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

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Received November 15, 2017; accepted March 16, 2018

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 possesses higher selectivity than PGI2 analogs for the IP 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)acetamide]. Selexipag and ACT-333679 relaxed rings of the isolated rat femoral artery contracted with either prostaglandin F2α (PGF2α) or the α1 adrenergic receptor (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., 2015). Restoration of IP receptor signaling compensates for the reduced production of PGI2 in PAH (Christman et al., 2015). Restoration of IP receptor signaling compensates for the reduced production of PGI2 in PAH (Christman et al., 2015).

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

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-thienylsulfonfonyl)acylamide; EFS, electrical field stimulation; EP3, prostaglandin E receptor 3; GR32191B, (4Z)-7-[(1R,2R,3S,5S)-5-[1,1′-biphenyl]-4-ylmethoxy]-3-hydroxy-2-[1-piperidinyl]cyclopentyl]-4-hetenoic acid; MCT, monocrotaline; PAH, pulmonary arterial hypertension; PGI2, prostacyclin (IP receptor); PH, pulmonary hypertension; SC51322, 8-chloro-2-[3-[2-furanyl methyl]thio]-1-oxopropanyl]hydrazide, dibenz[b,f][1,4]oxazepine-10-[11H]-carboxylic acid hydrazide; TP, thromboxane receptor; U46619, 9,11-dideoxy-9α,11α-metha-noepoxy prostaglandin F2α.
activity is increased in the legs of patients with PAH (Velez-Roa et al., 2004) and potent contractile synergy has been reported between α1-adrenoceptors and EP3 receptors in preclinical studies (Hung et al., 2006), a phenomenon that could contribute to the peripheral pain reported with PG12 analogs.

This study tested the hypothesis that selexipag and ACT-333679, unlike nonselective PG12 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 arteries were prepared from rats that were 30 days after injection of monocrystaline (MCT; 60 mg/kg, i.p.) into the Biotechnology and Animal Breeding Division (Harlan, Füllinsdorf, Switzerland). All rats were housed in climate-controlled conditions promulgated by the U.S. National Institutes of Health and were adopted and Guide for the Care and Use of Laboratory Animals as adopted and.

**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-μm 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 contraction with U46619 (9,11-dideoxy-9α,11α-metha-noxyepoxysulprostone) 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).

**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 μM). When the developed force had stabilized, relaxation to acetylcholine (10 μM) was measured.

**Experimental Protocols**

**Relaxation of the Femoral Artery to EFS.** Frequency–contraction curves (4–24 Hz) were first obtained in the absence or presence of tetrodotoxin (0.1 μM; 10 minutes) and prazosin (0.1 μM; 10 minutes) to establish that the smooth muscle contraction was neuronal in origin and mediated via activation of α1aARs (Zacharia et al., 2004). Contraction of the femoral artery to EFS was abolished by tetrodotoxin (0.1 μM) and prazosin (0.1 μM) (n = 3, data not shown).

In separate experiments, the influence of ACT-333679 or treprostinil (both at 10 μM) on EFS-induced contraction was measured. DBTSA (1 μM) and GR22191B (1 μM) 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 EP4 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 μM; 10 minutes), and the role of α1-adrenoceptors and EP3 receptors in this response was investigated by prior incubation with either prazosin (0.1 μM; 20 minutes) or DBTSA (1 μM; 20 minutes).

**Materials**

Selexipag, ACT-333679, and DBTSA were synthesized by Nippon Shinyaku Co. Ltd. (Kyoto, Japan). Iloprost, beraprost, treprostinil, manner to that described for the femoral artery. An initial resting force of 4.9 mN was applied to vessels.

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

**Frequency-contraction curves (4–24 Hz) were first obtained in the absence or presence of tetrodotoxin (0.1 μM; 10 minutes) and prazosin (0.1 μM; 10 minutes) to establish that the smooth muscle contraction was neuronal in origin and mediated via activation of α1aARs (Zacharia et al., 2004). Contraction of the femoral artery to EFS was abolished by tetrodotoxin (0.1 μM) and prazosin (0.1 μM) (n = 3, data not shown).

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**Materials**

Selexipag, ACT-333679, and DBTSA were synthesized by Nippon Shinyaku Co. Ltd. (Kyoto, Japan). Iloprost, beraprost, treprostinil,
SC51322, and sulprostone were obtained from Cayman Chemical (Ann Arbor, MI). Acetylcholine, GR32191B, phenylephrine, prostaglandin F$_{2\alpha}$, 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 ± 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). pEC$_{50}$ 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 PGI$_2$ 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 $\alpha_1$ adrenoceptor ($\alpha_1$AR) agonist phenylephrine. Acetylcholine (10 µM) 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).

**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 isoergic force development in the rat femoral artery were measured using rings precontracted with equi-effective concentrations of either prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) or the selective $\alpha_1$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$_{2\alpha}$ 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% ± 7.6% relative to KCl (60 mM). ACT-333679 (10 µM) significantly inhibited contraction of the femoral artery by EFS (4–24 Hz; area under the curve: 1234 ± 135.9 and 580.8 ± 69.3 for the control and ACT-333679, respectively; $P < 0.05$, n = 6; Fig. 2).

**PGI$_2$ Analogs Constrict the Femoral Artery**

The effects of PGI$_2$ 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$_{2\alpha}$ or phenylephrine. Although iloprost, beraprost, and treprostinil evoked concentration-dependent relaxation of the femoral artery contracted with PGF$_{2\alpha}$ (Fig. 3), these PGI$_2$ analogs did not cause vasorelaxation but rather induced further contraction in femoral arterial rings precontracted with phenylephrine (Fig. 3). The maximum contraction to

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**TABLE 2**

| Contractile agent | Selexipag | ACT-333679 |
|-------------------|-----------|------------|
| pEC$_{50}$        | 5.4 ± 0.1 | 5.5 ± 0.1 |
| PGF$_{2\alpha}$   | 5.5 ± 0.1 | 5.6 ± 0.1 |
| Phenylephrine     | 113.3 ± 5.4 | 126.9 ± 7.0 |
| $E_{max}$         | 116.6 ± 6.6 | 121.0 ± 6.6 |

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**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$_{2\alpha}$ or phenylephrine (n = 6/group).

**Fig. 2.** Effect of ACT-333679 (10 µM) on contraction of the rat femoral artery to EFS. *$P < 0.05$; **$P < 0.01$ (n = 6/group).
iloprost, beraprost, and treprostinil was 44.4% ± 15.1%, 78.4% ± 9.8%, and 34.6% ± 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 $\mu$M ($P < 0.05$, $n = 6$; Fig. 3C).

Iloprost, beraprost, and treprostinil caused weak vasorelaxation of the femoral artery contracted with phenylephrine in the presence of the EP$_3$ receptor antagonist (DBTSA, 1 $\mu$M) (Fig. 3). Relaxation to iloprost, beraprost, and treprostinil was 40.8% ± 5.6%, 51.8% ± 9.3%, and 37.6% ± 7.4%, respectively. Differences in area under the curve values for responses to all PGI$_2$ analogs tested in the absence and presence of DBTSA were statistically significant ($P < 0.05$, $n = 5$; Fig. 4). Antagonism of EP$_1$ (SC51322, 1 $\mu$M) and TP (GR32191B, 0.1 $\mu$M) receptors did not significantly modulate the reactivity of the femoral artery to PGI$_2$ analogs (Table 3).

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

α1ARs and EP3 Receptors Act Synergistically in the Femoral Artery

Since reactivity of the femoral artery to PGI2 analogs was only modulated during α1AR stimulation, the potential pharmacological interaction between contractile EP3 receptors and α1ARs was investigated. The EP1/3 receptor agonist sulprostone caused concentration-dependent contraction of the rat femoral artery (Fig. 6A; pEC50 = 6.4 ± 0.3, Pmax = 140.6% ± 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 μM) (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 μM) and prazosin (0.1 μM; selective α1AR antagonist) significantly reduced sulprostone-evoked contraction (Fig. 6B). Selective EP1 receptor antagonist SC51322 (1 μM) did not inhibit contraction to sulprostone (47.4% ± 10.5% and 38.5% ± 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 prostaglandin receptors. Relaxation of the femoral artery to selexipag and ACT-333679 was similar in rings precontracted with either PGF2α or the α1AR 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 PGE2α, 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 α1AR 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 α1ARs 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 α1ARs and EP3 receptors.

Marked contractile synergy between EP3 receptors and α1ARs 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;
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 α2ARs and its effect on vascular responsiveness to selexipag and analogs of PGI2. Synergy between α2ARs and EP3 receptors in the femoral artery was further supported by the observations that a subthreshold concentration of the EP3 receptor agonist sulprostone evoked significant contraction of the femoral artery only in the presence of phenylephrine. Activation of both EP3 and α2ARs receptors was required, since contraction to sulprostone was abolished by either DBTSA or prazosin. The contractile synergy between femoral EP3 receptors and α2ARs 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 PGL2 analogs (Tapson 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., 2017). Involvement of other lower limb arteries that are under adrenergic control (e.g., the popliteal artery) (Sada et al., 2017). Involvement of other lower limb arteries that are under adrenergic control (e.g., the popliteal artery) (Sada et al., 2017). Involvement of other lower limb arteries that are under adrenergic control (e.g., the popliteal artery) (Sada et al., 2017). Involvement of other lower limb arteries that are under adrenergic control (e.g., the popliteal artery) (Sada 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 PGL2 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 PGL2 in the rat femoral artery. Selexipag and ACT-333679 relaxed the femoral artery, whereas EP3 receptor-mediated contraction to PGL2 analogs was exacerbated during α2AR 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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