Differential Effects of Selexipag and Prostacyclin Analogs in Rat Pulmonary Artery

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ABSTRACT
(4-[[5,6-Diphenylpyrazin-2-yl]((isopropyl)amino)butoxy]acetic acid (ACT-333679) is the main metabolite of the selective prostacyclin (PGI₂) receptor (IP receptor) agonist selexipag. The goal of this study was to determine the influence of IP receptor selectivity on the vasorelaxant efficacy of ACT-333679 and the PGI₂ analog treprostinil in pulmonary artery under conditions associated with pulmonary arterial hypertension (PAH). Selexipag and ACT-333679 evoked full relaxation of pulmonary artery from control and monocrotaline (MCT)-PAH rats, and ACT-333679 relaxed normal pulmonary artery contracted with either endothelin-1 (ET-1) or phenylephrine. In contrast, treprostinil evoked weaker relaxation than ACT-333679 of control pulmonary artery and failed to induce relaxation of pulmonary artery from MCT-PAH rats. Treprostinil did not evoke relaxation of normal pulmonary artery contracted with either ET-1 or phenylephrine. Expression of prostaglandin E₃ (EP₃) receptor mRNA was increased in pulmonary artery from MCT-PAH rats.

Introduction
Selexipag [2-4-[[5,6-diphenylpyrazin-2-yl]((isopropyl)amino)butoxy]N-(methylsulfonyl)acetamide] is an orally available IP receptor agonist in clinical development for the treatment of PAH (Simonneau et al., 2012). Both selexipag and its active metabolite (4-[[5,6-diphenylpyrazin-2-yl]((isopropyl)amino)butoxy]acetic acid (ACT-333679) (previously known as MRE-269) possess high selectivity for the IP receptor over other prostanoid receptors and as such can be distinguished from PGI₂ analogs currently used in the management of PAH (Kuwano et al., 2007). Progression of PAH is associated with reduced PGI₂ production and its metabolite ACT-333679 is not modified under conditions associated with PAH, whereas relaxation to treprostinil may be limited in the presence of mediators of disease.

In contraction experiments, the selective EP₃ receptor agonist sulprostone evoked significantly greater contraction of pulmonary artery from MCT-PAH rats compared with control rats. The presence of a threshold concentration of ET-1 significantly augmented the contractile response to sulprostone in normal pulmonary artery. ACT-333679 did not evoke direct contraction of rat pulmonary artery, whereas treprostinil evoked concentration-dependent contraction that was inhibited by the EP₃ receptor antagonist (2E)-3-[(3',4'-dichlorobiphenyl-2-yl)-N-(2-thienylsulfonyl)acrylamide]. Antagonism of EP₃ receptors also revealed a relaxant response to treprostinil in normal pulmonary artery contracted with ET-1. These data demonstrate that the relaxant efficacy of the selective IP receptor agonist selexipag and its metabolite ACT-333679 is not modified under conditions associated with PAH, whereas relaxation to treprostinil may be limited in the presence of mediators of disease.

ABBREVIATIONS:
PGL₂, prostacyclin; IP receptor, PGI₂ receptor; EP₃ receptor, prostaglandin E receptor 1; ET-1, endothelin-1; EPA, extralobar pulmonary artery; ET-1, endothelin-1; IP receptor, therefore, is emerging as an important consideration in the management of PAH. For example, vasorelaxation to iloprost and beraprost is attenuated in pulmonary artery from monocrotaline (MCT)-PAH rats (Kuwano et al., 2008) via a mechanism that involves coactivation of the contractile EP₃ receptor. In contrast, ACT-333679 has at least 130-fold selectivity for the human IP receptor over other prostanoid receptors (Kuwano et al., 2007), and vasorelaxation of rat pulmonary artery evoked by ACT-333679 is not modulated by antagonism of IP receptors (Kuwano et al., 2008). Moreover, gastric side effects including cramping, nausea, and vomiting may develop via EP₃ receptor-dependent receptor signaling using analogs of PGL₂ is an effective strategy in the treatment of the disease (Gomberg-Maitland and Olschewski, 2008). Clinical efficacy and tolerability of PGI₂ analogs, however, may be compromised by concomitant activation of other prostanoid receptors. The selectivity of PGL₂ replacement therapies for the IP receptor, therefore, is emerging as an important consideration in the management of PAH.
mechanisms. Analogs of PGI₂ contract gastric smooth muscle via the stimulation of EP₃ receptors (Morrison et al., 2010). Activation of the EP₃ receptor subtype mediates disruption of gastric contractility (Pal et al., 2007; Forrest et al., 2009) and underlies the development of emesis (Kan et al., 2002). Selexipag does not evoke gastric contraction or disrupt gastric function in the rat stomach (Morrison et al., 2010). A role for EP₃ receptors has also been invoked in the development of peripheral pain reported in patients receiving treatment with PGI₂ analogs (Minami et al., 2001; Southall and Vasko, 2001). Thus, the development of a selective IP receptor agonist that is devoid of off-target effects may provide improved efficacy and tolerability in patients with PAH.

Infusion of treprostinil via the subcutaneous or intravenous routes is effective in the clinical management of PAH (Simonneau et al., 2002; Tapson et al., 2006). In addition, treprostinil reduces pulmonary arterial pressure, as measured by right ventricular systolic pressure and total pulmonary vascular resistance in the rat MCT-PAH model (Yang et al., 2010), but the contribution of dilatation of the pulmonary artery to this effect is not clear. Little information on the direct vasorelaxant efficacy of treprostinil in pulmonary artery ex vivo is available.

The present study sought to determine the influence of IP receptor selectivity of ACT-333679 and treprostinil on vasorelaxation of the rat pulmonary artery under pathological conditions associated with PAH. Experiments were performed with pulmonary artery isolated from the rat MCT-PAH model. Although development of neointimal and plexiform lesions, which are characteristic of the human disease (Tuder et al., 2007), is not a feature of this rat model, pulmonary hypertension in MCT-PAH rats is associated with sustained pulmonary arterial vasoconstriction (Oka et al., 2008). The pulmonary artery isolated from this model provides a suitable functional system in which to evaluate vasorelaxant properties of test compounds. Dysfunction of both the ET-1 and adrenergic systems underlies the development of PAH (Salvi, 1999; Rubens et al., 2001), and circulating levels of ET-1 and catecholamines are significantly increased in the MCT rat model of PAH (Clozel et al., 2006). Thus, the current study measured the ability of ACT-333679 and treprostinil to relax pulmonary artery from MCT-PAH rats and control pulmonary artery precontracted with ET-1 or the α-adrenoceptor agonist phenylephrine. The expression levels of mRNA encoding prostanoid receptors in the MCT rat model of PAH were also analyzed to provide a molecular mechanistic explanation for potential differences in arterial reactivity to the test compounds.

The data presented here highlight the importance of IP receptor selectivity for full vasorelaxant efficacy of IP receptor agonists under pathological conditions associated with PAH. Selexipag and its active metabolite ACT-333679 exert full relaxant efficacy, whereas the ability of treprostinil to evoke pulmonary arterial vasorelaxation is significantly diminished in disease. The limited relaxant efficacy of treprostinil observed in this study may result from coactivation of contractile EP₁ receptors, which are up-regulated in the MCT rat model of PAH. Potentiation of EP₁ receptor-mediated contraction by subthreshold concentrations of pathological mediators (ET-1 and catecholamines) in control pulmonary arteries substantiates the impact of this contractile mechanism in disease development.

### Materials and Methods

#### Animals

Male Wistar rats (12 weeks) were obtained from Harlan Laboratories B.V. (Venray, The Netherlands). All rats were housed in climate-controlled conditions with a 12-h light/dark cycle and had free access to normal pelleted rat chow and drinking water in accordance with local guidelines (Basel-Landschaft cantonal veterinary office). In certain experiments, PAH was induced in rats by a single injection of MCT (60 mg/kg i.p.). Vehicle control rats were treated in parallel. Disease was allowed to progress for 30 days before experimentation (Igarz et al., 2008). Development of disease was confirmed by an increase in the weight of right ventricle (right ventricle/ left ventricle + septum) ratio: control versus MCT-PAH, 0.33 ± 0.03 versus 0.81 ± 0.1 g; P < 0.05) and endothelial dysfunction as measured by attenuated relaxation to acetylcholine (pEC₅₀ values: control versus MCT-PAH, 7.23 ± 0.04 versus 6.06 ± 0.13, P < 0.0001; E_max values: control versus MCT-PAH, 82.33 ± 1.73 versus 39.79 ± 5.26%, P < 0.0001).

#### Rat Isolated Pulmonary Artery

After euthanasia by CO₂ asphyxiation, rings of extralobar pulmonary artery (EPA) and intralobar pulmonary artery (IPA) were prepared from rats using standard techniques. Two or four arterial rings, EPA (1.5 mm) and IPA (1.0 mm), respectively, were prepared from each animal. Vessels were suspended between stainless wires in either a standard tissue bath set-up (EPA) or a Mulvany-Halpern myograph system (IPA) containing modified Krebs-Henseleit buffer (10 ml) of the following composition: 115 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.5 mM KH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, and 10 mM glucose. Care was taken to avoid damage to the endothelium during the preparation of the arterial rings. In certain experiments, the vascular endothelium was removed by abrasion with the tip of a watchmaker’s forceps. Bathing solution was maintained at 37°C and aerated with 95% O₂/5% CO₂, pH 7.4. An initial resting force of 4.9 mN (EPA) or 3.9 mN (IPA) was applied to vessels, and changes in force generation were measured by using an isometric force recorder (Multi Wire Myograph System, model 610 M, version 2.2; DMT A/S, Aarhus, Denmark) coupled to a EMKA data acquisition system (EMKA Technologies Inc., Paris, France). Viability of the pulmonary artery was tested by measuring contraction to KCl (60 mM), and the presence of a functional endothelium was confirmed by measuring the ability of acetylcholine 10⁻⁶ M to relax arterial rings contracted with phenylephrine (10⁻⁶ M; EPA) or 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F₂α (U46619) (10⁻⁶ M; IPA).

#### Experimental Protocols

**Relaxation of Pulmonary Artery from Control and MCT-PAH Rats.** Rings of pulmonary artery were contracted with prostaglandin F₂α (10⁻⁵ M). When the developed force had stabilized, cumulative concentration-relaxation curves to selexipag, ACT-333679, beraprost, or treprostinil were obtained. The interval between additions of higher concentrations of compounds to the baths was determined by the time required for the response to reach plateau.

**Relaxation of Pulmonary Artery Contracted with ET₁ or Phenylephrine.** Rings of EPA and IPA, in the presence or absence of endothelium, were contracted with either ET₁ (3 × 10⁻¹⁰ M) or phenylephrine (1–3 × 10⁻⁶ M) before cumulative concentration-relaxation curves to ACT-333679 and treprostinil were obtained.

**Contraction of Pulmonary Artery.** Cumulative concentration-contraction curves to the EP₁/EP₂ receptor agonist sulprostone were obtained in the rings of EPA from control and MCT-PAH rats. Separate experiments measured the contractile responses of normal EPA and IPA to ACT-333679 and treprostinil. In experiments that sought to characterize the identity of the receptor mediating contraction to test compounds, rings of normal IPA were exposed...
to either drug vehicle or receptor antagonists for 20 min before cumulative concentration-response curves to agonists were obtained. The choices and concentrations of receptor antagonists were based on published data. The following receptor antagonists were used: (2E)-3-(3',4'-dichlorobiphenyl-2-yl)-N-(2-thienylsulfonyl)acrylamide (DBTSA) (EP3 receptor; Gallant et al., 2002; Kuwano et al., 2008); 8-chloro-2-[3-[(2-furanylmethyl)thio]-1-oxopropyl]hydrazide, dibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid (SC51322) (EP receptor; McCormick et al., 2010), and (4Z)-7-[(1R,2R,3S,5S)-5-[[1,1'-biphenyl]-4-ylmethoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid (GR32191B) (TP receptor; Lumley et al., 1989).

**Measurement of Receptor mRNA Expression**

Prostanoid receptor mRNA expression was measured by quantitative polymerase chain reaction in arteries from normal and MCT-PAH Wistar rats. Analyses were performed by using the main, left, and right branches of the pulmonary artery. Total RNA was isolated from arteries (30-mg samples) by using the RNeasy micro fibrous tissue kit according to the manufacturer’s protocol (QIAGEN GmbH, Hilden, Germany). Remaining genomic DNA was digested by using the DNase free kit (Ambion, Austin, TX). The quantity of RNA was analyzed by using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and RNA quality was assessed by using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Total RNA was reverse-transcribed with a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Quantitative polymerase chain reaction was performed on an ABI 7500 machine using TaqMan probes (Applied Biosystems). The TaqMan assays from Applied Biosystems used for mRNA detection were IP-Rn01764022_m1, DP1-Rn0423963_g1, EPI-Rn01432713_s1, EP3-Rn0121735_m1, TP-Rn00690601_m1; 18s-4319413E, B2M-Rn00560865_m1 and GUS-Rn00566655_m1 were used for normalization. Results were calculated by using the ΔΔCt method, which allows comparison between gene expression values for different genes based on an identical linear scale. A value of 1 was defined as no expression. An identical linear scale was used to present the PAH/control rat ratio.

**Materials**

Selexipag, ACT-333679, and DBTSA were synthesized by Nippon Shinyaku Co. Ltd (Kyoto, Japan). Beraprost, treprostinil, SC51322 and sulprostone were obtained from Cayman Chemical (Ann Arbor, MI). Acetylcholine, GR32191B, phenylephrine, prostaglandin F2 α, and U46619 were purchased from Sigma (St Louis, MO).

**Statistical Evaluation of Results**

Responses of rat pulmonary artery to test compounds are expressed either as a percentage of the precontraction or the reference contraction to KCl (60 mM). Contraction to phenylephrine and ET-1 was measured in millinewtons. Results are presented as mean ± S.E.M. In some experiments, the S.E.M. values are smaller than the data symbol. p values refer to the number of animals. pEC50 values are defined as the negative logarithm of the concentration of agonist that evoked half-maximal response. The effects of receptor antagonists on responses of pulmonary artery to analogs of PG12 were quantified by comparing calculated areas under the agonist concentration-response curves in the absence and presence of antagonists. Statistical comparisons between control and treated groups were performed by using either Student’s paired t test or Student’s unpaired t test (two-tailed). Significance was accepted at P < 0.05.

**Results**

Selexipag/ACT-333679, but Not Prostacyclin Analogs, Relax Pulmonary Artery from MCT-PAH Rats. The abilities of selexipag, metabolite ACT-333679, and the analogs of prostacyclin, beraprost, and treprostinil to relax rat pulmonary artery were measured. Arterial rings from control and MCT-PAH rats were contracted to equal degrees with PGF2 α (10⁻⁵ M) (control versus MCT-PAH; P > 0.05). Selexipag and ACT-333679 evoked concentration-dependent relaxation of EPA rings contracted with PGF2 α (10⁻⁵ M). Relaxation was similar in EPA from control and MCT-PAH rats (Table 1; Fig. 1) (areas under curves, P > 0.05). Beraprost and treprostinil evoked concentration-dependent relaxation of control rat EPA, but EPmax values were significantly less than that measured in response to selexipag and ACT-333679 (Table 1; Fig. 1). Likewise, relaxation of EPA from MCT-PAH rats to beraprost and treprostinil was significantly reduced compared with control EPA (Table 1; Fig. 1) (areas under curves: beraprost, P < 0.05; treprostinil, P < 0.0001). In particular, relaxation to treprostinil was abolished in EPA from MCT-PAH rats.

ACT-333679, but Not Treprostinil, Relaxes Pulmonary Artery Contracted with ET-1. ET-1 is a key mediator in the development of PAH (Rubens et al., 2001; Clozel et al., 2006). Thus, the ability of ACT-333679 and treprostinil to evoke vasorelaxation of pulmonary artery contracted with ET-1 was measured in both the presence and absence of functional endothelium. Rings of EPA and IPA from normal rats were contracted with ET-1 (3 × 10⁻¹⁰ M), and when tone had stabilized, cumulative concentration-response curves to ACT-333679 and treprostinil were obtained. Contraction to ET-1 was similar in the presence or absence of endothelium within each pulmonary artery group (Table 2). ACT-333679 evoked concentration-dependent relaxation of normal EPA and IPA rings (Table 3; Fig. 2). In contrast, treprostinil did not evoke relaxation of either EPA or IPA rings, even at the highest concentration tested (Table 3; Fig. 2). Removal of the endothelium did not influence the responsiveness of EPA and IPA rings to treprostinil. Removal of endothelial cells in IPA, however, caused a small, but statistically significant, decrease in the pEC50 value for ACT-333679 (Table 3).

ACT-333679, but Not Treprostinil, Relaxes Pulmonary Artery Contracted with Phenylnorephrine. Hyperactivation of the sympathetic nervous system is reported in

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**TABLE 1**

Summary of relaxation data for selexipag, ACT-333679, beraprost, and treprostinil in pulmonary artery from control and MCT-PAH rats

<table>
<thead>
<tr>
<th></th>
<th>Selexipag</th>
<th>ACT-333679</th>
<th>Beraprost</th>
<th>Treprostinil</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEC50</td>
<td>5.2 ± 0.1</td>
<td>5.9 ± 0.1</td>
<td>6.18 ± 0.2</td>
<td>6.88 ± 0.1</td>
</tr>
<tr>
<td>EPmax</td>
<td>98.4 ± 2.1</td>
<td>102.3 ± 0.7</td>
<td>81.1 ± 6.9*</td>
<td>72.8 ± 3.7**</td>
</tr>
<tr>
<td><strong>MCT-PAH rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEC50</td>
<td>4.9 ± 0.1</td>
<td>5.5 ± 0.1</td>
<td>4.7 ± 0.2</td>
<td>N.D.</td>
</tr>
<tr>
<td>EPmax</td>
<td>104.8 ± 2.4</td>
<td>104.5 ± 1.0</td>
<td>57.5 ± 7.8**</td>
<td>4.3 ± 2.3***</td>
</tr>
</tbody>
</table>

EPmax: Percentage of reference contraction to KCl (60 mM); N.D., not determined.

*, P < 0.05; **, P < 0.001; ***, P < 0.0001 comparison with EPmax value for ACT-333679.
PAH (Haneda et al., 1983; Salvi, 1999; Nagaya et al., 2000; Velez-Roa et al., 2004), and circulating levels of catecholamines are increased in the MCT model of PAH (Clozel et al., 2006). Vasorelaxation of EPA and IPA rings to selexipag (A), ACT-333679 (B), beraprost (C), and treprostinil (D). Rings of EPA were contracted with 

\[
PGF_{2\alpha} \times 10^{-5} \text{ M.} \]

Data are shown as mean ± S.E.M. (n = 4).

**TABLE 2**

<table>
<thead>
<tr>
<th>Contractile Agent/Relaxant</th>
<th>EPA With Endothelium</th>
<th>EPA Without Endothelium</th>
<th>IPA With Endothelium</th>
<th>IPA Without Endothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1/ACT-333679</td>
<td>8.9 ± 1.3</td>
<td>10.3 ± 1.1</td>
<td>3.5 ± 0.4</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>ET-1/treprostinil</td>
<td>8.1 ± 0.9</td>
<td>10.5 ± 1.0</td>
<td>2.7 ± 0.3</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Phenylephrine/ACT-333679</td>
<td>5.5 ± 0.8</td>
<td>7.6 ± 1.2</td>
<td>3.1 ± 0.2</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Phenylephrine/treprostinil</td>
<td>6.5 ± 0.9</td>
<td>6.7 ± 0.5</td>
<td>3.1 ± 0.1</td>
<td>3.4 ± 0.2</td>
</tr>
</tbody>
</table>

**TABLE 3**

<table>
<thead>
<tr>
<th>Contractile Agent</th>
<th>EPA With Endothelium</th>
<th>EPA Without Endothelium</th>
<th>IPA With Endothelium</th>
<th>IPA Without Endothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>5.0 ± 0.3</td>
<td>4.8 ± 0.3</td>
<td>5.4 ± 0.2</td>
<td>4.5 ± 0.2*</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>4.9 ± 0.1</td>
<td>4.7 ± 0.1</td>
<td>5.1 ± 0.2</td>
<td>4.5 ± 0.1*</td>
</tr>
</tbody>
</table>

*P < 0.05, comparison between IPA with and without endothelium.

**PAH** (Haneda et al., 1983; Salvi, 1999; Nagaya et al., 2000; Velez-Roa et al., 2004), and circulating levels of catecholamines are increased in the MCT model of PAH (Clozel et al., 2006). Vasorelaxation of EPA and IPA rings to ACT-333679 and treprostinil, in the presence and absence of endothelium, was therefore measured after contraction of rings with the \(\alpha_1\)-adrenoceptor agonist phenylephrine. ACT-333679 evoked concentration-dependent relaxation of normal EPA and IPA rings contracted with phenylephrine, whereas treprostinil did not evoke relaxation (Table 3; Fig. 3). Removal of the endothelium did not influence the responsiveness of EPA and IPA rings to treprostinil. A small, but statistically significant, decrease in the pEC\(_{50}\) value for ACT-333679 was measured upon removal of the endothelium in IPA (Table 3), but no significant differences in responses of IPA, with or without endothelium, were observed over the full range of concentrations tested (areas under curve: with and without endothelium, \(P > 0.05\)).

**Sulprostone Contracts Pulmonary Artery from Control and MCT-PAH Rats.** The potential role of EP\(_3\) receptors in modulating vasoreactivity of the pulmonary artery from MCT-induced PAH rats was investigated. First, the ability of the selective EP\(_3\) receptor agonist sulprostone to contract rat EPA from control and MCT-PAH rats was measured. Sulprostone evoked concentration-dependent contraction of EPA, which was significantly more pronounced in rings from MCT-PAH compared with control rats (Fig. 4) (\(E_{\text{max}}\) values control versus MCT-PAH: 62.7 ± 7.8 versus 168.5 ± 20.6%, \(P < 0.001\)).

**Differential Expression of Prostanoid Receptors from Control and MCT-PAH Rats.** The expression profile of prostanoid receptors in pulmonary artery from control and MCT-PAH rats was measured (Fig. 5). TP and EP\(_1\) showed the highest basal expression, IP and EP\(_3\) showed moderate expression, and DP\(_1\) showed low expression (data not shown). mRNA that encoded the IP receptor was significantly increased in all segments of pulmonary artery analyzed from MCT-PAH rats (\(P < 0.01\)). Likewise, EP\(_3\) was significantly up-regulated in the main branch of MCT-PAH pulmonary artery (\(P < 0.05\)). In contrast, DP\(_1\) (\(P < 0.05\)), EP\(_1\) (\(P < 0.001\)), and TP (\(P < 0.01\)) were significantly down-regulated in arteries from MCT-PAH rats.

**Effect of Threshold Concentration of ET-1 on Contraction of Pulmonary Artery to Sulprostone.** Because the function of the EP\(_3\) receptor subtype seems to be increased in pulmonary artery from MCT-PAH rats, the influence of ET-1 on contraction of control pulmonary artery to
Sulprostone and ET-1 each evoked concentration-dependent contraction of rat EPA (Fig. 6; sulprostone: pEC$_{50}$ 5.0 ± 0.4, $E_{\text{max}}$ 37.9 ± 8.3%; ET-1: pEC$_{50}$ 9.6 ± 0.09, $E_{\text{max}}$ 131.9 ± 4.3%). Next, the effect of the threshold concentration of ET-1 on contraction of rat EPA to a threshold concentration of sulprostone was tested. Sulprostone (3 × 10$^{-6}$ M) alone evoked a small contraction of rat EPA (3.9 ± 0.9%) (Fig. 6). Addition of sulprostone after an initial exposure of EPA to the threshold concentration of ET-1 (10$^{-10}$ M, contraction 2.5 ± 0.6%) evoked a significantly greater contraction than that observed in the absence of ET-1 (66.0 ± 11.2%; $P < 0.05$) (Fig. 6). Similar data were obtained when sulprostone was combined with a threshold concentration of phenylephrine (10$^{-8}$ M) in rat EPA (data not shown).

EP$_{1}$, EP$_{2}$, and TP Receptors and Contraction of Pulmonary Artery from Control Rats. Contraction of normal
rat EPA and IPA to ACT-333679 and treprostinil was measured. ACT-333679 did not contract rings of pulmonary artery, whereas treprostinil evoked concentration-dependent contraction of both EPA and IPA. Contraction to treprostinil was more pronounced in IPA than in EPA rings (E₂₅ max 22.5 ± 5.8%; IPA: E₂₅ max 62.8 ± 11.9%) (Fig. 7). The EP₃ receptor antagonist DBTSA (3 \times 10^{-6} M) abolished contraction of rat IPA to treprostinil (Fig. 7). The EP₁ receptor antagonist SC51322 (10^{-6} M) had no effect on contraction of IPA to treprostinil (control versus treated: pEC₅₀ 4.8 ± 0.15 versus 4.8 ± 0.15, P > 0.05; E₂₅ max, 52.5 ± 5.5 versus 50.8 ± 5.6%, P > 0.05). Likewise, antagonism of TP receptors with GR32191B (10^{-6} M) did not significantly inhibit contraction to treprostinil (control versus treated: pEC₅₀ 4.5 ± 0.4 versus 4.5 ± 0.3, P > 0.05; E₂₅ max, 34.8 ± 11.3 versus 22.0 ± 5.2%, P > 0.05).

**EP₃ Receptors and Relaxation of IPA from Normal Rats.** The influence of DBTSA on the relaxant response of rat IPA to treprostinil was measured next. Rings of IPA, in the absence or presence of DBTSA, were contracted to the same level with ET-1 (control versus DBTSA groups, 3.08 ± 0.5 versus 2.8 ± 0.6 mN; P > 0.05). In the absence of DBTSA, treprostinil evoked minimal relaxation of IPA (Fig. 8). Antagonism of EP₃ receptors with DBTSA, however, revealed a significant concentration-dependent relaxation of rat IPA to treprostinil (Fig. 8) (control versus treated: areas under curves, P < 0.05).

**Discussion**

Selexipag and the active metabolite ACT-333679 can be distinguished from analogs of PGI₂ based on vasorelaxant efficacy in the rat pulmonary artery. Specifically, both selipexag and ACT-333679, but not the PGI₂ analog treprostinil, evoke concentration-dependent relaxation of pulmonary artery under pathological conditions associated with PAH. Increased expression of mRNA that encodes EP₃ receptors and enhanced pharmacological activity of this receptor subtype are measured in pulmonary artery from the MCT rat model of PAH, but do not influence the relaxant efficacy of the selective IP receptor agonist ACT-333679. The limited vasorelaxant efficacy of treprostinil was partially restored after EP₁ receptor blockade, indicating that coactivation of contractile EP₃ receptors attenuates the relaxant action of treprostinil.

ACT-333679 is highly selective for the human IP receptor (Kuwano et al., 2007). The Kᵢ value for ACT-333679 at the IP receptor was 130-fold lower than that at other prostanoid receptors. The affinity value for ACT-333679 at the rat IP receptor was 2.2 \times 10^{-7} M and would be expected to activate IP receptors at the concentrations used in the current study. Treprostinil, however, is a nonselective agonist at prostanoid receptors (Whittle et al., 2012). In particular, treprostinil binds and activates DP₁ and EP₂ receptors in addition to the IP receptor. Treprostinil regulates macrophage function and promotes survival of bacteria via an EP₂ receptor-dependent mechanism (Aronoff et al., 2007). In addition, treprostinil activates contractile EP₃ receptors in gastric (Morrison et al., 2010) and vascular smooth muscle (Orie and Clapp, 2011).

The relaxant response to ACT-333679 was similar in pulmonary artery from control and MCT-PAH rats. These data confirm previous findings (Kuwano et al., 2008). In contrast, treprostinil evoked weaker relaxation than ACT-333679 in pulmonary artery from control rats and failed to relax pulmonary artery from MCT-PAH rats. Diminished relaxation of pulmonary artery from MCT-PAH rats to iloprost and beraprost has been reported (Kuwano et al., 2008), but treprostinil seems to be particularly sensitive to the pathological conditions associated with PAH. This possibility was further examined in experiments that measured the relaxant efficacy of ACT-333679 and treprostinil in pulmonary artery contracted with ET-1 or the α₁-adrenoceptor agonist phenylephrine. Dysfunction of both the ET-1 and adrenergic systems underlie the progression of PAH (Salvi, 1999; Rubens et al., 2001), and circulating levels of ET-1 and catecholamines are significantly increased in the rat MCT-PAH model (Clozel et al., 2006). The relaxant profile of ACT-333679 and treprostinil was remarkably similar to that observed in MCT-PAH rats: ACT-333679 evoked concentration-dependent relaxation of all arterial rings tested, whereas treprostinil was unable to relax pulmonary artery contracted with ET-1 or phenylephrine.

Treprostinil can activate contractile EP₃ receptors that counter the vasorelaxant properties of the drug (Orie and Clapp, 2011). Contraction evoked by the selective EP₁ receptor agonist sulprostone (e.g., Dong et al., 1986) is significantly greater in pulmonary artery from MCT-PAH rats than in control rats and is consistent with previous findings (Kuwano et al., 2008). Hyper-responsiveness of the MCT-PAH pulmonary artery to sulprostone may reflect an increase in the number of EP₃ receptors. Receptor density is a key determinant of both potency and efficacy of an agonist (Neubig et al., 2003). Molecular analyses of pulmonary artery from control and MCT-PAH rats revealed a modest, but statistically significant, up-regulation of the expression of mRNA encoding EP₃ receptors in the disease condition. Thus, both
expression and pharmacological activity of contractile EP\textsubscript{3} receptors are enhanced in pulmonary artery from MCT-PAH rats. A differential regulation of IP and TP receptor mRNA expression was also noted: an up-regulation of IP receptor mRNA whereas down-regulation of mRNA encoding TP receptors was recorded. These data are suggestive of a pharmacological compensatory mechanism to combat disease progression through improved PGI\textsubscript{2} signaling (IP receptor) and diminished deleterious effects of thromboxane A\textsubscript{2} (TP receptor) (Christman et al., 1992; Tuder et al., 1999). Published information on the regulation of prostanoid receptors in PAH is, however, contradictory. Down-regulation in expression of IP receptors is reported in pulmonary arterial smooth muscle cells cultured from MCT-PAH rats (Lai et al., 2008) and pulmonary arterial smooth muscle cells and nondefined lung tissue from human patients with PAH (Lai et al., 2008; Falcetti et al., 2010). In contrast, a genomewide RNA expression study did not observe significant regulation of IP or EP\textsubscript{3} receptor mRNA in lung tissue samples from patients with PAH (Rajkumar et al., 2010). Differences in the observations between studies may reflect the experimental approaches adopted. For example, in the present study receptor mRNA

Fig. 6. Contraction of rat pulmonary artery (EPA) to sulprostone and ET-1. A and B, concentration-dependent contraction of EPA to sulprostone (A) and ET-1 (B). C and D, representative traces of contractile responses of EPA to sulprostone (3 $\times$ 10\textsuperscript{-6} M) alone (C) or in combination with ET-1 (10\textsuperscript{-10} M) (D). E, quantification of contractile data shown in C and D. Data are shown as mean $\pm$ S.E.M. ($n$ = 4). *, $P$ < 0.05.

Fig. 7. Contraction of rat pulmonary artery to ACT-333679 and treprostinil. A and B, concentration-dependent contraction of EPA and IPA to ACT-333679 (A) and treprostinil (B). C, DBTSA (3 $\times$ 10\textsuperscript{-6} M) inhibits contraction of IPA to treprostinil. Data are expressed as mean $\pm$ S.E.M. ($n$ = 4). *, $P$ < 0.05.
was measured in arterial blood vessels that were directly isolated from MCT-PAH rats. An additional cell culture stage was included in previous work and may have incurred changes in receptor expression (e.g., Boess et al., 2003). In addition, nonvascular components in snap-frozen lung tissue may have altered the receptor expression profile. Despite the variability of results, any potential changes in the expression of IP receptors should affect the interaction with selexipag and treprostinil equally, because both drugs bind the human IP receptor with similar affinity ($K_i$ values, 2 × 10^{-8} and 3.2 × 10^{-8} M, respectively) (Kuwano et al., 2007; Whittle et al., 2012). Vascular responsiveness to a nonselective prostanooid receptor agonist, such as treprostinil, however, may be more susceptible to changes in the overall expression pattern and pharmacological activity of prostanooid receptors.

Increased pharmacological activity of the EP$_3$ receptor was also evident in combination experiments that measured contraction to sulprostone and ET-1 or phenylephrine in pulmonary arteries isolated from control rats. Contraction of pulmonary artery to sulprostone was significantly augmented in the presence of a threshold concentration of ET-1 or phenylephrine and raises the possibility of a synergistic interaction between the EP$_3$ and endothelin and adrenergic receptor systems. Contractile synergism between ET-1 and serotonin is reported in the human mammary artery (Yang et al., 1997), and significant synergy exists between the α-adrenoceptor and EP$_3$ receptors in rat femoral artery (Hung et al., 2006). Coactivation of EP$_3$ receptors under pathological conditions of PAH may limit the therapeutic efficacy of treprostinil. The current study and other work (Orie and Clapp, 2011) demonstrate a clear role for the EP$_3$ receptor subtype in vasoconstriction to treprostinil. The beneficial effects of IP receptor signaling on vascular wall remodeling in PAH can potentially be tempered by excessive vasoconstriction. Indeed, the EP$_3$ receptor mediates contraction of human pulmonary artery (Maddox et al., 1985; Haye-Legrand et al., 1987; Norel et al., 1991; Qian et al., 1994; Walch et al., 1999) and may modulate relaxant IP receptor function (Jones et al., 1997; Chan and Jones, 2004). In addition, EP$_3$ receptor signaling is profibrotic (White et al., 2008; Bozyk and Moore, 2011; Harding and LaPointe, 2011) and may limit the beneficial effects of treprostinil in PAH.

Failure of treprostinil to evoke relaxation of the pulmonary artery from MCT-PAH rats may not be fully explained by the small down-regulation of mRNA encoding relaxant DP$_1$ receptors in this model. A role for EP$_2$ receptors can similarly be discounted because no change in the expression of mRNA encoding this receptor subtype is measured in MCT-PAH rats (Lai et al., 2008). Indeed, agonism at DP$_1$ and EP$_2$ receptors does not evoke relaxation of human pulmonary artery (Walch et al., 1999).

The vasorelaxant efficacy of ACT-333679 measured here in rat pulmonary artery ex vivo reflects the high selectivity of this compound for the relaxant IP receptor (Kuwano et al., 2007). Furthermore, vasorelaxation evoked by ACT-333679 under pathological conditions associated with PAH (absence of endothelium, ET-1, and α-adrenoceptor stimulation) supports the therapeutic profile of selexipag for the treatment of PAH. The combined vasodilator and antiproliferative properties of ACT-333679 (Kuwano et al., 2007, 2008) probably lead to the prolonged survival of MCT-PAH rats (Kuwano et al., 2008) and contribute to the significant decrease in pulmonary vascular resistance in patients with PAH (Simonneau et al., 2012). In contrast, the present data suggest that the therapeutic benefits of treprostinil in the management of PAH may not be attributed solely to direct vasodilatation. Indeed, only a small, but statistically significant, reduction in right ventricular systolic pressure is reported in the MCT-PAH rat (Yang et al., 2010). Infusion of treprostinil in a therapeutic regimen effectively prevents smooth muscle proliferation and progression of PAH in the rat MCT-PAH model (Yang et al., 2010), although the impact on survival is not reported. In addition, treprostinil exerts antiproliferative effects via an IP receptor-dependent mechanism in a recombinant cell system (Falcetti et al., 2007).

In conclusion, these data demonstrate that selexipag/ACT-333679 and treprostinil differ in their vasorelaxant effects in pulmonary artery. Selexipag/ACT-333679 relax pulmonary artery under conditions associated with PAH, whereas treprostinil fails to evoke relaxation. The relaxant efficacy of treprostinil depends on the nature of the agonist used to contract the pulmonary artery and is also attenuated by off-target agonism at EP$_3$ receptors. Thus, a high degree of selectivity for the target IP receptor may help maintain therapeutic efficacy and minimize unwanted side effects of IP receptor agonists.

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