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Dissecting the Cellular Mechanism of Prostacyclin Analog Iloprost in Reversing Vascular Dysfunction in Scleroderma

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Objective. Intravenous iloprost improves Raynaud's phenomenon (RP) and promotes healing of digital ulcers in systemic sclerosis (SSc; scleroderma). Despite a short half-life, its clinical efficacy lasts weeks. Endothelial adherens junctions, which are formed by VE-cadherin clustering between endothelial cells (ECs), regulate endothelial properties including barrier function, endothelial-to-mesenchymal transition (EndoMT), and angiogenesis. We undertook this study to investigate the hypothesis that junctional disruption contributes to vascular dysfunction in SSc, and that the protective effect of iloprost is mediated by strengthening of those junctions.

Methods. Dermal ECs from SSc patients and healthy controls were isolated. The effect of iloprost on ECs was examined using immunofluorescence, permeability assays, Matrigel tube formation, and quantitative polymerase chain reaction.

Results. Adherens junctions in SSc were disrupted compared to normal ECs, as indicated by reduced levels of VE-cadherin and increased permeability in SSc ECs ($P < 0.05$). Iloprost increased VE-cadherin clustering at junctions and restored junctional levels of VE-cadherin in SSc ECs (mean ± SD 37.3 ± 4.3 fluorescence units) compared to normal ECs (mean ± SD 29.7 ± 3.4 fluorescence units; $P < 0.05$), after 2 hours of iloprost incubation. In addition, iloprost reduced permeability of monolayers, increased tubulogenesis, and blocked EndoMT in both normal and SSc ECs ($n \geq 3$; $P < 0.05$). The effects in normal ECs were inhibited by a function-blocking antibody that prevents junctional clustering of VE-cadherin.

Conclusion. Our data suggest that the long-lasting effects of iloprost reflect its ability to stabilize adherens junctions, resulting in increased tubulogenesis and barrier function and reduced EndoMT. These findings provide a mechanistic basis for the use of iloprost in treating SSc patients with RP and digital ulcers.

INTRODUCTION

Systemic sclerosis (SSc; scleroderma) is an autoimmune disease characterized by immune activation, widespread fibrosis, and a structural and functional vasculopathy (1). Vascular involvement includes Raynaud's phenomenon (RP), digital ulcers, scleroderma renal crisis, and pulmonary arterial hypertension. RP is the most typical vascular manifestation that occurs earliest in SSc and generally precedes organ involvement. Digital ulcers are present in ~50% of SSc patients and responsible for significant disability and poor quality of life.

Iloprost is a synthetic analog of prostacyclin (prostaglandin I_2 [PGI_2]). Similar to PGI_2, iloprost has vasodilatory and antiplatelet effects but it is more stable than PGI_2, with a longer half-life (20–30 minutes) and better solubility (2). Intravenous (IV) iloprost is marketed in Europe for multiple indications, including the treatment of patients with severe, disabling RP that is unresponsive to other therapies. In addition, 2017 European Scleroderma Trials and

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Research recommendations assigned iloprost a grade A recommendation for treatment of severe SSc-related RP attacks and for treatment of digital ulcers (3). It is also widely used in the management of peripheral vascular complications unrelated to SSc-related RP. Pharmacologically, iloprost activates PGI_2 receptors, which stimulate adenylate cyclase to produce cAMP. PGI_2 receptors on smooth muscle cells and platelets inhibit smooth muscle constriction and platelet aggregation. PGI_2 receptors are also expressed on endothelial cells (ECs), where they initiate numerous protective effects, including augmentation of endothelial adherens junctions and reduced monolayer permeability (4,5).

Vascular and endothelial pathology is present in SSc patients. These functional and structural defects include increased vascular permeability, reduced nitric oxide (NO) activity, elevated inflammation, EC apoptosis, impaired angiogenesis, endothelial-to-mesenchymal transition (EndoMT), intravascular fibrosis, and microvascular rarefaction (6–13). ECs also have lower VE-cadherin expression (12). Adherens junctions, which are formed by clustering of VE-cadherin on neighboring ECs, regulate numerous endothelial properties, including cell morphology, signaling, and phenotype. The vascular-protective effects of adherens junctions include increased barrier function, amplified NO signaling, inhibited apoptosis, and reduced inflammation (14). In contrast, when junctions are disrupted, VE-cadherin and β-catenin are disengaged from the cell membrane and contribute to vascular dysfunction and EndoMT. It has been demonstrated that VE-cadherin clustering at adherens junctions is increased by iloprost and PGI_2, via activation of PGI_2 receptors (4,15).

Despite its short half-life, iloprost is beneficial for RP and the healing of digital ulcers, with effects extending for weeks after cessation of treatment (16). To dissect the mechanisms involved, we hypothesized that vascular dysfunction in SSc reflects disruption of EC adherens junctions and that the vascular-protective effect of iloprost is mediated by the strengthening of these junctions in SSc ECs. In this study, we demonstrate the beneficial effect of iloprost in SSc ECs. Iloprost was shown to enhance the impaired barrier dysfunction in these cells, promote angiogenesis, and inhibit EndoMT, all of which were potentially dependent on increased VE-cadherin clustering at adherens junctions, as blockade of VE-cadherin in normal ECs blocked these effects.

PATIENTS AND METHODS

Patients and controls. All patients who were recruited met the 2013 American College of Rheumatology/European League Against Rheumatism criteria for the classification of SSc (17). We obtained two 4-mm punch biopsy specimens from the distal forearm of subjects for EC isolation. The healthy controls and patients were matched according to age, ethnicity, and sex (Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41536/abstract). Thirteen healthy controls were recruited (mean ± SEM age 50.8 ± 3.8 years). All 10 patients had diffuse cutaneous SSc (mean ± SEM age 54.9 ± 4.9 years), and the mean ± SEM disease duration was 2.6 ± 0.4 years. Subjects' skin scores ranged from 0 to 28, with a mean ± SEM score of 11.1 ± 2.9. All patients had RP, but none had active digital ulcers at the time of biopsy. These patients were being treated with immunosuppressive drugs, vasodilators, or proton pump inhibitors, among others, at the time of biopsy (Supplementary Table 1). This study was approved by the University of Michigan Institutional Review Board.

Cell culture and treatment. Dermal ECs were isolated from skin biopsy specimens obtained from the forearms of subjects. Although the skin scores ranged from 0 to 28 in SSc patients, the skin scores at the site of biopsies ranged between 0 and 2 (6 patients with a score of 0, 3 with a score of 1, and 1 with a score of 2). The cells from patients were randomized in each experiment, with ≥3 patient-derived lines used in each assay. Skin digestion and cell purification were described previously (6,18). A CD31 MicroBead Kit (Miltenyi Biotech) was used to purify ECs. Cells were maintained in endothelial basal medium 2 (EBM-2) supplemented with growth factors (Lonza). For all experiments, ECs between passages 3 and 6 were used. Before all experiments, cells were cultured in endothelial growth medium supplemented with bovine brain extract for ≥1 day. Cells were treated with 150 n*M* iloprost (Cayman) at various time points. There are currently no published pharmacokinetic studies on SSc patients receiving iloprost by IV infusion. In healthy subjects, infusion of iloprost at doses of 1 ng/kg/minute and 3 ng/kg/minute achieved steady-state concentrations of 0.13 n*M* and 0.37 n*M*, respectively (19). However, SSc patients showed increased systemic exposure to iloprost after oral doses, suggesting reduced clearance of the drug, with a 1.8-fold increase in maximum concentration after 8 days of iloprost administration (20). The concentration of iloprost in this study was chosen based on published in vitro studies (21–23).

Inhibition of VE-cadherin clustering at endothelial junctions was achieved by pretreating cells with 25 μg/ml VE-cadherin function–blocking antibody (BV9; LSBio) for 30 minutes in culture. This approach prevents new VE-cadherin *trans* interactions without affecting existing junctional or monolayer integrity (21). To induce EndoMT in normal ECs, cells were treated with 10 ng/ml transforming growth factor β (TGFβ) and/or 150 n*M* iloprost for 3 days. For the groups incorporating BV9, the cells were pretreated with 25 μg/ml of this antibody for 30 minutes before TGFβ and/or iloprost were added. BV9 was present for the duration of the experiment.

Immunofluorescence staining. ECs were cultured in gelatin-coated chambers and treated with iloprost for up to 2 hours. Anti–VE-cadherin antibodies (R&D Systems), anti–β-catenin antibodies (Abcam), and Texas Red-X Phalloidin (ThermoFisher) were used to visualize VE-cadherin, β-catenin, and F-actin, respectively. VE-cadherin and β-catenin were then

probed using Alexa Fluor secondary antibodies. The nuclei were stained using DAPI. Fluorescence was detected using a Nikon A1 confocal microscope. Visualization and analysis of images were performed using the ND2 reader plugin in ImageJ.

Permeability assay. Permeability was assessed by measuring horseradish peroxidase (HRP) movement through EC monolayers in a Transwell system (Cell Biologics). Briefly, ECs were plated at 50,000 cells/ml in the Transwells and allowed to grow to confluence, then cultured in EBM-2 media with 1% fetal bovine serum (FBS) in the upper and lower chambers. Treatments, including iloprost (150 n*M*) and/or TGFβ (10 ng/ml), were added to the upper chambers along with HRP, and aliquots of the media in the lower chambers were collected at various time points. When analyzing the effect of the function-blocking antibody to VE-cadherin, cells were pretreated with BV9 (25 μg/ml) for 30 minutes before the addition of iloprost and/or TGFβ. BV9 was present throughout the experiment. The amount of HRP was quantified by the addition of 3,3',5,5'-tetramethylbenzidine and stop solution and measured at 450 nm in a plate reader.

Matrigel tube formation assay. To examine whether iloprost affects EC angiogenesis, we pretreated ECs with iloprost for 24 hours and performed Matrigel tube formation assays. Growth factor–reduced Matrigel (BD Biosciences) was coated in 8-well Lab-Tek chambers before adding treated ECs, which were suspended in EBM-2 with 1% FBS in the presence of

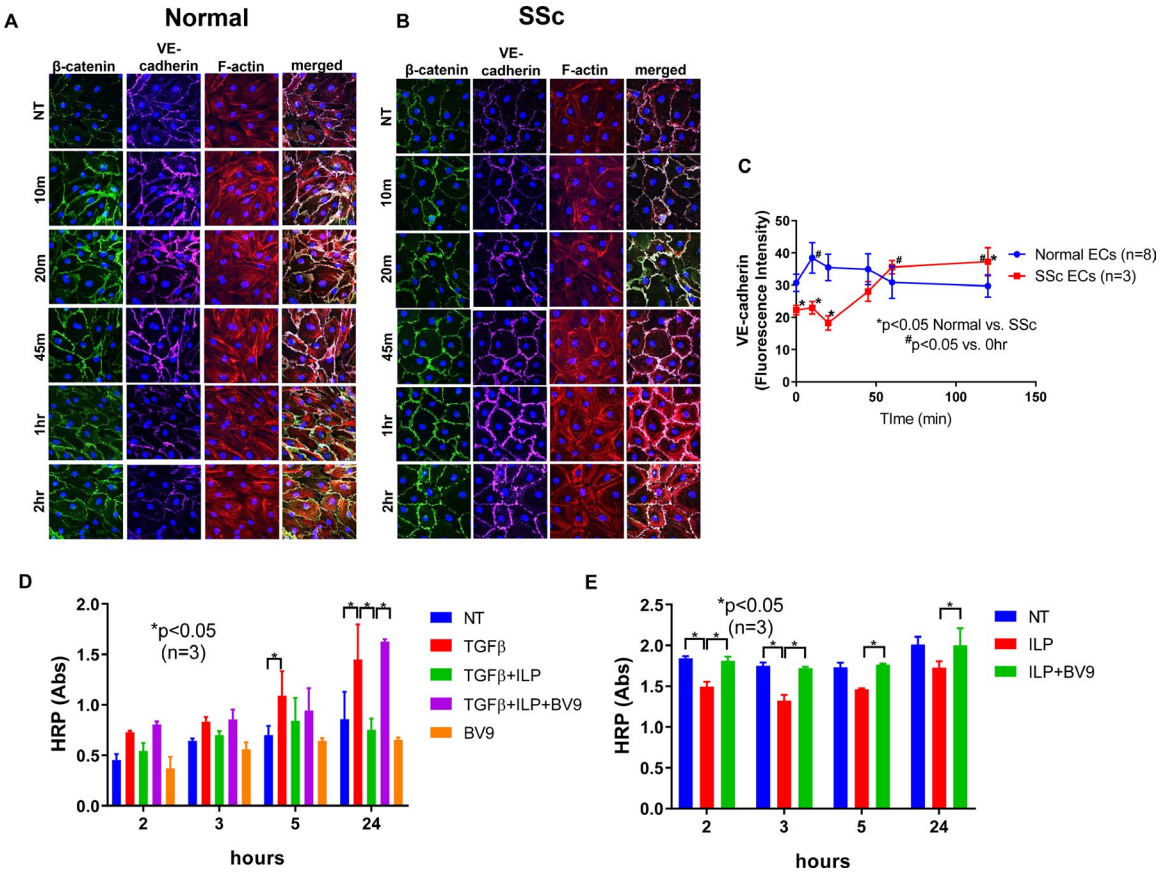

Figure 1. Effect of iloprost (ILP) on VE-cadherin localization and cell permeability. Immunofluorescence of VE-cadherin, β-catenin, and F-actin in endothelial cells (ECs) was visualized using a Nikon A1 confocal microscope. Cell permeability was assessed by measuring horseradish peroxidase (HRP) movement through EC monolayers using an Endothelial Transwell Permeability Assay Kit. Cells were treated with iloprost (150 n*M*) and/or transforming growth factor β (TGFβ; 10 ng/ml) at various time points. BV9 (25 μg/ml) was used to pretreat the cells for 30 minutes before iloprost and TGFβ were added. **A**, Iloprost increased junctional clustering of VE-cadherin and β-catenin with as little as 10 minutes of incubation in normal ECs. Original magnification × 600. **B**, Iloprost had a delayed but more prolonged effect on VE-cadherin and β-catenin clustering in systemic sclerosis (SSc) ECs. Disorganized F-actin filaments in SSc ECs were also observed. Original magnification × 600. **C**, Quantification of fluorescent signal of VE-cadherin showed significant reduction of VE-cadherin in SSc ECs compared to normal ECs at baseline. After iloprost treatment, VE-cadherin intensity was greater in SSc ECs compared to normal ECs. **D**, In normal ECs, TGFβ increased permeability as measured by HRP movement through EC monolayers. Iloprost inhibited permeability of EC monolayers, while blockade of VE-cadherin by BV9 reversed it. **E**, In SSc ECs, iloprost inhibited the increased permeability of these cells, while the function-blocking antibody to VE-cadherin (BV9) prevented the effects of iloprost therapy. Experiments were performed with ≥3 subject-derived lines. Values in **C**–**E** are the mean ± SD. NT = not treated; Abs = absorbance.

iloprost. Cells were cultured for 6 hours before they were fixed and stained. Pictures were taken using the EVOS XL Core Cell Imaging System. The Angiogenesis Analyzer function in ImageJ was used to quantify the tubes. In a separate experiment, we adopted a different approach, in which blocking antibodies to VE-cadherin (25 μg/ml) were added in the Matrigel-coated chambers with the ECs for 30 minutes before adding iloprost. The ECs were then cultured in the presence of iloprost and/or BV9 for an additional 8–10 hours.

Messenger RNA (mRNA) extraction and quantitative reverse transcriptase–polymerase chain reaction. Extraction of RNA was performed using a Direct-zol RNA Mini-Prep Kit and complementary DNA (cDNA) was prepared using a Verso cDNA synthesis kit. Primers were mixed with Power SYBR Green PCR master mix (Applied Biosystems). The ViiA 7 Real-Time PCR System was used to quantify cDNA.

Statistical analysis. Results are expressed as the mean ± SD, except when stated otherwise. To determine the significance of differences between the groups, Mann-Whitney U test, Kruskal-Wallis test, or two-way analysis of variance was performed using GraphPad Prism, version 6. *P* values less than 0.05 were considered significant.

RESULTS

Disruption of adherens junctions in SSc ECs compared to normal ECs. To examine the effect of iloprost on adherens junctions, we treated ECs with 150 n*M* iloprost for various periods of time, and VE-cadherin, β-catenin, and F-actin were visualized via immunofluorescence. Under control conditions, in the absence of iloprost (untreated or at baseline [0 minutes]), adherens junctions were disrupted and F-actin filaments were disorganized in SSc ECs compared to normal ECs (Figures

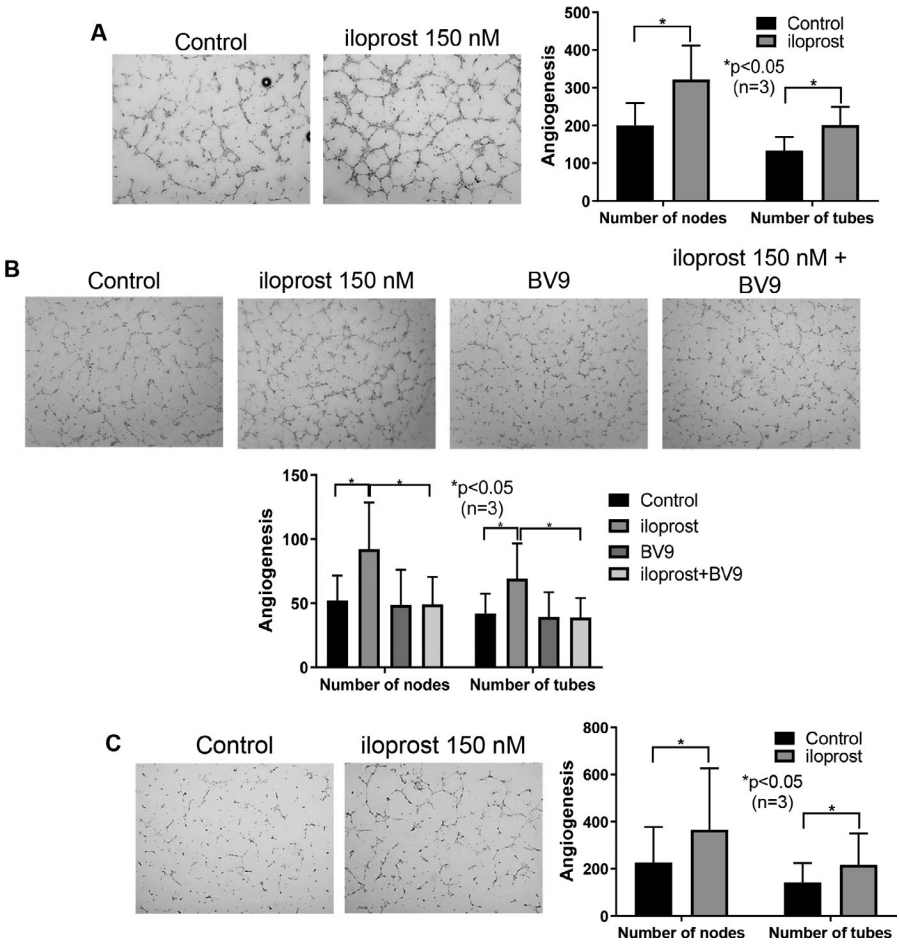

Figure 2. Effect of iloprost on endothelial cell (EC) angiogenesis. Angiogenesis of ECs was measured using an in vitro Matrigel tube formation assay. **A** and **B**, Iloprost increased tubulogenesis in normal ECs, and this was reduced by a function-blocking antibody to VE-cadherin (BV9). **C**, Iloprost induced tubulogenesis in systemic sclerosis (SSc) ECs. Original magnification × 40. In **A** and **C**, ECs were pretreated with iloprost (150 n*M*) overnight before they were plated on Matrigel. After 6 hours, cells were fixed and stained. In **B**, BV9 (25 μg/ml) was used to pretreat the ECs for 30 minutes before iloprost was added. The cells were cultured for 8 hours before they were fixed and stained. Experiments were performed with 3 subject-derived lines. Values are the mean ± SD.

1A and B). Indeed, junctional levels of VE-cadherin were significantly lower in SSc ECs compared to normal ECs at baseline (Figure 1C). In normal ECs, iloprost stimulation caused a transient increase in the clustering of VE-cadherin and β-catenin at cell junctions, which peaked at 10 minutes and returned to control levels by 60 minutes (Figure 1A). VE-cadherin was significantly elevated at 10 minutes of iloprost stimulation compared to baseline ($P < 0.05$) (Figure 1C). Staining of F-actin showed that iloprost induced accumulation of peripheral F-actin at the adherens junctions (Figure 1A).

In contrast, in SSc ECs, iloprost caused a delayed but more sustained increase in the clustering of β-catenin and VE-cadherin at cell junctions (Figures 1B and C). The increase in VE-cadherin clustering was evident at 60 minutes and remained significantly elevated after 120 minutes (Figure 1C). After iloprost treatment, the clustering of VE-cadherin at cell junctions in SSc ECs was not only normalized but was significantly higher than was observed in normal ECs (Figure 1C). Consistent with the increased clustering of VE-cadherin and strengthening of adherens junctions, iloprost stimulation also promoted sustained increases in cortical F-actin in SSc ECs (Figure 1B).

Effect of iloprost on endothelial monolayer permeability. To examine the effect of iloprost on permeability of EC monolayers, we first treated normal ECs with TGFβ, which is known to increase permeability in ECs (24). TGFβ increased HRP permeability significantly across EC monolayers at 5 and 24 hours (Figure 1D). Coincubation of iloprost prevented the TGFβ-induced increase in permeability at 24 hours. To examine whether VE-cadherin is involved in the response to iloprost, we used BV9, a blocking antibody to VE-cadherin that prevents it from clustering at EC junctions. Pretreatment with BV9 did not affect EC monolayer integrity (Figure 1D). However, the effect of iloprost in reducing the TGFβ-induced increase in permeability was prevented by BV9 at 24 hours, suggesting that the protective effect of iloprost on EC monolayer integrity was dependent on VE-cadherin clustering at adherens junctions.

Because microvascular abnormalities and vascular leakage are prominent hallmarks of SSc (25), we postulated that SSc ECs would show barrier dysfunction in vitro. Indeed, under control conditions in the absence of iloprost, HRP permeability was significantly higher in SSc ECs compared to normal ECs (mean ± SD HRP absorbance 1.843 ± 0.027 versus 0.454 ± 0.059 at 2 hours; 1.751 ± 0.038 versus 0.644 ± 0.022 at 3 hours; 1.733 ± 0.054 versus 0.700 ± 0.091 at 5 hours; 2.010 ± 0.096 versus 0.855 ± 0.272 at 24 hours; $P < 0.005$ for all), confirming that SSc ECs have impaired endothelial barrier function. Iloprost treatment significantly decreased the permeability of SSc ECs at 2 and 3 hours, an effect that was prevented by BV9 (Figure 1E). Taken together, these results suggest that iloprost potentiates formation of adherens junctions, augmenting endothelial barrier function and reducing the barrier dysfunction of SSc ECs in a VE-cadherin–dependent manner.

Effect of iloprost on EC tubulogenesis. In normal ECs, iloprost significantly increased tube formation (Figure 2A). This angiogenic effect was prevented by BV9 (Figure 2B), suggesting that it was dependent on the clustering of VE-cadherin at endothelial junctions. We have previously shown that angiogenesis is deficient in SSc ECs (6,18). Pretreatment of SSc ECs with iloprost enhanced their ability to form tubes on Matrigel (Figure 2C).

Effect of iloprost on EndoMT. EndoMT appears to be a prominent feature of SSc, with SSc ECs having increased expression of EndoMT markers and reduced expression of EC marker proteins (12). During EndoMT, ECs lose cell–cell adhesion, disrupting endothelial junction stability and increasing vascular permeability. We hypothesized that iloprost, by enhancing VE-cadherin clustering at the adherens junction, could reduce EndoMT in SSc. We first tested this hypothesis in normal ECs. TGFβ treatment for 72 hours in ECs significantly increased mesenchymal markers at the mRNA level, including *ACTA2* (encoding for α-smooth muscle actin [αSMA]) and *S100A4,* while significantly down-regulating EC markers *PECAM1* and *CDH5* (encoding for CD31 and VE-cadherin) (Figure 3A). In addition, TGFβ induced *SNAI1* (encoding for SNAIL), a transcription factor for EndoMT. However, TGFβ did not affect *FLI1*. Cotreatment with iloprost significantly reduced TGFβ-mediated EndoMT by decreasing the up-regulated mesenchymal markers and *SNAI1*, while increasing the down-regulated EC markers. These findings suggest that iloprost inhibits EndoMT induced by TGFβ. This protective effect of iloprost was markedly reduced by the function-blocking antibody to VE-cadherin, which enabled restoration of the EndoMT response to TGFβ (Figure 3A). This indicates that iloprost inhibits EndoMT by increasing the clustering of VE-cadherin at endothelial junctions. Similarly, in SSc ECs, iloprost reduced the increased levels of EndoMT in these cells, significantly reducing expression of mesenchymal markers (*COL1A1*, *ACTA2*, *S100A4*) and *SNAI1* (Figure 3B).

DISCUSSION

In this study, we have provided a mechanistic basis for the use of iloprost in treating SSc patients with RP and digital ulcers. We have demonstrated that iloprost stabilizes endothelial adherens junctions, increases barrier function, promotes EC tubulogenesis, and inhibits EndoMT, all of which would be expected to have important therapeutic benefits in SSc (Figure 4). These protective effects of iloprost appear to be dependent on increased clustering of VE-cadherin at endothelial junctions, because they were markedly reduced by a function-blocking antibody to VE-cadherin. Since enhanced VE-cadherin clustering also occurs in response to PGI_2 itself and other PGI_2 analogs (4,15), we believe the effects observed in this study are not limited to iloprost itself but apply to other PGI_2 analogs and agonists.

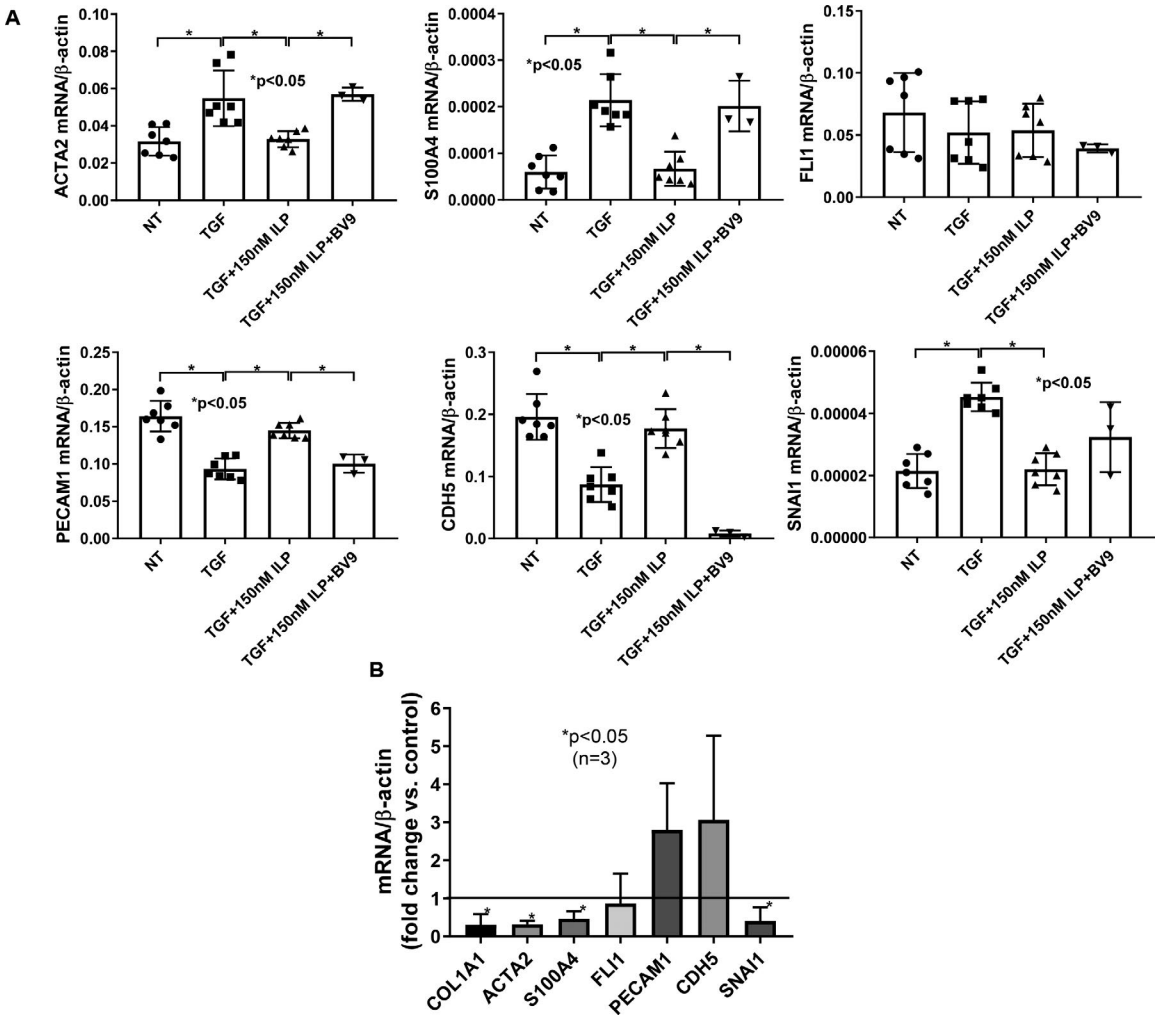

Figure 3. Effect of iloprost on endothelial-to-mesenchymal transition (EndoMT) in ECs. Normal ECs were treated with 10 ng/ml TGFβ and/or 150 n*M* iloprost for 3 days. BV9 was added 30 minutes before the addition of other specified treatments. Cellular markers for EndoMT were measured by quantitative polymerase chain reaction. **A**, Iloprost inhibited TGFβ-induced EndoMT in normal ECs, and the effect was blocked by BV9, a VE-cadherin antibody. **B**, Iloprost inhibited the EndoMT phenotype in SSc ECs. Experiments were performed with 3–7 subject-derived lines. Symbols represent individual subjects; bars show the mean ± SD. See Figure 1 for other definitions.

Pathologically, endothelial injury is a pivotal initial event in SSc pathogenesis. Persistent activation of SSc ECs by currently unknown sources results in early functional changes and alterations in vasculature including vascular leakage (26). Indeed, electron microscopy of the nailfold in patients with early SSc revealed decreased capillary loops with intercellular gaps, associated with interstitial edema (9–11). The regression of the small vessels in SSc is partly due to destabilization of vessels and defect in angiogenesis. As demonstrated by us and others, SSc ECs showed reduced angiogenic properties in in vitro studies (6,7). These cells also showed intrinsic defect in NO production due to down-regulation of endothelial NO synthase (8). Although these endothelial events precede tissue fibrosis, leakage of the blood vessels fuels later tissue fibrosis by involving abnormal ECs, activated inflammatory cells, and fibroblasts. In addition, in the presence of TGFβ and immune and profibrotic mediators, SSc ECs acquire a promigratory and profibrotic phenotype through EndoMT, where they differentiate into collagen-producing/αSMA-positive cells that contribute to intravascular and extravascular fibrosis (12). All of these events illustrate the critical involvement of endothelial dysregulation in SSc pathogenesis. Therapeutic intervention aiming to stabilize ECs and reverse endothelial dysfunction may not only inhibit vascular complications in SSc patients, but also attenuate fibrosis.

Adherens junctions are largely composed of VE-cadherin that binds to several partners, including β-catenin, via its cytoplasmic domain. Junctional clustering of VE-cadherin and β-catenin directly or indirectly modulates various proteins and signaling pathways including Rac1, tyrosine kinase receptors, protein tyrosine phosphatases, Akt, RhoA/ROCK, Wnt, and Notch signaling (14,27,28). These complex signaling events reflect the wide range of biologic effects in which adherens junctions are involved.

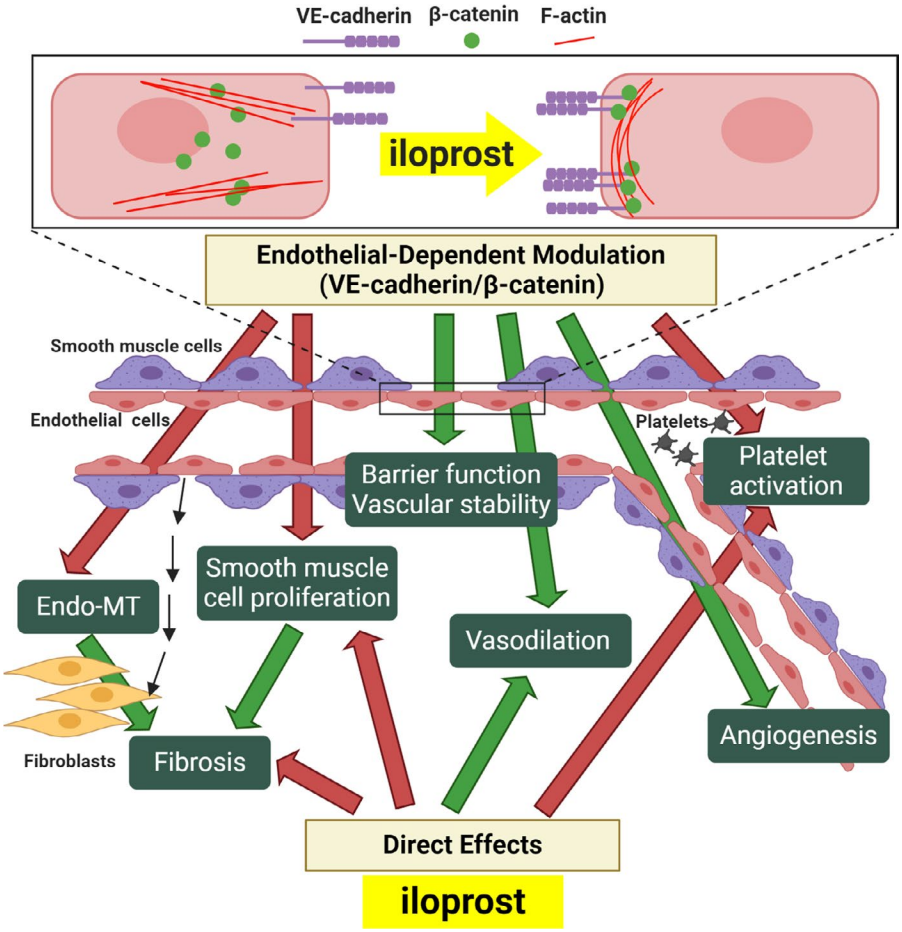

Figure 4. Vascular protective effect of iloprost in systemic sclerosis (SSc). In SSc endothelial cells, iloprost increases VE-cadherin and β-catenin clustering at the adherens junction, accompanied by accumulation of peripheral F-actin. Increased interaction and signaling of VE-cadherin/β-catenin promotes protective endothelial functions and vascular stability including an increase in barrier function, promotion of angiogenesis, inhibition of endothelial-to-mesenchymal transition (EndoMT), and increased nitric oxide activity, which contributes to vasodilation, inhibition of platelet activation, and blockade of smooth muscle cell proliferation. EndoMT and smooth muscle proliferation can contribute to intravascular and extravascular fibrosis, and to microvascular rarefaction. In addition to these endothelial-dependent modulatory effects, iloprost can act directly on platelets, fibroblasts, and smooth muscle cells to inhibit platelet activation and fibrosis and promote vasodilation. Inhibitory effects are indicated with **red arrows**, and positive effects are indicated with **green arrows**.

They not only promote endothelial barrier function but maintain EC identity by inhibiting EndoMT, promoting NO production, inhibiting apoptosis, blocking leukocyte extravasation and inflammation, and promoting endothelial and vascular stability (14). Indeed, when adherens junctions are disrupted, increased permeability, impaired NO production, inflammatory cell infiltration, enhanced transcription of proinflammatory mediators, and increased EndoMT are well documented. Based on this evidence, disruption of adherens junctions could lead to serious pathologic consequences in the vasculature, many of which are characteristic of vascular complications seen in SSc. Interestingly, immunohistochemistry staining of skin biopsy samples showed that CD31-positive ECs were accompanied by a loss of VE-cadherin expression in SSc patients (13). In vitro experiments, including those in this study, also confirmed the down-regulation of VE-cadherin in SSc ECs (12).

Our findings showing that the barrier-protective effect of iloprost in SSc ECs was mediated by enhancing VE-cadherin adherens junctions echo those of Birukova et al (21). This is also demonstrated in pulmonary ECs and human umbilical ECs using PGI_2; the barrier-protective effect of PGI_2 was mediated by cAMP and downstream pathways, which ultimately led to enhancement of adherens junctions (4,15). In several follow-up studies, the protective effect of iloprost on barrier dysfunction has been further highlighted (29–31). Iloprost not only protected against ventilator-induced acute lung injury in mice by improving lung endothelial barrier function, it also enhanced barrier function in cultured human lung microvascular ECs (29). The beneficial effect of iloprost in a model of septic lung injury was due, in part, to attenuating barrier dysfunction in the lung (30,31). Additionally, in human pulmonary artery ECs, iloprost

attenuated the disruption of the endothelial monolayer and suppressed the activation of p38 MAPK, NF-κB, and Rho signaling after lipopolysaccharide challenge. These studies using PGI_2 and its analogs in lungs and pulmonary ECs further support the benefits of administering these drugs to patients with pulmonary arterial hypertension.

We postulate that the mechanisms behind the prolonged effect of iloprost on barrier protection in SSc ECs are multifactorial. The cAMP/exchange protein directly activated by the cAMP/Rap1 pathway involved in barrier function might be impaired in SSc ECs. In addition, lower levels of VE-cadherin in SSc ECs might require more time to cluster at the junctions. We previously demonstrated that RhoA/Rock expression and activity are elevated in SSc ECs compared to normal ECs (18). Since activation of the RhoA/ROCK signaling pathway induced VE-cadherin internalization in ECs (32), it is possible that the activated RhoA/ROCK signaling in SSc ECs reduces VE-cadherin localization at cell junctions. In addition, EZH2, a histone methyltransferase that catalyzes repressive H3K27me3 marks, might also play a role. We previously showed that, in SSc ECs, both EZH2 and H3K27me3 are up-regulated compared to normal ECs (33). A study by Morini et al suggested that clustered VE-cadherin anchors EZH2 at the cell membrane, allowing active gene transcription by impeding the recruitment of EZH2 to the polycomb repressive complex to promoters of genes that strengthen endothelial junctions (34). Down-regulation of VE-cadherin at cell junctions and nuclear localization of EZH2 in SSc ECs could both contribute to the vascular leakage and barrier dysfunction in this disease.

In addition to barrier enhancement, we showed that iloprost induced EC angiogenesis on Matrigel. The proangiogenic potential of iloprost was also shown in dental organ cultures (35), endothelial progenitor cells (36), and mouse models of cornea neovascularization (37,38). Early studies suggested that the proangiogenic properties of iloprost depend on its action on peroxisome proliferator–activated receptors, which occurs via a vascular endothelial growth factor–dependent mechanism (37,38). Although additional mechanisms may be involved, the findings of the present study suggest that the effect of iloprost in increasing junctional clustering of VE-cadherin plays a fundamental role in the angiogenic response of this drug.

In SSc, EndoMT is evident and likely contributes to intravascular and extravascular fibrosis and microvascular rarefaction (12,39). Both TGFβ and endothelin 1 treatments induced EndoMT in normal and SSc ECs in a Smad-dependent manner (39). In addition, SSc ECs have lower levels of endothelial markers and increased expression of fibroblast markers such as αSMA (12). These cells also show increased ability to contract collagen gels, a major characteristic of myofibroblast function. While the canonical TGFβ/Smad pathway is a major regulator for EndoMT, TGFβ-induced EndoMT can also be impacted by crosstalk with other pathways, including Wnt/β-catenin, endothelin 1, and Notch (40). Moreover, modulators of TGFβ, such as thrombospondin 1, and proteins regulated by TGFβ, such as connective tissue growth factor (CTGF), can also contribute to EndoMT directly or indirectly (41,42). As disruption of adherens junctions is a key initial step of EndoMT (43) and many of these aforementioned pathways interact directly or indirectly with adherens junctions (14,28), the effect of iloprost on EndoMT inhibition in ECs is not surprising. Indeed, increased clustering of VE-cadherin/β-catenin at adherens junctions can inhibit the nuclear localization of β-catenin and block its EndoMT-promoting effect (14).

PGI_2 is synthesized from arachidonic acid by sequential actions of cyclooxygenase and PGI_2 synthase. It acts directly on platelets and vascular smooth muscle cells to reduce platelet aggregation and induce vascular relaxation through the cAMP pathway. These effects will be amplified by the effects of PGI_2 on ECs. Increased VE-cadherin clustering at adherens junctions can amplify NO production and reverse endothelial NO dilator dysfunction (14), which is present in SSc (8). Because NO–cGMP signaling acts synergistically with PGI_2–cAMP signaling to induce vasodilation, as well as inhibition of platelet activation and thrombosis, the ability of PGI_2 to stimulate both cGMP and cAMP pathways in the vasculature will provide added benefit for SSc patients. The vascular-protective effect of PGI_2 analogs, therefore, likely stems from their combined action on multiple cell types. Likewise, iloprost acts directly on fibroblasts to block CTGF production and collagen synthesis (44). The elevated levels of CTGF in skin blister fluid from SSc patients were also reduced by 5 days of iloprost therapy. The antifibrotic effect of iloprost was further shown in an animal model of heart failure and pulmonary fibrosis. Figure 4 summarizes the effects of iloprost.

One potential limitation of this study is the lack of recruitment of patients with limited cutaneous SSc. We routinely recruit patients with diffuse cutaneous SSc for EC isolation, since this is the most severe form of disease and ECs from these patients show prominent impairment in endothelial phenotypes (6,18,33,45). It has been shown that the vascular effect of iloprost was evident in both diffuse and limited cutaneous SSc patients, with or without digital ulcers (46), suggesting that iloprost does not discriminate between these patient groups. Another potential drawback is the variability stemming from patient disease activity and medication differences between patients and controls. Although the overall skin scores ranged from 0 to 28, the skin scores at the site of biopsy ranged from 0 to 2. We randomized patient cells into various experiments and also controlled the experiments by using age-, sex, and ethnicity-matched healthy subjects. As the cells were cultured for ≥3 passages before they were used in experiments, and medications taken at the time of skin biopsy had reversible mechanisms of action, they would have been removed during cell processing. Therefore, our experimental findings on endothelial function are unlikely to have been directly affected by the medications. In this study, BV9 was incorporated in all experiments using normal ECs as a validation that the effect of iloprost on VE-cadherin clustering was critical in improving those same functional end points. Although we included BV9 control studies on SSc ECs in the permeability

assay only, the effect of VE-cadherin blockade was similar to what was observed in normal ECs. We postulate that the effect of BV9 in tube formation and EndoMT shown with SSc ECs would show similar results if performed with normal ECs. We recognize that the iloprost concentration that we used in this study (150 n*M*) is significantly higher than the steady-state concentrations reported in healthy controls receiving iloprost infusion (0.13 n*M* and 0.37 n*M*) (19). Clinically, for SSc patients with RP or digital ulcers, iloprost is most commonly administered and titrated to the highest tolerated dose, between 0.5 ng/kg/minute and 2 ng/kg/minute, for 6–8 hours of infusion on day 1 and continued for 5 consecutive days (47). It is possible that for short-term iloprost treatments in vitro, a higher dose is needed to achieve therapeutic effects as opposed to the prolonged infusion treatment that patients typically receive. In addition, the dose used in this study is similar to what was used in in vitro systems published previously (21–23).

In summary, our data provide novel insight into the vascular effects of iloprost treatment in SSc. Endothelial adherens junctions function as an amplification nexus for protective EC signaling, including positive feedback that strengthens the junctions (14). The prolonged clinical endothelial-protective effect of PGI_2 analogs may therefore stem from their ability to stabilize EC adherens junctions resulting in vasculoprotection, including improved barrier function, normalization of dysregulated angiogenesis, and inhibition of EndoMT. The present findings, together with their antiplatelet, vasodilatory, and potential antifibrotic effects, indicate that the therapeutic use of PGI_2 analogs in treating SSc-associated RP and digital ulcers is warranted.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Tsou had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Tsou, Flavahan, Khanna.
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