Selective Prostacyclin Receptor Agonist Selexipag, in Contrast to Prostacyclin Analogs, Does Not Evoke Paradoxical Vasoconstriction of the Rat Femoral Artery

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ABSTRACT

Selexipag [2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-N-(methylsulfonyl)acetamide] is a selective nonprostanoid prostacyclin (PGI₂) receptor (IP receptor) agonist that is approved for the treatment of pulmonary arterial hypertension (PAH). In contrast to selexipag, PGI₂ analogs used in the clinic are nonselective agonists at prostanoid receptors and can also activate contractile prostaglandin E receptor 3 (EP₃) receptors. Leg pain is a common side effect in patients receiving treatment with PGI₂ analogs and peripheral vasoconstriction can be responsible for side effects related to muscular ischemia. This study tested the hypothesis that PGI₂ analogs could cause paradoxical vasoconstriction of the femoral artery via EP₃ receptor activation but that only vasorelaxation would be observed in response to selexipag and its active metabolite ACT-333679 [[4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy]acetic acid]. Selexipag and ACT-333679 relaxed rings of the isolated rat femoral artery contracted with either

prostaglandin F_{2α} (PGF_{2α}) or the α₁ adrenoceptor (α₁AR) agonist phenylephrine. ACT-333679 also inhibited contraction of the femoral artery to sympathetic nerve stimulation. In contrast, PGI₂ analogs (iloprost, beraprost, and treprostinil) caused additional contraction of arterial rings precontracted with phenylephrine, which was reverted to relaxation by antagonism of EP₃ receptors. Treprostinil augmented contraction of the femoral artery to sympathetic nerve stimulation in an EP₃ receptor-dependent manner. Mechanistically, concomitant EP₃ and α₁AR receptor activation synergistically constricted femoral arteries. It is concluded that selexipag and ACT-333679 are vasorelaxants of the rat femoral artery and, unlike PGI₂ analogs, do not cause paradoxical vasoconstriction via activation of EP₃ receptors. EP₃ receptor-mediated vasoconstriction may contribute to the well documented peripheral muscle pain reported in patients with PAH receiving PGI₂ analogs. Leg pain may be less in patients treated with selexipag.

Introduction

Selexipag [2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-N-(methylsulfonyl)acetamide] is a selective and orally bioavailable prostacyclin (PGI₂) receptor (IP receptor) agonist (Kuwano et al., 2007) that is approved for the treatment of pulmonary arterial hypertension (PAH). Selexipag lowered the risk of the primary composite end point of death or a complication related to PAH in newly treated patients or in patients already treated with one or two other classes of PAH therapies compared with patients who received placebo in the GRIPHON phase 3 clinical trial (Sitbon et al., 2015). Restoration of IP receptor signaling compensates for the reduced production of PGI₂ in PAH (Christman et al., 1992; Tudor et al., 1999) through mechanisms that include vasodilatation and inhibition of exaggerated vascular smooth muscle cell proliferation (Fetalvero et al., 2007; Smyth et al.,

2009). Selexipag and its active metabolite ACT-333679 [[4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy]acetic acid; previously known as MRE-269] have nonprostanoid structures and possess higher selectivity than PGI₂ analogs for the IP receptor over other prostanoid receptor subtypes in binding and functional cellular assays (Kuwano et al., 2007; Gatfield et al., 2016). In contrast, PGI₂ analogs used in the clinical management of PAH are not selective for the IP receptor and can activate other prostanoid receptor subtypes (Abramovitz et al., 2000; Kuwano et al., 2008; Whittle et al., 2012) as the vasorelaxant efficacy of treprostinil and beraprost, but not selexipag, is reduced via activation of contractile prostaglandin E receptor 3 (EP₃) receptors in the pulmonary artery under conditions associated with PAH (Kuwano et al., 2008; Morrison et al., 2012).

Leg pain is a common side effect in patients receiving treatment with PGI₂ analogs and although the effect may be neuropathic in origin (Pagani-Estévez et al., 2017), an additional vascular component can also be considered. Adrenergic

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ABBREVIATIONS: α₁AR, α₁ adrenergic receptor; ACT-333679, {4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}acetic acid; DBTSA, (2E)-3-(3',4'-dichlorobiphenyl-2-yl)-N-(2-thienylsulfonyl)acrylamide; EFS, electrical field stimulation; EP₃, prostaglandin E receptor 3; GR32191B, (4Z)-7-[(1*R*,2*R*,3*S*,5*S*)-5-[(1,1'-biphenyl)-4-ylmethoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-hetenoic acid; MCT, monocrotaline; PAH, pulmonary arterial hypertension; PGF_{2α}, prostaglandin F_{2α}; PGI₂, prostacyclin (IP receptor); PH, pulmonary hypertension; SC51322, 8-chloro-2-[3-[(2-furanylmethyl)thio]-1-oxopropyl]hydrazide, dibenz[*b,f*][1,4]oxazepine-10(11*H*)-carboxylic acid hydrazide; TP, thromboxane receptor; U46619, 9,11-dideoxy-9α,11α-metha-neoepoxy prostaglandin F_{2α}.

activity is increased in the legs of patients with PAH (Velez-Roa et al., 2004) and potent contractile synergy has been reported between α_1 -adrenoceptors and EP₃ receptors in preclinical studies (Hung et al., 2006), a phenomenon that could contribute to the peripheral pain reported with PGI₂ analogs.

This study tested the hypothesis that selexipag and ACT-333679, unlike nonselective PGI₂ analogs (iloprost, beraprost, and treprostinil), cause only relaxation of the femoral artery without paradoxical EP₃ receptor-mediated vasoconstriction.

Materials and Methods

Animals

Original studies in animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and were approved by the local Basel-Landschaft cantonal veterinary office (Switzerland). Twelve-week-old male Wistar rats were obtained from the Biotechnology and Animal Breeding Division (Harlan, Füllinsdorf, Switzerland). All rats were housed in climate-controlled conditions with a 12-hour light/dark cycle and had free access to normal pelleted rat chow and drinking water. In certain experiments, pulmonary hypertension (PH) was induced in rats by a single injection of monocrotaline (MCT; 60 mg/kg, i.p.). Vehicle control rats were treated in parallel. Endothelial function was tested 30 days after injection of MCT (Iglarz et al., 2008).

Rat Isolated Femoral Artery

After euthanasia, rings of the rat femoral artery were prepared using a standard technique. Briefly, the right and left femoral arteries were isolated. Two arterial rings (1.5 mm) were prepared from each artery, and vessels were suspended between 40- μ m stainless steel wires in a Mulvany-Halpern myograph system (10 ml) containing modified Krebs-Henseleit buffer with 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. Bathing solution was maintained at 37°C and aerated with 95% O₂/5% CO₂ (pH 7.4). An initial resting force of 3.9 mN was applied to the vessel (Duckles et al., 1985), and changes in force generation were measured 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 femoral 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 μ M) to relax arterial rings contracted with U46619 (9,11-dideoxy-9 α ,11 α -metha-noepoxy prostaglandin F_{2 α} ; 1 μ M). Mean relaxation to acetylcholine was 81.6% $\pm$ 1.2% for all rings tested. In certain experiments, contraction of the femoral artery to electrical field stimulation (EFS) was measured. Arterial rings were placed between platinum electrodes and stimulated every 5 minutes (17 V, 0.5-millisecond pulse width, 10 seconds, 4–24 Hz) using an electrical stimulator (EMKA Technologies Inc.). Two frequency-contraction curves were obtained in each vessel: an initial control response, followed (after a period of recovery) by a second curve in the presence of drug vehicle or test compound(s). Contraction in the presence of test compound was expressed as a percentage of the maximal contraction in the first control response.

Rat Isolated Pulmonary Artery

Rings of the extralobar pulmonary artery were prepared from rats using standard techniques. Vessels were suspended between stainless steel wires in a 10-ml tissue bath set-up and processed in a similar

TABLE 1

Relaxation to acetylcholine (10 μ M) in the pulmonary and femoral artery from control and MCT-PH rats

Data are presented as the mean $\pm$ S.E.M.

Group	Pulmonary Artery	Femoral Artery
	%	
Control	81.2 $\pm$ 4.2	82.9 $\pm$ 3.4
MCT-PH	25.3 $\pm$ 6.9*	90.7 $\pm$ 2.3

* P < 0.0001 compared with the control pulmonary artery.

manner to that described for the femoral artery. An initial resting force of 4.9 mN was applied to vessels.

Experimental Protocols

Relaxation of the Pulmonary and Femoral Artery from Control and MCT-PH Rats. Rings of the pulmonary and femoral artery were contracted with phenylephrine (1 μ M). When the developed force had stabilized, relaxation to acetylcholine (10 μ M) was measured.

Relaxation of the Femoral Artery. Rings of the femoral artery were contracted with either prostaglandin F_{2 α} or phenylephrine (3.5 $\pm$ 0.9 and 3.0 $\pm$ 0.5 μ M, respectively) to give matched submaximal contraction relative to KCl (60 mM) (50.8% $\pm$ 2.5% and 50.4% $\pm$ 2.7%, respectively). Cumulative concentration-relaxation curves to selexipag, ACT-333679, iloprost, beraprost, or treprostinil were obtained when the developed force had stabilized. The interval between additions of higher concentrations of compounds to the baths was determined by the time required for the response to reach plateau. In experiments that sought to characterize the identity of the receptor mediating responses to test compounds, rings of the femoral artery were exposed to either vehicle or receptor antagonists for 30 minutes prior to obtaining cumulative concentration-response curves to agonists. The choice and concentrations of the following receptor antagonists were based on published data: DBTSA [(2*E*)-3-(3',4'-dichlorobiphenyl-2-yl)-*N*-(2-thienylsulfonyl)acrylamide; EP₃ receptor] (Gallant et al., 2002; Kuwano et al., 2008), SC51322 (8-chloro-2-[3-[(2-furanylmethyl)thio]-1-oxopropyl]hydrazide, dibenz[*b,f*][1,4]oxazepine-10(11*H*)-carboxylic acid hydrazide; EP₁ receptor) (McCormick et al., 2010), and GR32191B [(4*Z*)-7-[(1*R*,2*R*,3*S*,5*S*)-5-[(1,1'-biphenyl)-4-ylmethoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-hetenoic acid; thromboxane receptor (TP receptor) TP receptor] (Lumley et al., 1989).

Contraction of the Femoral Artery to EFS. Frequency-contraction curves (4–24 Hz) were first obtained in the absence or presence of tetrodotoxin (0.1 μ M; 10 minutes) and prazosin (0.1 μ M; 10 minutes) to establish that the smooth muscle contraction was neuronal in origin and mediated via activation of α_1 ARs (Zacharia et al., 2004). Contraction of the femoral artery to EFS was abolished by tetrodotoxin (0.1 μ M) and prazosin (0.1 μ M) (n = 3, data not shown).

In separate experiments, the influence of ACT-333679 or treprostinil (both at 10 μ M, 20-minute incubation) on EFS-induced contraction was measured. DBTSA (1 μ M) and GR32191B (1 μ M) were added to the bath 20 minutes prior to the addition of treprostinil.

Contraction of the Femoral Artery to Agonists. Cumulative concentration-contraction curves to the EP_{1/3} receptor agonist sulprostone were obtained in rings of the femoral artery. The ability of a subthreshold concentration of sulprostone to contract the femoral artery was measured after exposure of the artery to phenylephrine (0.1 μ M; 10 minutes), and the role of α_1 -adrenoceptors and EP₃ receptors in this response was investigated by prior incubation with either prazosin (0.1 μ M; 20 minutes) or DBTSA (1 μ M; 20 minutes).

Materials

Selexipag, ACT-333679, and DBTSA were synthesized by Nippon Shinyaku Co. Ltd. (Kyoto, Japan). Iloprost, beraprost, treprostinil,

Fig. 1. (A and B) Relaxation to selexipag (A) and ACT-333679 (B) in the rat femoral artery contracted with equi-effective concentrations of either PGF_{2α} or phenylephrine (*n* = 6/group).

SC51322, and sulprostone were obtained from Cayman Chemical (Ann Arbor, MI). Acetylcholine, GR32191B, phenylephrine, prostaglandin F_{2α}, and U46619 were purchased from Sigma (St Louis, MO).

Statistical Analyses

Relaxation of the rat femoral artery to test compounds is expressed as a percentage of the contraction, and contractile responses are expressed as a percentage of the reference contraction to KCl (60 mM). Results are presented as the mean $\pm$ S.E.M. In some experiments, the S.E.M. values are smaller than the data symbol. *n* values refer to the number of animals. Best-fit analyses of graphs were performed using GraphPad Prism software (version 7.02 for Windows, www.graphpad.com; GraphPad Software Inc., La Jolla, CA). pEC₅₀ values are defined as the negative logarithm of the concentration of agonist that evokes a half maximal response. The effects of receptor antagonists on responses of the femoral artery to analogs of PGI₂ were quantified by comparing calculated areas under the agonist concentration-response curves in the absence and presence of antagonists. Calculation of the area under the curve is an integrated analytical method for quantifying the response to an agonist over the whole range of concentrations tested (Hermann et al., 2003; Liang et al., 2010; Morrison et al., 2012). Statistical comparisons between control and treated groups were performed using the paired *t* test (two-tailed). Differences were considered significant at *P* < 0.05.

Results

Endothelial Function of the Pulmonary and Femoral Artery in MCT-Induced PH Rats

Relaxation of the extralobar pulmonary and femoral artery to acetylcholine was measured using rings precontracted with the selective α_1 adrenoceptor (α_1 AR) agonist phenylephrine. Acetylcholine (10 μ M) relaxed rings of the femoral artery from both control and MCT-PH rats, whereas relaxation of the pulmonary artery to acetylcholine was significantly less in arterial rings from MCT-PH rats (Table 1).

TABLE 2
Relaxation of the femoral artery to selexipag and ACT-333679
Data are presented as the mean $\pm$ S.E.M.

Contractile agent	Selexipag	ACT-333679
pEC ₅₀		
PGF _{2α}	5.4 $\pm$ 0.1	5.5 $\pm$ 0.1
Phenylephrine	5.5 $\pm$ 0.1	5.6 $\pm$ 0.1
E _{max}		
PGF _{2α}	113.3 $\pm$ 5.4	126.9 $\pm$ 7.0
Phenylephrine	116.6 $\pm$ 6.6	121.0 $\pm$ 6.6

Selexipag and ACT-333679 Relax the Femoral Artery

As femoral arteries from MCT-PH rats displayed a normal endothelial function, the remaining experiments were conducted in femoral arteries from healthy Wistar rats. The effects of selexipag and its metabolite ACT-333679 on isometric force development in the rat femoral artery were measured using rings precontracted with equi-effective concentrations of either prostaglandin F_{2α} (PGF_{2α}) or the selective α_1 AR agonist phenylephrine. Both selexipag (Fig. 1A) and ACT-333679 (Fig. 1B) relaxed the femoral artery. No statistically significant difference in relaxation (area under the curve) to either selexipag or ACT-333679 was observed in the femoral artery contracted with PGF_{2α} or phenylephrine (Fig. 1; Table 2). EFS (4–24 Hz) contracted the femoral artery via endogenously released norepinephrine (Fig. 2). The maximum contraction under control conditions was 91.1% $\pm$ 7.6% relative to KCl (60 mM). ACT-333679 (10 μ M) significantly inhibited contraction of the femoral artery by EFS (4–24 Hz; area under the curve: 1234 $\pm$ 135.9 and 580.8 $\pm$ 69.3 for the control and ACT-333679, respectively; *P* < 0.05, *n* = 6; Fig. 2).

PGI₂ Analogs Constrict the Femoral Artery

The effects of PGI₂ analogs on the rat femoral artery were compared with those of selexipag and ACT-333679 in rings precontracted with equi-effective concentrations of either PGF_{2α} or phenylephrine. Although iloprost, beraprost, and treprostinil evoked concentration-dependent relaxation of the femoral artery contracted with PGF_{2α} (Fig. 3), these PGI₂ analogs did not cause vasorelaxation but rather induced further contraction in femoral arterial rings precontracted with phenylephrine (Fig. 3). The maximum contraction to

Fig. 2. Effect of ACT-333679 (10 μ M) on contraction of the rat femoral artery to EFS. **P* < 0.05; ***P* < 0.01 (*n* = 6/group).

Fig. 3. (A–C) Responses to iloprost (A), beraprost (B), and treprostinil (C) in the rat femoral artery contracted with equi-effective concentrations of either PGF_{2α} or phenylephrine. **P* < 0.05 for comparison of area under the curve values at concentrations above 1 μ M; ***P* < 0.01 for comparison of area under the curve values over the full range of concentrations tested (*n* = 6/group).

iloprost, beraprost, and treprostinil was $44.4\% \pm 15.1\%$, $78.4\% \pm 9.8\%$, and $34.6\% \pm 12.1\%$, respectively. Differences in area under the curve values for responses to iloprost and beraprost (over the full range of concentrations tested) were statistically significant (*P* < 0.05, *n* = 6; Fig. 3, A and B), whereas area under the curve values for responses to treprostinil were significantly different only at concentrations above 1 μ M (*P* < 0.05, *n* = 6; Fig. 3C).

Iloprost, beraprost, and treprostinil caused weak vasorelaxation of the femoral artery contracted with phenylephrine in

Fig. 4. (A–C) Effects of the EP₃ receptor antagonist DBTSA (1 μ M) on responses of the rat femoral artery to iloprost (A), beraprost (B), and treprostinil (C). Arterial rings were contracted with phenylephrine. **P* < 0.05 for comparison of area under the curve values (*n* = 5/group).

the presence of the EP₃ receptor antagonist (DBTSA, 1 μ M) (Fig. 4). Relaxation to iloprost, beraprost, and treprostinil was $40.8\% \pm 5.6\%$, $51.8\% \pm 9.3\%$, and $37.6\% \pm 7.4\%$, respectively. Differences in area under the curve values for responses to all PGI₂ analogs tested in the absence and presence of DBTSA were statistically significant (*P* < 0.05, *n* = 5; Fig. 4). Antagonism of EP₁ (SC51322, 1 μ M) and TP (GR32191B, 0.1 μ M) receptors did not significantly modulate the reactivity of the femoral artery to PGI₂ analogs (Table 3).

In direct contrast to ACT-333679, treprostinil (10 μ M) significantly increased contraction to EFS (maximum contraction: $94.5\% \pm 10.2\%$ and $188.6\% \pm 9.2\%$ for the control and treprostinil, respectively; *P* < 0.05, *n* = 4; Fig. 5). This

TABLE 3

Effect of SC51322 and GR32191B on responses of the femoral artery to PGI₂ analogs

Data are presented as the mean $\pm$ S.E.M. area under the curve.

Group	Iloprost	Beraprost	Treprostinil
Control	684.7 $\pm$ 50.7	742.5 $\pm$ 44.2	822.5 $\pm$ 47.7
SC51322	704.1 $\pm$ 44.5	769.1 $\pm$ 25.6	841.0 $\pm$ 43.7
Control	752.4 $\pm$ 21.2	729.4 $\pm$ 17.3	870.0 $\pm$ 27.0
GR32191B	724.6 $\pm$ 28.3	756.7 $\pm$ 14.0	857.2 $\pm$ 12.5

augmented contraction was significantly reduced by the EP₃ receptor antagonist DBTSA, with area under the curve values of 1398 ± 162.5 and 2586 ± 199.6 for the control and treprostinil, respectively ($P < 0.01$) compared with 1512 ± 376.8 for treprostinil and DBTSA ($P < 0.05$ vs. treprostinil alone; $n = 4$; Fig. 5). Antagonism of TP receptors with GR32191B ($1 \mu\text{M}$) did not significantly inhibit the effect of treprostinil on contraction to EFS, with area under the curve values of 1398 ± 162.5 and 2470 ± 182.3 for the control and treprostinil ($P < 0.01$), respectively, and 1954 ± 374.5 for treprostinil and GR32191B ($P > 0.05$ vs. treprostinil alone, $n = 4$).

α_1 ARs and EP₃ Receptors Act Synergistically in the Femoral Artery

Since reactivity of the femoral artery to PGI₂ analogs was only modulated during α_1 AR stimulation, the potential pharmacological interaction between contractile EP₃ receptors and α_1 ARs was investigated. The EP_{1/3} receptor agonist sulprostone caused concentration-dependent contraction of the rat femoral artery (Fig. 6A; $\text{pEC}_{50} = 6.4 \pm 0.3$, $E_{\text{max}} = 140.6\% \pm 15.6\%$). Sulprostone at a concentration that did not cause contraction by itself (subthreshold concentration of 1 nM) was able to contract the femoral artery in the presence of phenylephrine ($0.1 \mu\text{M}$) (Fig. 6B). Next, the identity of the receptor subtype involved in the exaggerated contraction to sulprostone in the presence of phenylephrine was determined. EP₃ receptor antagonist DBTSA ($1 \mu\text{M}$) and prazosin ($0.1 \mu\text{M}$; selective α_1 AR antagonist) significantly reduced sulprostone-evoked contraction (Fig. 6B). Selective EP₁ receptor antagonist SC51322 ($1 \mu\text{M}$) did not inhibit contraction to sulprostone ($47.4\% \pm 10.5\%$ and $38.5\% \pm 6.9\%$ for the control and treated groups, respectively; $P > 0.05$, $n = 4$).

Discussion

The results of this study demonstrate the functional impact of the selectivity of selexipag and its metabolite for the IP receptor over other prostanoid receptors. Relaxation of the femoral artery to selexipag and ACT-333679 is not modulated by coactivation of contractile EP₃ receptors, nor is it dependent on the nature of the contractile agent used to raise vascular tone. In contrast, PGI₂ analogs activate EP₃ receptors to contract the femoral artery in the presence of phenylephrine, and treprostinil augments contraction to nerve-released norepinephrine.

We established that endothelial function was preserved in the femoral, but not pulmonary, artery from MCT-PH rats, demonstrating the vascular selectivity of this PH model. Further experiments using the femoral artery were therefore

Fig. 5. Effect of treprostinil ($10 \mu\text{M}$) on contraction of the rat femoral artery to EFS in the absence and presence of the EP₃ receptor antagonist DBTSA. ** $P < 0.01$; *** $P < 0.001$ ($n = 6/\text{group}$).

performed in the presence of a functional vascular endothelium. The femoral artery was chosen for investigation since its occlusion contributes to leg pain in patients with peripheral artery disease (Beard 2000). This artery is also predominantly used in preclinical models of leg ischemia (Krishna et al., 2016; Queme et al., 2017).

Vasorelaxation of the femoral artery to selexipag and ACT-333679 was similar in rings precontracted with either PGF_{2 α} or the α_1 AR agonist phenylephrine. These data are in good agreement with previous findings in the pulmonary artery (Kuwano et al., 2008; Morrison et al., 2012). Reactivity to analogs of PGI₂ was markedly different from that measured in response to selexipag and ACT-333679. PGI₂ analogs relaxed the femoral artery precontracted with PGF_{2 α} but caused further contraction of the femoral artery precontracted with phenylephrine. This augmented contraction to PGI₂ analogs might be caused by activation of contractile EP₃ receptors, since antagonism of EP₃ receptors revealed modest relaxation to all PGI₂ analogs tested. Contraction of the femoral artery to PGI₂ analogs measured during α_1 AR activation contrasted with the weak relaxation observed under the same conditions in pulmonary artery (Morrison et al., 2012). These data suggest an important synergy between EP₃ receptors and the adrenergic system in the femoral artery.

Differential effects of ACT-333679 and analogs of PGI₂ were also observed after transmural sympathetic nerve stimulation. ACT-333679, at a concentration that evoked maximal relaxation of the femoral artery, inhibited arterial contraction to EFS. This inhibitory effect of ACT-333679 is considered to be mediated via postsynaptic IP receptors in a manner similar to that observed for PGI₂ in the rabbit mesenteric artery (Armstrong et al., 1979). The same concentration of treprostinil, however, significantly augmented contraction to EFS in an EP₃ receptor-dependent manner. Sensitivity of EFS-induced contraction to tetrodotoxin and prazosin confirmed the nerve origin and critical involvement of α_1 ARs in this response (Zacharia et al., 2004). Thus, the ability of treprostinil to augment contraction of the femoral artery to endogenously released norepinephrine is consistent with postsynaptic interplay between α_1 ARs and EP₃ receptors.

Marked contractile synergy between EP₃ receptors and α_1 ARs has been described in the rat femoral artery (Hung et al., 2006). This artery receives dense sympathetic innervation and possesses high norepinephrine content (Todd 1980;

Fig. 6. Contraction of the rat femoral artery to sulprostone in the absence or presence of phenylephrine. (A) Sulprostone causes concentration-dependent contraction of the femoral artery. (B) A subthreshold concentration of sulprostone (1 nM) contracts the femoral artery in the presence of phenylephrine (0.1 μ M). DBTSA (1 μ M) and prazosin (0.1 μ M) inhibit contraction to sulprostone in the presence of phenylephrine. * $P < 0.05$; ** $P < 0.01$ ($n = 6$ /group).

Duckles et al., 1985; Stassen et al., 1998). Thus, the femoral artery is suitable for study of the potential pharmacological interplay between EP₃ receptors and α_1 ARs and its effect on vascular responsiveness to selexipag and analogs of PGI₂. Synergy between α_1 ARs and EP₃ receptors in the femoral artery was further supported by the observations that a subthreshold concentration of the EP_{1/3} receptor agonist sulprostone evoked significant contraction of the femoral artery only in the presence of phenylephrine. Activation of both EP₃ and α_1 ARs receptors was required, since contraction to sulprostone was abolished by either DBTSA or prazosin. The contractile synergy between femoral EP₃ receptors and α_1 ARs described here and by others (Hung et al., 2006) may contribute to the well documented peripheral muscle pain (myalgia) reported in patients with PAH receiving treatment with PGI₂ analogs (Tapon et al., 2012, 2013; Pagani-Estévez et al., 2017). Involvement of other lower limb arteries that are under adrenergic control (e.g., the popliteal artery) (Sada et al., 1985) cannot be excluded. Although the development of pain is considered to arise from IP and EP₃ receptor-dependent sensitization of sensory afferent neurons (Nakae et al., 2005; Southall and Vasko, 2001), our data support an additional vascular mechanism. Leg ischemia is commonly associated with pain in the calf and thigh muscles while walking, due to restriction of blood flow through the femoral artery (Beard 2000). In addition, reduced blood flow and tissue oxygenation, as occur after exaggerated vasoconstriction, promote the production of metabolism-derived pain mediators (Queme et al., 2017).

The high selectivity of selexipag and its metabolite for the prostacyclin IP receptor precludes EP₃ receptor-mediated vasoconstriction and sensitization of afferent neurons, which might translate into improved tolerability over PGI₂ analogs in patients with PAH. In conclusion, this study described differences in the pharmacology of the selective prostacyclin IP receptor agonists selexipag and ACT-333679 and non-selective analogs of PGI₂ in the rat femoral artery. Selexipag and ACT-333679 relaxed the femoral artery, whereas EP₃ receptor-mediated contraction to PGI₂ analogs was exacerbated during α_1 AR stimulation.

Authorship Contributions

Participated in research design: Morrison, Iglarz, Clozel.

Conducted experiments: Haag, Ernst.

Performed data analysis: Morrison, Haag, Ernst.

Wrote or contributed to the writing of the manuscript: Morrison, Iglarz, Clozel.

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