SS ion-treated VSMC cultures were also examined for TSP1 expression (fig. 2b). The effect of SS ions on extracellular matrix (type I collagen and ED-A FN) production by VSMCs was also examined (fig. 2b, c). Results show that TGF-β activity, TSP1 and extracellular matrix proteins are indeed upregulated by SS ion treatment of VSMCs.

SS Ions, TSP1, and TGF-β Reduce Expression of VSMC Contractile Protein Desmin

Loss of VSMC contractile proteins is characteristic of the switch to the synthetic phenotype. Consistent with the induction of increased extracellular matrix and TSP1 by SS ions, SS ions also decrease expression of the VSMC contractile protein, desmin (fig. 2d). Desmin levels are also reduced by treatment with either TSP1 or active TGF-β (fig. 2d). Furthermore, SS ion-treated VSMCs acquire a rhomboid phenotype with loss of the contractile cytoskeletal protein calponin indicative of the synthetic phenotype, as compared to acid control-treated cells which are more spindle shaped and have strong staining for calponin (fig. 3).
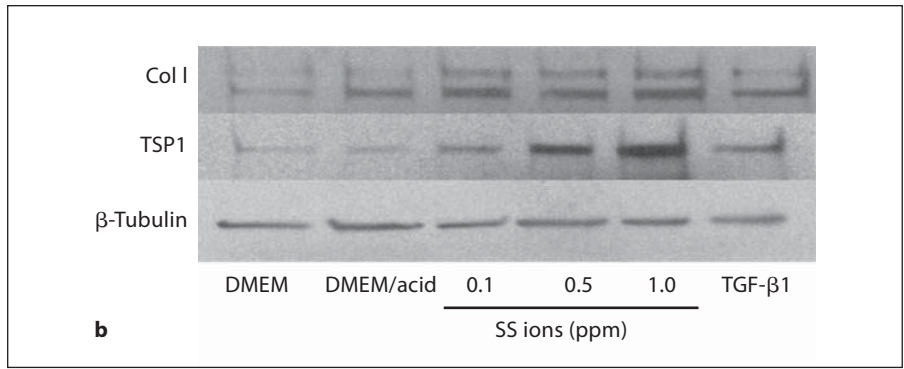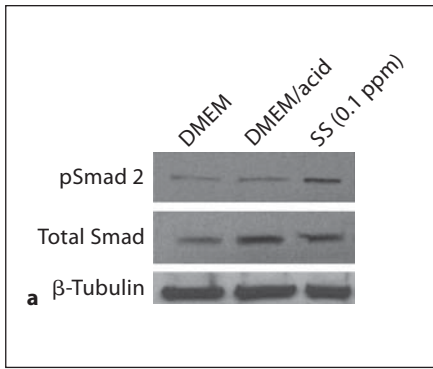
Fig. 2. SS ions stimulate increased TGF-β activity, TSP1, extracellular matrix production and reduce desmin expression. VSMCs (p3–4) were cultured in the presence of 0.1–1.0 ppm SS ion cocktail in dilute acid, TGF-β (200 pmol/l) or TSP1 (32 nmol/l) for 24 h. Pooled cell lysates from triplicate samples were harvested and proteins were immunoblotted for pSmad 2 and total Smad 2/3 (n = 3) (**a**) or type I collagen (n = 4), TSP1 (n = 3) (**b**) and ED-A fibronectin (n = 3) or α-SMA (n = 3) (**c**). Blots were stripped and reprobed for β-tubulin as a loading control. **d** Pooled cell lysates of triplicate samples were harvested at 48 h of treatment and immunoblotted for desmin (n = 4). Blots were stripped and reprobed for β-tubulin.
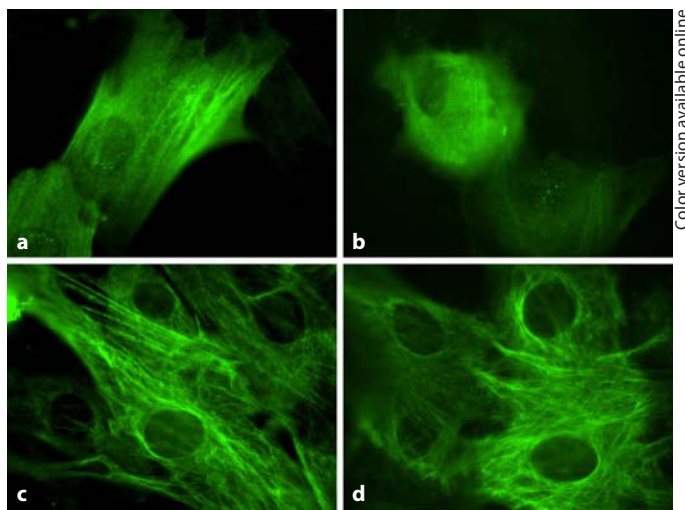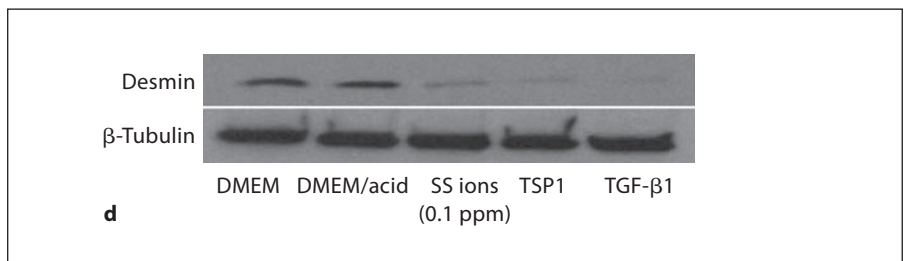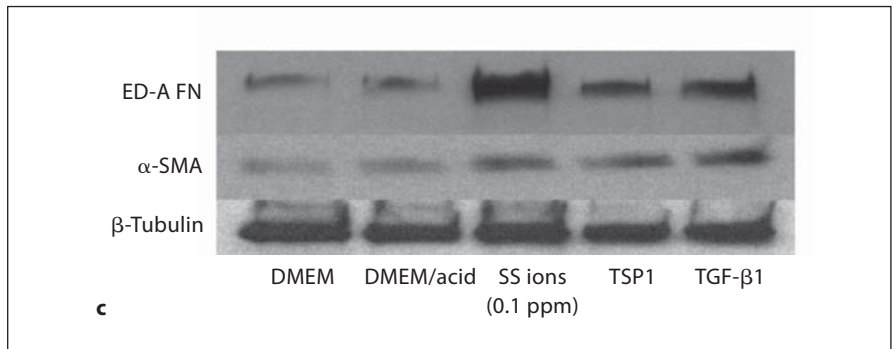

Fig. 3. SS ion treatment induces the synthetic phenotype in VSMCs. VSMCs were cultured for 24 h in presence of dilute acid (**a**, **c**) or 0.1 ppm SS ions (**b**, **d**). Cells were fixed and permeabilized and then stained for calponin (**a**, **b**) and vimentin (**c**, **d**) by indirect immunofluorescence with an Alexa Fluor 488 secondary antibody. 40×.
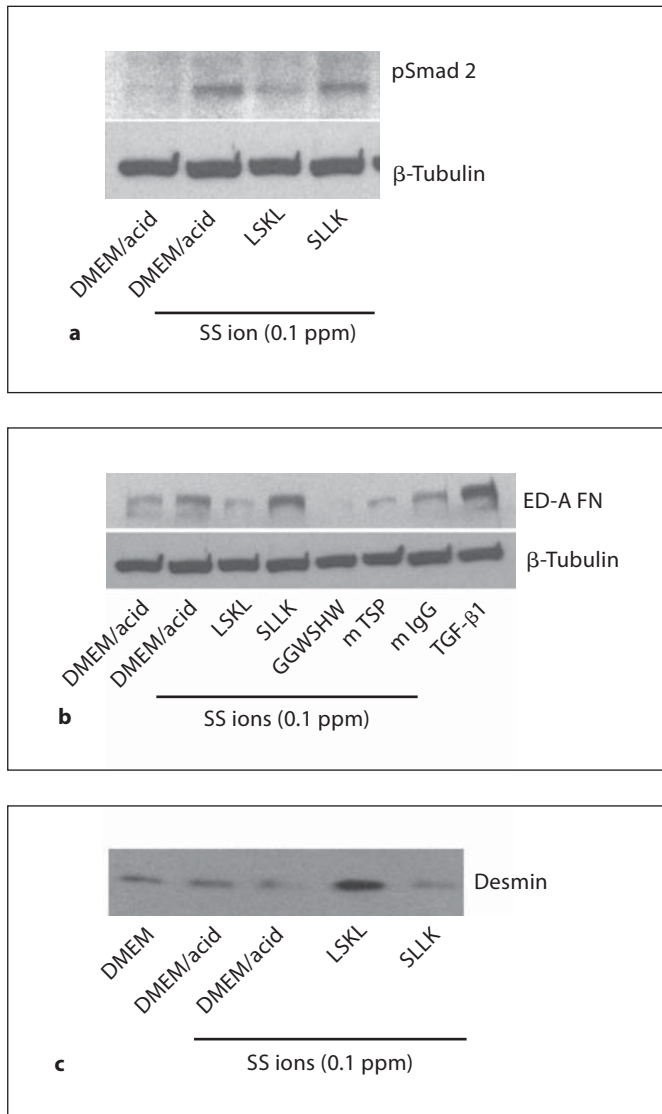
Fig. 4. Blockade of TSP1-dependent TGF-β activation reduces TGF-β signaling and matrix expression due to SS ions and increases desmin expression. VSMCs (p3) were treated with 0.1 ppm SS ions for 24 h in the presence or absence of LSKL or SLLK (1 μmol/l), GGWSHW (20 μmol/l) or Mab 133 monoclonal antibody to TSP1 or nonimmune mouse IgG (25 μg/ml). Active TGF-β (200 pmol/l) was used as a positive control. Pooled cell lysates from triplicate samples were harvested, separated by SDS-PAGE and proteins analyzed by immunoblotting for phospho-Smad 2 (n = 3) (**a**) or ED-A fibronectin (n = 3) (**b**). Membranes were stripped and reprobed for β-tubulin. **c** In separate experiments, cells were treated with 0.1 ppm SS ions for 48 h in the presence of either 1 μmol/l LSKL or SLLK peptide and cell lysates were harvested and immunoblotted for desmin (n = 3).

Blockade of TSP1-Dependent TGF-β Activation Inhibits SS Ion Stimulation of TGF-β Signaling and ED-A Fibronectin Expression and Prevents Desmin Downregulation

Previously, we established that the LSKL peptide acts as a competitive antagonist of TSP1-dependent TGF-β activation in vitro and in vivo [20, 22]. VSMCs treated with SS ions in the presence of 1 μM LSKL peptide had reduced TGF-β signaling as measured by pSmad 2 and expression of the synthetic/myofibroblastic protein, ED-A FN (fig. 4a, b). Control peptide, SLLK, had no effect. Another TSP1 antagonist peptide, GGWSHW, and a monoclonal antibody to TSP1 (Mab133) similarly reduce ED-A FN expression. Furthermore, the action of SS ion-stimulated TSP1 in reducing desmin expression is reversed by the LSKL peptide, but not by the control SLLK peptide (fig. 4c).

SS Ions Increase Oxidant Stress

ISR and metal ions are both known to stimulate increased oxidative stress and a reduction in anti-oxidant enzymes [37]. VSMCs treated with SS ions were measured for the generation of hydrogen peroxide (H_2O_2). SS ions stimulated increased levels of H_2O_2 by 1 h of treatment, which was sustained during the 4-hour duration of the assay (fig. 5a; data not shown). Catalase, which causes H_2O_2 decomposition, blocked SS ion generation of H_2O_2 (fig. 5b). In contrast, direct stimulation of VSMCs with either TSP1 or active TGF-β failed to stimulate H_2O_2 (fig. 5a). Blockade of TSP1-dependent TGF-β activation with either inhibitory peptide or a monoclonal antibody had no effect on H_2O_2 generation by SS ions (data not shown), suggesting that the increase in H_2O_2 is a direct effect of SS ions and not secondary to either TSP1 or TGF-β activity stimulation. Interestingly, iron, nickel and, to a greater extent, manganese, appear to be involved in H_2O_2 generation (fig. 5c).

Since oxidative stress is increased by treatment with SS ions and both TSP1 and TGF-β activity are regulated by ROS, we assessed the role of H_2O_2 in these mechanisms. To determine the role of H_2O_2 in SS ion induction of TSP1, TGF-β and ED-A FN, VSMCs were treated with catalase which decomposes H_2O_2 to water and molecular oxygen. Catalase reduced both TSP1 and ED-A FN expression and also decreased TGF-β activity (p-Smad 2) induced by SS ion treatment (fig. 6).

SS Ions Decrease PKG Activity

NO-dependent PKG activity is a key factor regulating the contractile phenotype of VSMCs and loss of PKG ac-

Fig. 5. SS ions increase H_2O_2 in VSMCs. H_2O_2 levels were measured using the Amplex Red Hydrogen Peroxide/Peroxidase assay according to the manufacturer's instructions. **a** VSMCs (p5) were treated with SS ions (0.1 ppm), TSP1 (32 nmol/l) or active TGF-β (200 pmol/l) for 1–4 h. Hydrogen peroxide was measured as fluorometric emission at 580 nm following excitation at 530 nm using a Cytofluor 4000 microplate reader. Results are expressed as mean fluorometric units ± SD of triplicate samples read at 3 h. **b** In the same experiment, VSMCs (p5) were treated with SS ions in the presence or absence of 100 units of catalase or with catalase alone. Results are expressed as mean fluorometric units ± SD of triplicate samples read at 4 h. **c** VSMCs (p5) were treated with cocktails of SS ions, each lacking one of the metal ion components. Results are expressed as mean fluorometric units ± SD of triplicate samples read at 4 h. All experiments were performed in triplicate in at least 2 separate experiments. * $p < 0.027$ vs. SS ion treatment; ** $p < 0.001$ vs. SS ion treatment. n.s. = Not significant.
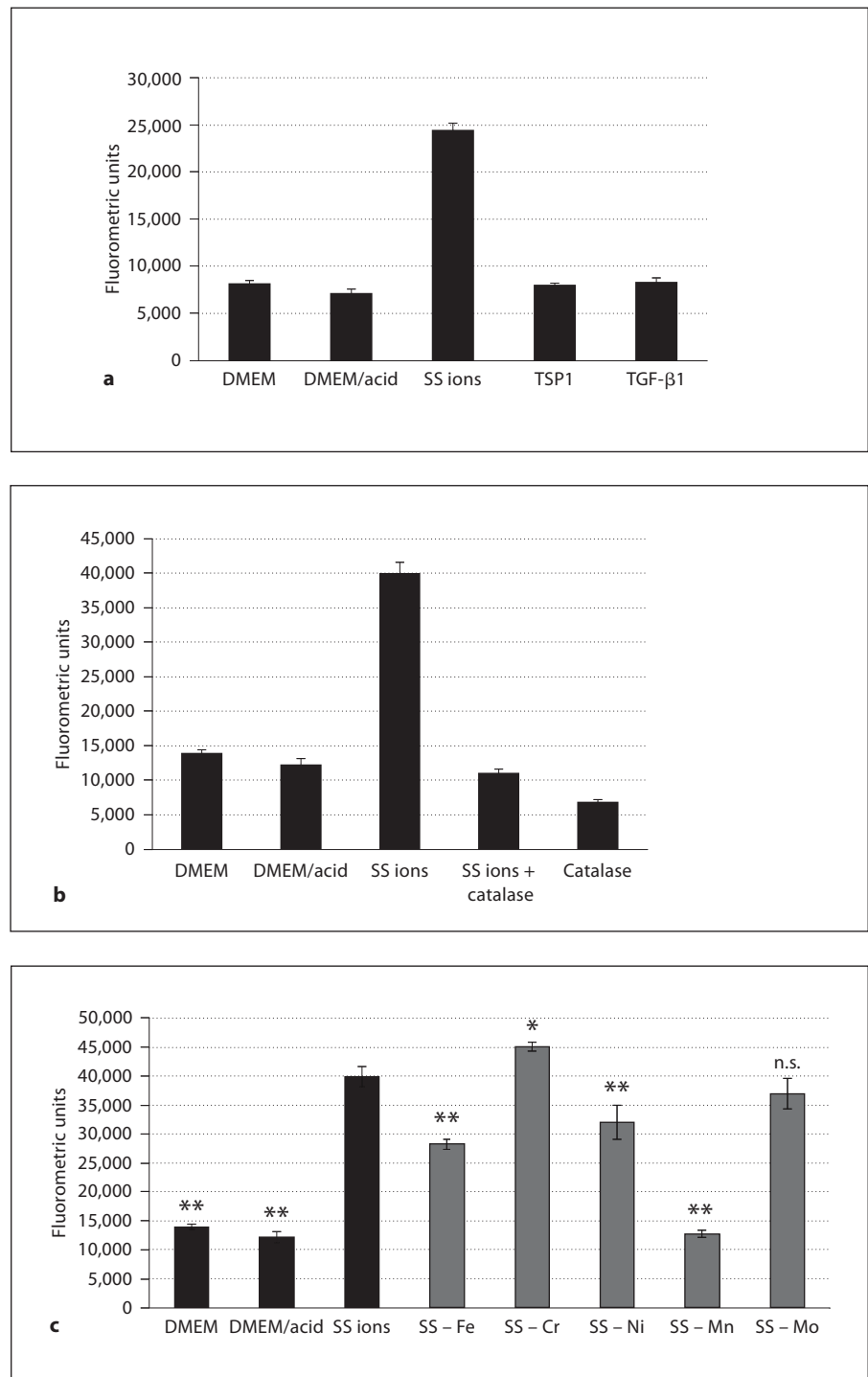
tivity is associated with transition to the synthetic phenotype [38]. Since PKG represses TSP1 expression by VSMCs and also TSP1 transcription by blocking the upregulation of the transcription factor USF2 under high-glucose conditions [25, 36], we hypothesized that SS ions might attenuate PKG protein or activity leading to an increase in TSP1 expression and TGF-β activation. Since SS ions increase ROS, especially H_2O_2, it is possible that SS ions attenuate PKG activity through depletion of bioavailable NO and cGMP. Thus, PKG protein was evalu-
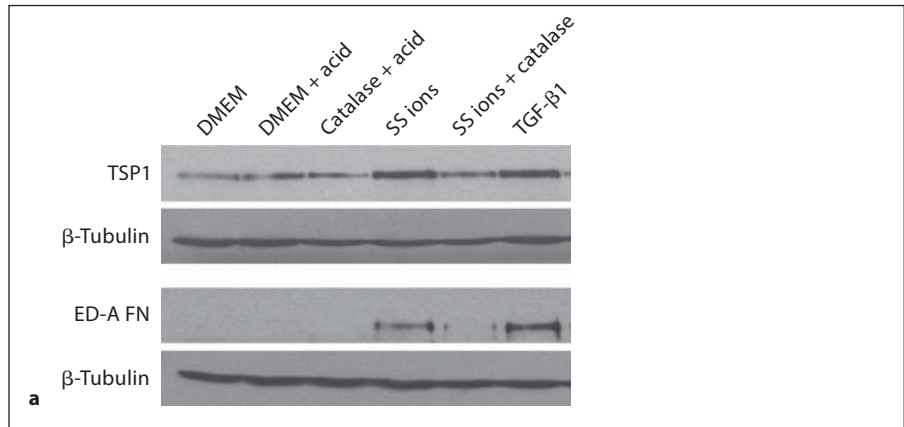
Fig. 6. Catalase reduces SS ion-induced TSP1 and ED-A FN expression and TGF-β activity in VSMCs. VSMCs (p4) were treated with SS ions (0.1 ppm) in the presence or absence of catalase (100 units/ml). Media with dilute acid was used as a control for the SS ion preparation. Cells analyzed for pSmad 2 were treated with both 100 units/ml and 500 units/ml catalase. Active TGF-β (200 pmol/l) or the PKG activator, 8pCPT-cGMP (1 mmol/l), were used as positive controls for stimulation of pSmad 2 and PKG activity, respectively. **a** Cells harvested after 24 h of treatment were assayed for TSP1 and ED-A FN (n = 5). **b** Cells harvested at 6 h were analyzed for pSmad 2 (n = 3) by immunoblotting of pooled cell lysates from triplicate samples. Blots were reprobed for β-tubulin.
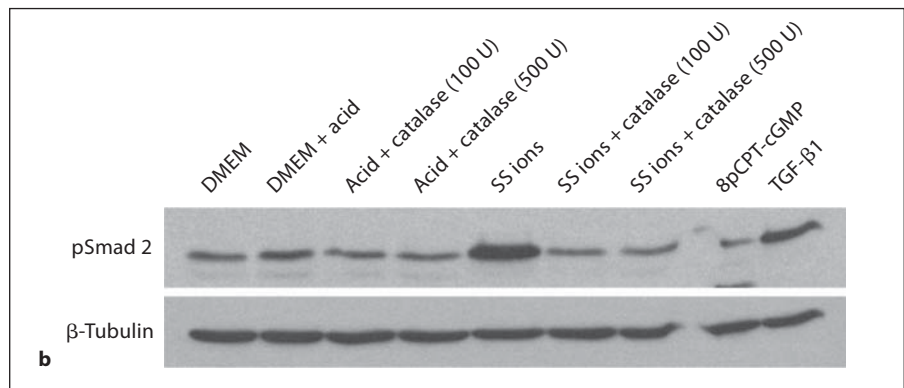
Fig. 7. SS ions reduce PKG-dependent VASP phosphorylation in VSMCs. VSMCs (p2) were treated with DMEM, dilute acid in DMEM or 0.1 ppm SS ions in dilute acid for 1 or 6 h. Pooled cell lysates of triplicate samples were harvested and immunoblotted with rabbit anti-PKG and rabbit anti-phospho-VASP (ser239) antibodies (n = 3). Cells treated with an activator of PKG, 8pCPT-cGMP (1 mmol/l), were used as a positive control for VASP phosphorylation. Blots were stripped and reprobed for β-tubulin as a loading control.
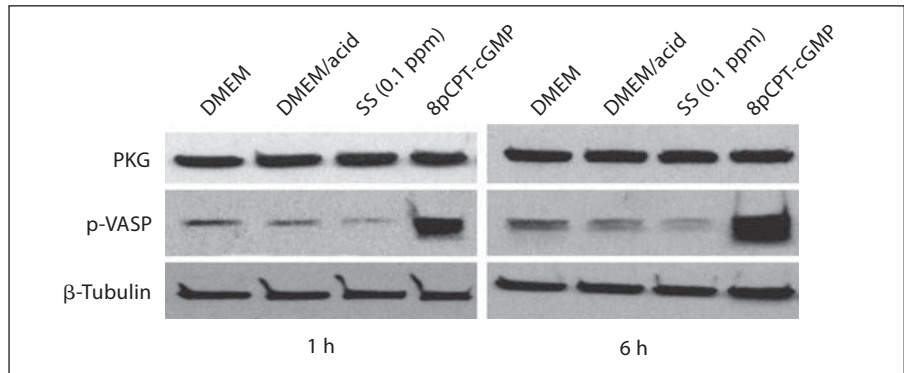

Fig. 8. Catalase blocks SS ion reduction in PKG-dependent VASP phosphorylation in VSMCs. VSMCS (p4) were treated with SS ions (0.1 ppm) for 6 h in the presence or absence of either 100 units/ml or 500 units/ml catalase. Dilute acid is a control for SS ion preparation. 8pCPT-cGMP (1 mmol/l) is a positive control for PKG-dependent VASP phosphorylation. Cell lysates of pooled triplicate samples were analyzed for phospho-VASP (ser239) by immunoblotting (n = 3). Blots were stripped and reprobed for β-tubulin.
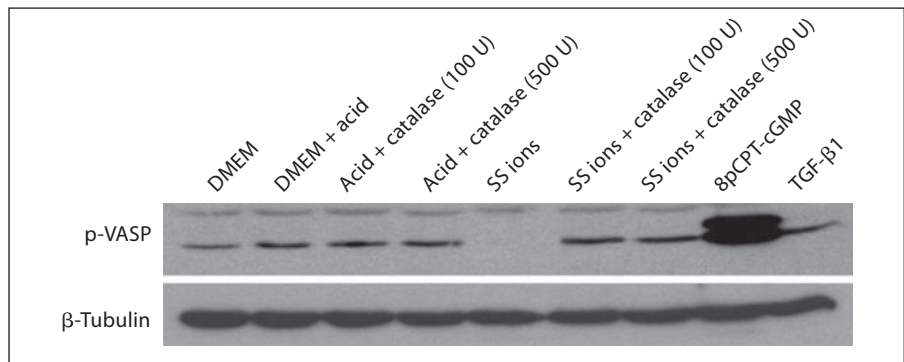
ated following SS ion treatment of VSMCs and PKG activity was determined by immunoblotting for the PKG substrate, phosphorylated VASP (fig. 7). These results show that SS ions do not affect PKG protein expression, but that SS ions do reduce PKG activity as detected by decreased VASP phosphorylation at the PKG-specific site (serine 239). Reduced VASP phosphorylation was detected by 1 and 6 h. Consistent with reduced PKG activity due to lack of NO-generated cGMP under high-ROS conditions, catalase blocked SS ion downregulation of PKG activity (phospho-VASP; fig. 8).

Discussion

Our current studies show that SS ions stimulate increased TSP1 expression and TSP1-dependent increases in TGF-β activity in VSMCs, which stimulates the phenotypic switch to the synthetic phenotype. The SS ions stimulate increased TSP1 expression through a hydrogen peroxide-mediated attenuation of PKG activity to downregulate PKG-mediated repression of TSP1 synthesis. These results provide novel insights into how biodegradation products from stent alloys can influence VSMC responses to drive ISR.

TGF-β has complicated effects on the vasculature: on the one hand, it is considered atheroprotective because it stimulates endothelial proliferation through its ALK 1 receptor, and it is thought to enhance plaque stability by increasing extracellular matrix production in the fibrous cap [39, 40]. TGF-β also stimulates VSMC differentiation by increasing expression of α-SMA, SMMHC and calponin, and it inhibits SMC proliferation [41]. However, TGF-β can also induce PDGF expression to stimulate SMC proliferation and phenotype switching [39]. Different TGF-β concentrations in vitro can elicit contradictory responses, making generalizations about TGF-β function difficult. However, TGF-β expression is rapidly increased following vascular injury in both experimental animals and human specimens, and evidence clearly indicates that TGF-β contributes to restenosis [42–44]. Furthermore, activators of TGF-β, including TSP1, are similarly upregulated at sites of injury in a porcine coronary angioplasty model [16, 24]. TGF-β and its receptors are increased at sites of stenting in a porcine coronary artery model [45]. Increases in TGF-β mRNA were observed as early as 5 days following stent deployment and protein was detectable in tissue at 28 days. TGF-β was predominantly localized around stent struts, which are known sites of increased VSMC migration [45]. The im-

portance of TGF-β in vascular remodeling has been established using in vivo models, which either deliver active TGF-β or block its action. Expression of constitutively active TGF-β induced by gene transfer in uninjured porcine arteries induced collagen and proteoglycan synthesis as well as both intimal and medial hyperplasia [17]. In contrast, neutralizing anti-TGF-β1 antibodies suppressed both extracellular matrix accumulation and neointimal hyperplasia in a rat model of carotid balloon injury [46]. Soluble TGF-β type II receptor antagonist also decreased negative remodeling and intimal lesions in a rat model of balloon catheterization, but the receptor antagonist had no adverse effect on re-endothelialization [47]. Not surprisingly, therapeutic approaches aimed at blocking the fibroproliferative effects of TGF-β on restenosis and ISR have been tested. In pigs undergoing coronary stent implantation, tranilast treatment attenuated TGF-β and TGF-β receptor expression, reduced leukocyte accumulation and significantly reduced neointimal hyperplasia [45]. Others have used pioglitazone to treat ISR in rabbits through reducing TGF-β and MCP-1 [48]. Our current studies suggest that regulating TGF-β activity through attenuation of TSP1-dependent TGF-β activation may represent a new therapeutic approach to treating restenosis.

TSP1 is a multifunctional matricellular protein that is upregulated after arterial injury and has been implicated in the process of restenosis [23, 24]. In addition to activating TGF-β, TSP1 also stimulates endothelial cell apoptosis and acts as a mitogen and chemoattractant for VSMCs [49–51]. Release from platelet α-granules and activated macrophages are potential sources of TSP1 in early vascular injury and stent placement. However, both PDGF and TGF-β induce TSP1 expression by VSMCs [52]. There are multiple lines of evidence to suggest that TSP1 is important in restenosis after balloon angioplasty and stent placement. TSP1 expression as measured by gene array and by immunohistochemistry is markedly enhanced in atherectomy-isolated neointima of arteries from patients with ISR [53]. Similarly, VSMCs obtained from coronary atherectomy specimens of patients with ISR had 1.7-fold increases in TSP1 as compared to normal medial VSMCs [54]. Our staining of TSP1 in the neointima of arteries from peri-stent regions also indicates TSP1 expression by neointimal VSMC-like cells, although others failed to observe TSP1 immunostaining in restenotic lesions following balloon angioplasty [55]. Furthermore, an in vitro study evaluating differential gene expression of circulating peripheral blood cells after 90 min exposure to SS stents found that TSP1 was the most markedly (>1,500-

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fold) upregulated gene [23]. Antibody blockade of TSP1 accelerates re-endothelialization and reduces neointimal formation, potentially acting by inhibiting endothelial cell apoptosis and VSMC proliferation [56]. The role of TSP1 as an activator of latent TGF-β in restenosis has not been previously investigated. Our data strongly suggest that TSP1-dependent TGF-β activation plays a role in restenotic vascular remodeling due to stent alloy corrosion.

There are also contrasting reports which suggest alternate roles for TSP1 in vascular responses to injury. Neointima formation following carotid artery ligation is reduced in TSP1 null mice, with decreased α-SMA-positive cells, but increased collagen and osteopontin deposition [57]. Others observed no difference in vascular response to wire-induced injury between wild-type and TSP1 null mice, but more thrombus formation [58]. Because TSP1 has multiple functions apart from regulation of TGF-β activation, including regulating MMP activity and NO signaling, these null phenotypes might reflect the absence of multiple, sometimes contradictory functions. It is also likely that cellular responses to TSP1 vary with the agent inducing vascular injury, the oxidative milieu and any underlying disease such as hypertension, diabetes and atherosclerosis.

Factors regulating VSMC phenotype switching are not completely understood. PKG is a major regulator of the contractile phenotype in adult smooth muscle cells [38]. Loss of PKG results in downregulation of contractile elements and increased expression of extracellular matrix proteins such as TSP1 and osteopontin [38]. Anderson et al. [59] showed that arterial PKG expression is decreased following balloon angioplasty and that loss of PKG coincides with increased osteopontin expression. Furthermore, expression of constitutively active PKG reduces neointima formation in a model of ISR [60]. PKG activity is dependent on NO-stimulated cGMP generation, which can be decreased in the presence of elevated ROS. PKG protein itself can also be downregulated by high levels of cAMP, cGMP or inflammatory mediators [38]. Previously, we showed that culture of mesangial cells or VSMCs with 30 mM glucose, which reduces bioavailable NO, downregulates PKG activity with increased TSP1 expression and TGF-β activity [61; S. Wang, T. Lincoln and J. Murphy-Ullrich, manuscript in preparation]. Our data now provide a link between metal ion-induced oxidative stress, PKG signaling and induction of the TSP1-TGF-β axis, suggesting that PKG regulation of TSP1 expression and the consequent TGF-β activation are involved in VSMC phenotype regulation by SS ions.

Interestingly, PKG activity has also been shown to inhibit TGF-β signaling in VSMCs and endothelial cells through inhibition of pSmad nuclear translocation, but not by inhibition of Smad phosphorylation [62, 63].

NO deficiency is associated with restenosis. Stenting also increases ROS, including hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{•-}$) and hydroxyl radical ($OH^•$) [15]. Antioxidant enzymes are decreased in rabbit tissues surrounding both titanium and SS implants [64]. High H_2O_2 and decreased catalase activity in response to implant debris can activate NFκB and initiate cytokine production [13]. Furthermore, macrophage attachment and H_2O_2 generation on SS surfaces was increased as compared to macrophages attached to diamond-like carbon film-coated steel surfaces, indicating that SS surfaces elicit ROS [65]. Inhibition of ROS signaling by various antioxidant approaches has been tested as a therapeutic approach to ISR. NO donors or stimulation of endogenous NO via L-arginine limits ISR in rabbit and rat aortas through increasing NO, cGMP and by inhibiting VSMC proliferation [66]. These data underscore the importance of ROS in mediating ISR.

Oxidation of specific amino acids on LAP leads to a conformational change in the latent complex, resulting in release of active TGF-β [67]. Furthermore, NAD(P)H oxidase 4 (NOX4), an enzyme which generates superoxide, mediates TGF-β1 conversion of fibroblasts to myofibroblasts [68]. The free radical scavenger NO inhibits TSP1 synthesis [61]. Conversely, TSP1 binding to its receptors CD47 or CD36 inhibits NO signaling by preventing cGMP accumulation [69]. This suggests that TSP1 is elevated when free radicals are high and NO is low, and that TSP1 itself might contribute to its auto-induction in a feed-forward loop. Furthermore, it is well established that H_2O_2 and oxidants generated by high glucose increase TGF-β expression and activity and that NO downregulates TSP1 expression in mesangial cells [70, 71]. Our data are supportive of a role for H_2O_2 in regulating PKG activity, TSP1 expression and TGF-β activity in VSMCs due to SS ions. Interestingly, MnTBAP, a mimetic of superoxide dismutase which converts superoxide anion to H_2O_2, actually increased ED-A FN and α-SMA production by mesenchymal stem cells in the presence or absence of SS ions (data not shown). The H_2O_2 generation assays demonstrated a requirement for manganese, iron and nickel ions. Of these ions, manganese alone was sufficient to generate H_2O_2 by VSMCs (data not shown), which is consistent with reports that manganese catalyzes H_2O_2 from dioxygen [72]. Furthermore, manganese chloride can induce the release of H_2O_2 from microglial cells [14].

In summary, these studies identify a molecular pathway involved in vascular smooth muscle cell-driven restenotic responses activated by the metal ions. These studies support the idea that corrosion of endovascular stent metals contributes to the problem of ISR through the release of metal ions to the vascular wall. These data suggest that methods to reduce stent metal corrosion or, alternately, to block the metal ion-triggered TSP1-TGF-β activation pathway might represent novel approaches to treat ISR.

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