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Effects of a new PAR1 antagonist, F 16618, on smooth muscle cell contraction

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ABSTRACT

This study evaluated the effects of two PAR1 antagonists on vessels contracted by SFLLR. ER 121958 antagonized the SFLLR-induced contraction on rat denuded superior mesenteric artery and pig coronary artery in a non-competitive manner (IC_{50} values were 22 [7.5–43.6] nM and 2.9 [2.09–4.02] nM, respectively). F 16618 inhibited the SFLLR-induced superior mesenteric arteries and coronary arteries contraction in a competitive manner (pA_2 values of 7.3 and 6.2, respectively). PAR1 antagonists do not affect vessel resting tension or haemodynamic parameters in anaesthetized rats. Thus, PAR1 antagonists could have beneficial effects against vasospasm due to vessel injury.

Contents

1. Introduction

Protease activated receptors (PARs) belong to the G protein coupled receptor superfamily, and include four different members. PAR1, PAR3 and PAR4 are activated by thrombin whereas PAR-2 is cleaved mainly by trypsin (Macfarlane et al., 2001; Hirano et al., 2005). Activation of PAR involves a cleavage of the N-terminal exodomain, unmasking a new N-terminal domain which acts as a tethered ligand, auto-activating the receptor (Vu et al., 1991). It is well established that thrombus formation participates in the development of vascular disorders (Nelken et al., 1992; Ku and Dai, 1997). Thus, thrombin, a serine protease acting at the end of the coagulation cascade, plays a key role in thrombus formation. Under physiological conditions, thrombin mediates endothelium-dependent vasorelaxion *in vitro* (Hamilton and Cocks, 2000; Mizuno et al., 1998) but has also been reported to induce endothelium-dependent vessel contraction

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(Ku and Zaleski, 1993). Under pathological conditions, the direct activation of PAR1 in smooth muscle cells induces a vasoconstriction (Ku and Dai 1997). In addition, it has been reported that PAR1 is up-regulated in several pathological conditions, in particular after balloon angioplasty or during the development of atherosclerotic plaques (Andrade-Gordon et al., 2001; Takada et al., 1998). This up-regulation has been correlated with the increase of vascular contractile responses (Ku and Dai, 1997; Fukunaga et al., 2006) and is thought to participate in the development of vascular lesion. This hypercontractile response to thrombin can be antagonized by treatment with a PAR1 antagonist, E5555, in a model of subarachnoid haemorrhage (Maeda et al., 2007; Kai et al., 2007).

We recently characterized a new non peptidic small molecule PAR1 antagonist, F 16618, currently in preclinical phase. We proposed herein to test the vascular properties of F 16618 with ER 121958 as a reference compound using isometric tension experiments on denuded vessels.

2. Materials and methods

Animals were housed and tested in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility in strict compliance with all applicable regulations and the protocol was carried out in compliance with French regulations and local Ethical Committee guidelines for animal research.

2.1. Calcium release assay

CHO cells were assayed for Ca^{2+} responses 48 h post-seeding upon 1 h loading with the Ca^{2+} indicator dye Fluo-3 (2 μM) in a 96-well format. Fluorescent readings were made every 2 s for a 6 min time period using a fluorometric image plate reader (FLIPR, Molecular Devices) as described previously (Pauwels and Colpaert, 2000). Compounds were applied 2 min prior to application of PAR1 agonist SFLLR.

2.2. Tissue preparation

2.2.1. Rat superior mesenteric arteries

Male OFA–SPF rats weighing 250–270 g were euthanized by injection of sodium pentobarbital (160 mg/kg, i.p.). The superior mesenteric artery was removed and placed in ice-cold Krebs solution (NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $NaHCO_3$, 25 mM, glucose 10 mM, pH 7.4). Arteries were cleaned of fat tissue and cut into 2 mm segments. Rat artery rings were stripped of endothelium by gently rubbing the inner surface with a wire shaft. Arteries were mounted in a 5 ml wire myograph for resistance arteries (DMT 610 M, Danish MYO Technology, Denmark) with a resting tension of 5 mN. The Krebs solution was maintained at 37 °C and bubbled with 95% O_2/5% CO_2 to maintain the pH at 7.4. Vessels were allowed to stabilize for half an hour. Artery viability was tested using a potassium-rich solution (80 mM) and reproducibility of the response was assessed with a second KCl challenge.

2.2.2. Pig coronary artery

The pig coronary artery preparations (left anterior descending coronary artery (LAD)) were obtained from male Landrace pigs weighing 18–35 Kg (GAEC la Jonjaune, France) 48 h after a myocardial ischemia experiment resulting from a left circumflex coronary artery occlusion. After endothelium denudation, left anterior descending coronary strips of 5 mm in length were mounted between two triangle wire hooks and suspended in organ baths (Emka technology, France) containing 20 ml of Krebs with a resting tension of 10 grams. Artery viability was then tested using a potassium-rich solution (80 mM).

2.3. Tension measurement experiments

Cumulative concentration–response curves were obtained with PAR1 agonist peptide SFLLR (0.1 μM–100 μM with half log dose increment). PAR1 antagonists ER 121958, F 16618 or corresponding vehicle (DMSO 0.1%) were pre-incubated 30 min before the SFLLR concentration–response curve. The amplitude of the tension was measured irrespective of the time for each concentration.

2.4. Haemodynamic studies

Male OFA–SPF rats (Iffa-Credo, l'Arbresle, France) weighing 300–350 g were anaesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg, Sanofi–Aventis Laboratories, Libourne, France). Rats underwent tracheotomy and were mechanically ventilated. Catheters were inserted into a femoral vein and artery for infusing drugs and continuously measuring arterial pressure, respectively.

Following stabilisation of haemodynamic and blood gases, the treatments (vehicle/ER-121958/F 16618 at 1.25 mg/kg) were intravenously administered over 5 min (as 1 ml/kg solutions). The doses of reference compounds injected are in terms of their corresponding bases.

2.5. Data analysis

For tension measurement experiments, results are expressed as % mean (E_{max}) ± S.E.M. where E_{max} was obtained with the higher concentration of SFLLR (100 μM). Concentration response curve were fitted using origin 7.5 software to calculate EC_{50}, IC_{50} and pA_2 values. One-way analysis of variance (ANOVA) followed by a Dunnett's test, were performed to compare each group. Differences were considered when $P<0.05$.

2.6. Drugs

SFLLR was synthesised in the Laboratoire des Amino Acides, Peptides et Proteins, directed by Jean Martinez, Faculté de Pharmacie, Montpellier. ER 121958 (2-(2-(3,5-di-tert-butyl-4-hydroxyphenyl)-2-oxoethyl)-6-ethoxy-3-imino-N-methylisoindoline-5-carboxamide) and F 16618 ((2-[5-Oxo-5-(4-pyridin-2-yl-piperazin-1-yl)-penta-1,3-dienyl]-benzonitrile hydrochloride) were synthesized by the Division of Medicinal Chemistry IV, (Centre de Recherche Pierre Fabre, Castres, France). All drugs were dissolved in double-distilled water except ER 121958 and F 16618 which were dissolved in DMSO 0.1% in double-distilled water for *in vivo* studies and in 40% polyethyleneglycol 300 in sterile saline for haemodynamic studies.

3. Results

3.1. Calcium fluorescence

In a CHO cell line, which constitutively expressed PAR1, F 16618 and ER 121928 inhibit the SFLLR-induced calcium release. This inhibition was clearly competitive for F 16618 and allowed to calculate a pA_2 value of 6.6 whereas ER121958 exhibits a non competitive profile with an IC_{50} value of 0.75 μM [0.53; 0.99] (Fig. 1A). In overexpressing system, F 16618 blocked the TFLLR induced PAR1 activation with a p*K*b value of 5.6 and the SFLLR induced PAR1 activation with a p*K*b value of 5.9 whereas it only weakly interacted with SLIGRL-activated PAR2 and with AYPGKF-activated PAR4 (p*K*b 4.39 and 4.93, respectively). Altogether, these data suggest that F 16618 is potently more selective for PAR1 vs PAR2 and PAR4.

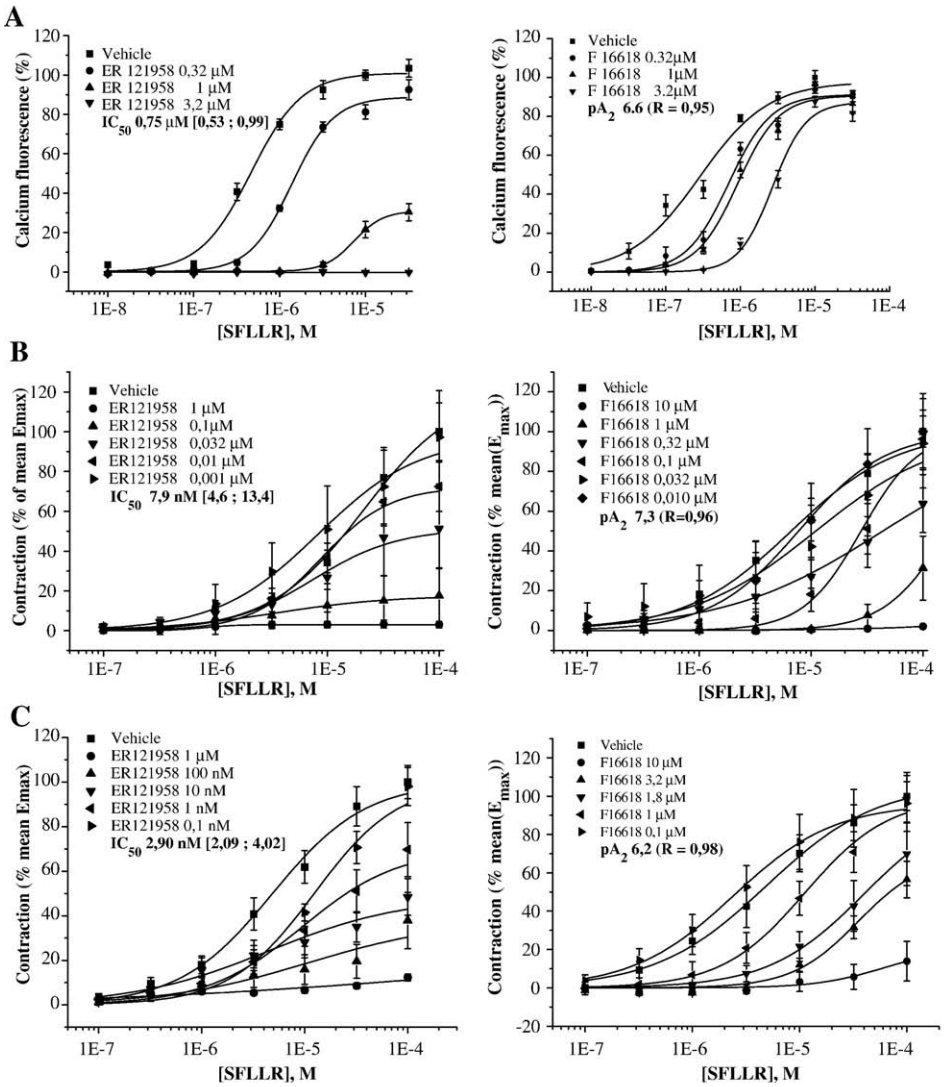

Fig. 1. Effects of ER 121958 and F 16618 on SFLLR-induced calcium release in CHO cells (A) and contraction on endothelium denuded rat superior mesenteric arteries (B) and pig coronary arteries (C). ER 121958 and F 16618 concentration-dependently inhibit the SFLLR-induced calcium release as well as contraction of rat superior mesenteric arteries and pig coronary arteries. Data are means ± SEM of $n = 5–15$.

3.2. Isometric tension experiments

As the presence of the endothelium does not allow to produce SFLLR-induced contraction (Kawabata et al., 2004), we performed experiments on endothelium-denuded vessels.

3.2.1. Rat isolated superior mesenteric artery

SFLLR induced a concentration-dependent constriction of the denuded superior mesenteric arteries (EC_{50} value of 21.1 [12.1; 128] µM (Fig. 1B)). Neither ER 121958 nor F 16618 affected baseline tension value even when tested at the higher concentration (Table 1). Nevertheless, both ER 121958 and F 16618 blocked the SFLLR-induced rat denuded superior mesenteric arteries contraction. ER 121958 inhibited the SFLLR-induced contraction in a non-competitive manner (IC_{50} value of 7.9 [4.6; 13.4] nM (Fig. 1B)) whereas F 16618 shifts the SFLLR concentration response curve to the right, suggesting a competitive inhibition (pA_2 value of 7.3, $R = 0.96$ (Fig. 1B)).

3.2.2. Pig isolated coronary artery

Neither ER 121958 nor F 16618 affected the baseline tension (Table 1). SFLLR induced a concentration-dependent constriction of the denuded coronary rings (EC_{50} value of 4.8 [3.7; 6.3] µM (Fig. 1C)). ER 121958 concentration-dependently blocked the SFLLR-induced denuded pig coronary arteries in a non-competitive manner (IC_{50} value of 2.90 [2.09; 4.02] nM). F 16618 also inhibited the SFLLR-induced pig coronary contraction in a concentration-dependent and competitive manner. The competitive profile allowed us to calculate a pA_2 value of 6.2, $R = 0.98$ (Fig. 1C).

Table 1

Vascular bed	Resting tension	Resting tension after 30 min incubation with ER l21958
Rat superior mesenteric artery	3.92 ± 0.33 mM	3.85 ± 0.34 mN (ns)
Pig coronary artery	10.45 ± 0.96 g	9.87 ± 0.34 g (ns)
	Resting tension	Resting tension after 30 min incubation with F 16618
Rat superior mesenteric artery	4.34 ± 0.44 mM	3.64 ± 0.77 mN (ns)
Pig coronary artery	9.47 ± 0.52 g	8.30 ± 0.89 g (ns)

3.3. Haemodynamic effects of PAR1 antagonists in anesthetized rat

To test haemodynamic effects of PAR1 antagonists, we chose a dose that exhibits an anti-thrombotic effect in our models (1.25 mg/kg, data not shown). Neither ER 121958 nor F 16618 significantly affected mean arterial pressure (vehicle in mmHg: 110 ± 6 ($n = 12$), ER 121958 (1.25 mg/kg) 107 ± 7 ($P =$ NS, $n = 8$) and F 16618 (1.25 mg.kg): 116 ± 4 ($P =$ NS, $n = 10$)) or heart rate (vehicle in bpm: 402 ± 12 ($n = 12$), ER 121958 (1.25 mg/kg) 410 ± 10 ($P =$ NS, $n = 8$) and F16618 (1.25 mg.kg): 399 ± 7 ($P =$ NS, $n = 10$), suggesting that in normal vasculature, these compounds are devoid of haemodynamic effects.

4. Discussion

The present report characterizes the functional effect of PAR1 antagonists ER 121958 and F 16618 on SFLLR-induced vascular contraction in two different vascular beds. Both ER 121958 and F 16618 antagonized vasoconstriction induced by PAR1 agonist SFLLR. We showed that PAR1 antagonists are potent anti-vasoconstrictors. Our model mimics the abnormal stimulation of PAR1 involved in lesionned vessels. PAR1 antagonists had no effect on the basal tension of the vessels suggesting they preferentially act locally at the site of the lesion where the PAR1 are exposed to the vessel lumen and where high concentrations of thrombin are produced (Hatton et al., 1989). Moreover, PAR1 antagonists do not affect the basal functioning of PAR (resting tone) in intact vessels. This property is confirmed by the absence of haemodynamic effects in normal vasculature. In addition to their anti-thrombotic properties, these results illustrate the putative beneficial effect of PAR1 antagonists to prevent vasospasm caused by arterial injury. Indeed, this antivasoconstricting effect allowed PAR1 antagonists to locally modulate the vasoconstriction induced by thrombin at the site of the lesion that contributes to vessel occlusion during vascular thrombosis. These properties have been suggested recently in a model of sub-arachnoid haemorrhage in which hypercontractility to thrombin is reduced by pre-treatment with PAR1 antagonist E5555 (Maeda et al., 2007; Kai et al., 2007). In a rabbit model of restenosis after balloon angioplasty showing PAR-dependent hypercontractile responses, SCH97797, a potent PAR1 antagonist, was not efficient against thrombin hyperresponsiveness (Fukunaga et al., 2006). Nevertheless, further additional in vivo studies are required to completely elucidate the haemodynamic effect of PAR antagonists.

We were unable to produce PAR2 (SLIGLR) and PAR4 (AYPGKF) dependant contraction of vessels to assess PAR1 antagonist specificity. Nevertheless, Using over-expression system of PAR1, PAR2 and PAR4 in CHO cells, we have demonstrated that F 16618 antagonism is more potent on PAR1 (whatever the agonist used SFLLR or TFLLR) compared to PAR2 and PAR4. These data allow demonstrating that the vascular effects of F 16618 are principally mediated by a PAR1 dependant mechanism.

Isometric tension experiments revealed a non-competitive inhibitory profile for ER 121958, whereas F 16618 is a competitive antagonist. These data point to different mechanisms of action for both molecules. In the case of F 16618, the competitive profile suggests that the interaction site is located near the receptor N-terminal extremity and directly blocks the interaction between thrombin and PAR. It could also be located on the third extracellular loop, which is central part for receptor activation after cleavage by thrombin. In contrast, ER 121958 showed a non-competitive profile suggesting an interaction which could be independent of the activation state of the receptor. This difference in the mechanism of action could explain, in part, why F 16618 is less potent on the pig coronary arteries compared to the rat superior mesenteric arteries, suggesting that the expression density of PAR might be of major importance for a competitive antagonist.

In conclusion, the present study characterized for the first time the vascular effects of F 16618, a new PAR1 antagonist in a model of vascular reactivity. These data suggest that in addition to the anti-thrombotic properties, F 16618 presents an additional beneficial effect in the treatment of vasospasm caused by vascular injury.

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