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EV-077 *in vitro* inhibits platelet aggregation in type-2 diabetics on aspirin

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ABSTRACT

Introduction: This study aimed to characterize the *in vitro* effect of EV-077, a compound that antagonises the binding of prostanoids and isoprostanes to the thromboxane receptor (TP) and inhibits the thromboxane synthase (TS), on platelet aggregation of patients with type-2 diabetes and coronary artery disease (CAD) on chronic aspirin treatment. The effect of EV-077 on 8-iso-PGE_2-mediated TP receptor contraction of human arteries was also investigated.

Materials and Methods: Fifty-two type-2 diabetics with CAD on chronic aspirin (100 mg) treatment were studied. Arachidonic acid-induced platelet aggregation was measured by impedance aggregometry in platelet-rich plasma (PRP) and whole blood anticoagulated with hirudin, and by light transmission aggregometry in citrate-anticoagulated PRP following 10-min *in vitro* exposure to EV-077 (100 nmol/l) or control. The effect of EV-077 was measured on isometric contraction of 24 human umbilical arteries induced by isoprostane 8-iso-PGE_2.

Results: Arachidonic acid (1 mmol/l) induced substantial aggregation in hirudin-anticoagulated whole blood (63 ± 4 AU), which was significantly reduced by *in vitro* exposure to EV-077 (38 ± 3 AU, $P<0.001$). Virtually no arachidonic acid-induced aggregation in citrate-anticoagulated or hirudin-anticoagulated PRP was observed. EV-077 potently, competitively and reversibly inhibited TP mediated contraction of umbilical arteries by 8-iso-PGE_2 ($P<0.01$).

Conclusions: Aspirin did not completely inhibit arachidonic acid-induced platelet aggregation in whole blood from type-2 diabetics with CAD. This aggregation is likely induced by prostanoids and/or isoprostanes produced by leukocytes, because it was significantly reduced by EV-077. The TP receptor-mediated contraction of human arteries induced by isoprostane 8-iso-PGE_2 was effectively inhibited by EV-077.

Introduction

Thromboxane receptors (TP) are expressed in several tissues and cell types, including platelets, endothelium and smooth muscle cells and have multiple physiological and pathophysiological roles [1–3]. The TP receptor is activated by several endogenous agonists, including the prostanoids thromboxane A_2 (TxA_2), PGG_2 and PGH_2 [4], isoprostanes [5,6], and some hydroxyeicosatetraenoic acids (HETEs) [7]. These TP receptor agonists, and in particular TxA_2, are prominent inducers of platelet aggregation [1].

Isoprostanes are a group of stable prostaglandin-like compounds produced by free radical-catalysed, non-enzymatic peroxidation of arachidonic acid [8,9], which are used as markers of oxidative stress in several human diseases, including diabetes [10]. In addition, isoprostanes are biologically active compounds involved in contraction of vascular smooth muscle and activation of platelets [11]. It is generally accepted that increased levels of prostanoids and isoprostanes play important roles in initiation and development of diabetic complications, including platelet hyper-reactivity and atherothrombosis [1,12].

Activation of TP receptors influences haemostasis in several ways. Platelet activation causes platelet aggregation, leading to thrombus formation [13]. Endothelial cell TP receptor activation inhibits angiogenesis, induces apoptosis and the expression of receptors for monocyte adhesion, which favours their migration into the intima, where they mature into macrophages [14,15]. TP receptor activation on vascular smooth muscles mediates vasoconstriction, hypertension and smooth muscle cell proliferation [16–18]. These processes may lead to chronic vascular inflammation and oxidative stress and ultimately cause atherosclerotic lesions [19,20].

Therefore, the availability of drugs inhibiting TxA_2 production and TP receptors have great potential utility for treatment of vascular disorders. Recently EV-077, a novel synthetic dual TP receptor antagonist and TS inhibitor, has been shown to potently inhibit arachidonic acid-, the

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TxA_2 mimetic U-46619- and collagen-induced platelet aggregation in platelet-rich plasma (PRP) from healthy volunteers; the inhibition was significantly greater than that by aspirin [21]. EV-077 is a significantly more potent TP receptor antagonists and TS inhibitor than picotamide, which reduces cardiovascular events by dual antagonism of platelet activation and progression of vascular arterial lesions [22].

Agents that inhibit both TxA_2 production and TP receptors may be particularly useful in patients with type 2 diabetes mellitus. Vascular inflammation and oxidative stress are significantly increased in type-2 diabetic patients, resulting in increased formation of isoprostanes and prostanoids and hyper-reactive platelets as compared to healthy individuals [1,23–25].

The aim of the present study is to characterize the *in vitro* effect of EV-077 on TP-mediated effects in platelets and human umbilical artery: 1) the effects of EV-077 was studied on platelet aggregation of patients with type-2 diabetes mellitus and CAD on chronic aspirin treatment; 2) the effects of EV-077 was studied on umbilical artery contraction using the isoprostane 8-iso-PGE_2, which has been demonstrated to be the most potent vasoconstrictor isoprostane on the human umbilical artery [5] and vein [26,27], activating the TPα isoform, which is present on the human umbilical vein and platelets [5,26–29].

Table 1
Characteristics of the enrolled patients with type-2 diabetes mellitus.

	Median (range)	Mean ± SD
N	52	52
Gender (M/F)	42/10	42/10
Age (years): All	69 (52–90)	70 ± 9
Female	69 (53–87)	71 ± 11
Male	70 (52–90)	69 ± 9
Red Blood Cells ($\times 10^6$/μl)	4.6 (3.6–5.6)	ND
Haemoglobin (g/dl)	13.8 (9.6–18.1)	13.7 ± 1.8
Haematocrit (%)	40.1 (30.0–52.9)	40.1 ± 4.7
Platelets ($\times 10^3$/μl)	195 (100–327)	202 ± 57
Mean platelet volume (fl)	8.5 (7.3–11.1)	8.8 ± 0.9
White Blood Cells ($\times 10^3$/μl)	7.3 (4.6–14.0)	ND
S-Glucose (mmol/l)	8.2 (4.1–23.3)	ND
HbA1c (%)	7.2 (4.9–10.8)	7.5 ± 1.2
Triglycerides (mg/dl)	147 (49–382)	158 ± 94
Total Cholesterol (mg/dl)	146 (92–222)	150 ± 34
HDL (mg/dl)	39 (24–62)	40 ± 8
LDL (mg/dl)	98 (52–156)	98 ± 30
Hs-C-Reactive Protein (mg/l)	1.6 (0.2–26.3)	3.4 ± 4.6
Patients on statins (%)	67	67
Patients on antihypertensives (%)	90	90

ND: not normally distributed.

Materials and methods

Materials

8-iso-PGE_2 (Cayman, Ann Arbour, MI, U.S.A.) was dissolved in DMSO, and EV-077 (Evolva) [21] in bi-distilled water; stock solutions, were further diluted with bi-distilled water. 5-Hydroxytryptamine creatinine sulphate complex (Research Biochemical, Natick, MA, U.S.A.) was prepared in bi-distilled water. U-46619 (Biomol Research, Plymouth Meeting, PA, U.S.A.) stock solutions were made in ethanol and subsequently diluted in bi-distilled water. Arachidonic acid and the stable TxA_2 analogue U-46619 were from Sigma (St Louis, MO, USA).

EV-077 is a TP receptor antagonist (Ki = 0.1–0.5 nM) and TS inhibitor (Ki = 4.8 μM), that inhibited platelet aggregation measured by light transmission aggregometry induced by arachidonic acid (1 mM), U-46619 (7 μM) and collagen (1 mg/ml) with IC_{50} values of 6.3, 24.3, and 8.0 nM, respectively [21].

Platelet aggregation was induced by arachidonic acid (1.0 mmol/l), and U-46619 (7 μM) (final concentrations).

All stock solutions were stored frozen in aliquots, thawed and diluted daily. Control experiments in the presence of corresponding vehicle controls were performed in order to rule out any possible non-specific action on tonus or contractility of the artery preparation.

Patient population

Patients were eligible for inclusion if they were above 18 years of age and had type-2 diabetes with significant CAD verified by coronary angiography. Our aim was to provide a study population of stable CAD patients without any selection for risk factors. Patients were excluded if they had had any ischemic events or revascularization procedures within the previous 6 months, if their platelet count was $<120\times10^9$ per litre, or if they took warfarin or drugs known to affect platelet function two weeks before blood sampling.

Fifty-two patients were enrolled in the study: 8 (15.3%) had coronary stenosis, 39 (75%) underwent percutaneous coronary intervention, 6 (11.5%) underwent coronary artery by-pass grafting and 33 (63.4%) had hypercholesterolemia. All patients were on chronic daily treatment (at least 1 year) with a daily single dose of 100 mg enteric coated acetylsalicylic acid (Cardioaspirin, Bayer, Germany), 43 (82.7%) with oral hypoglycaemic agents, and 12 (23%) with insulin. The complete characteristics of the patients are reported in Table 1.

The study was approved by the ethics committee of Ospedale San Paolo, and all patients provided informed consent.

Blood sampling and platelet aggregation - Light transmission aggregometry

Venous blood (10 ml) was collected from each subject 24 h after the last dose of aspirin, using a 21-gauge needle and no tourniquet in home-made tubes containing tri-sodium citrate as the anticoagulant (the first 3 ml of blood were placed in a tube without anticoagulant for serum TxB_2 determination). Free calcium is crucial for the magnitude of platelet aggregation. Since citrate remains in the plasma phase, calcium (and citrate) concentrations are directly determined by the haematocrit. Therefore the concentration of plasma sodium citrate was adjusted to 20 mmol/l according to the following formula [30]:

$$\mathbf{V_c = V(9.2–0.18\%Hct)/88},$$

where

- V_c = volume of 109 mmol/l tri-sodium citrate added to blood sample anticoagulated with 1/10 volume 109 mmol/l tri-sodium citrate
- V = volume of anticoagulated blood sample
- %Hct = haematocrit

PRP was obtained by centrifugation of whole blood samples at $200\times g$ at room temperature for 10 min. Autologous platelet-poor plasma (PPP) was obtained by further centrifugation at $1.400\times g$ for 15 min at room temperature [31].

After incubation with a concentration of EV-077 (100 nmol/l) that has previously been shown to maximally inhibit platelet aggregation induced by arachidonic acid and U-46619 [21] or vehicle at 37 °C for 10 min, 270 μl PRP was placed in a light transmission aggregometer (Chrono-Log 560, Havertown, PA, USA) and continuously stirred at 1.000 rpm for 1 min [32]. Autologous PPP was used to set the instrument's 100% light transmission, while the un-stimulated PRP was used to set 0% light transmission. The individual platelet count of the PRPs was not adjusted to a pre-determined range, because this procedure may induce artefacts [31]. Subsequently, the platelet aggregation agonist (10 μl), either 7 μmol/l U-46619, or 1.0 mmol/l arachidonic acid was added and the aggregation response was recorded for 5 min. All aggregation tests were performed within 3 h of blood collection. Maximal aggregation response to each agonist was considered after 3 min.

Blood sampling and platelet aggregation—impedance aggregometry

Venous blood (12 ml) was drawn from each patient 2 h after the intake of 100 mg enteric-coated aspirin at around 11:00, using a 21-gauge needle and no tourniquet in hirudin (25 μg/ml, 16.000 ATU/mg) (Refludan ®, Lepirudin, Pharmion, Summit, NJ, USA) anticoagulant. The choice of hirudin as anticoagulant was to decrease the variability of the results based on the recommendations of the manufacturer of the impedance aggregometry device. Compared to citrate, the use of hirudin allows studying platelet function at physiological concentrations of ionized calcium.

Whole blood or PRP (prepared as for transmission aggregometry) samples (300 μl) were diluted 1:1 with 0.9% sodium chloride, then placed into the Multiplate® platelet aggregation device (Verum Diagnostica, Munich, Germany), continuously stirred at 800 rpm for 3 min at 37 °C and then stimulated with arachidonic acid (1 mmol/l).

Whole blood samples were pre-incubated with 6 μl of EV-077 (100 nmol/l) or vehicle at 37 °C for 7 min before placing into the Multiplate® Analyser.

Platelet aggregation was induced and monitored for 6 min and expressed as maximum aggregation in Arbitrary Unit (AU).

Thromboxane B_2 (TxB_2) measurements

TxB_2, the stable metabolite of TxA_2, was measured using an enzyme immunoassay (Thromboxane B2 EIA kit, Cayman) in serum and in the supernatant of PRP or whole blood samples. Serum was prepared from non-anticoagulated blood after 1 h of incubation at 37 °C by centrifugation at $1400 \times g$ for 15 min at room temperature. Supernatants were obtained by centrifuging whole blood or PRP at the end of the observation period of aggregation induced by arachidonic acid at $13.000 \times g$ for 1 min at room temperature.

Preparation of human umbilical artery rings

Human umbilical cords ($n = 24$) were collected from healthy and normotensive women after full-term vaginal or caesarean deliveries [33,34]. Artery preparations with intact endothelium were cut in rings of approximately 3 mm width and mounted in 10 ml organ baths in modified Krebs solution of the following composition (mmol/l): NaCl: 119, KCl: 4.7, $NaHCO_3$: 25, KH_2PO_4: 1.2, $CaCl_2$: 2.5, $MgSO_4$:1.0, EDTA: 0.004 and D-glucose: 11. The isometric tension was adjusted to 20–40 mN throughout the 150 min equilibration period. Only one concentration-response curve was performed on each ring.

The reported investigations were carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. The use of umbilical cord samples was approved by written informed consent from each parturient.

EV-077 antagonism of isoprostane-induced TP arterial contraction

Cumulative concentration-response curves to 8-iso-PGE_2 were obtained by addition to the organ bath in 0.25 log10 increments (1, 1.7, 3, 5.5, and 10 nmol/l etc. to 30 μmol/l). Concentration-response curves to 8-iso-PGE_2 were obtained alone and in the presence of EV-077 (1, 3, 10 and 30 nmol/l) added to the solution 30 min before the agonist. At the end of each concentration-response curve, serotonin (5-HT, 10 μmol/l) was applied to determine the tissue maximal contractile response [33].

EV-077 agonism on TP-mediated arterial contraction

Cumulative concentration-response curves to U-46619 (0.1 nmol/l - 1 μmol/l) and to EV-077 (0.1 nmol/l - 100 μmol/l) were obtained by cumulative addition of the compound to the solution in 0.50 log10 increments. At the end of each concentration-response curve, serotonin (5-HT) 10 μmol/l was applied to determine the tissue maximal contractile response [33].

Data calculation for arterial ring contraction

Four or eight rings from each artery were typically used. Responses are expressed as the percentage of tissue maximum response elicited by 5-HT (10 μmol/l).

The agonist concentration–response curves were characterised assuming one binding site by an equation describing an hyperbolic function containing two constants, $y = P_1 \times x \,/\, (EC_{50} + x)$ where y is the evoked contraction, x is the agonist concentration, P_1 is the maximal contraction asymptotically approached at 'infinitely' high agonist concentration, and EC_{50} is the agonist concentration giving the half-maximal contraction. The constants were calculated using a non-linear regression computer program (GraphPad Prism, La Jolla, CA, USA).

When the criteria for competitive antagonism were satisfied, that is when the antagonist produced a parallel rightward shift of the agonist curve without attenuation in the maximum, antagonist pA_2 values and slope of Schild regressions were calculated [35]. Agonist log concentration ratio (r) was determined by subtracting the pEC_{50} value of the agonist in the presence of the antagonist from the pEC_{50} in control preparation. When the slope of the Schild plot was not significantly different from unity, it was constrained to unity and the pK_B calculated [36].

Statistical analysis

Statistical analysis of the pEC_{50} values was performed using one-way analysis of variance followed by Tukey's post-test. *P*-values lower than 0.05 were taken to indicate significant differences between means.

Normal distribution of the platelet aggregation data was tested by the Kolmogorov-Smirnov- and the Shapiro-Wilk test, which showed that not all of the parameters were normally distributed. Since t-tests require proximally normally distributed variables, statistical testing was done by the non-parametric Wilcoxon test.

Data are presented as median with range and mean ± SD, or mean ± SEM.

Results

Serum TxB_2 levels

The median serum levels of TxB_2 in 52 aspirin-treated patients with type-2 diabetes and CAD, 24 h after the last intake of aspirin were 2.5 (range: 0.2-12.1 ng/ml). Only one of the patients had a serum TxB_2 level above 12 ng/ml, which some consider the cut-off value for aspirin responsiveness [37].

Effect of EV-077 on platelet aggregation in citrate anticoagulated PRP measured by light transmission aggregometry

EV-077 significantly and almost completely inhibited U-46619-induced platelet aggregation of aspirin-treated diabetic patients (Control: 72 ± 1%, $n = 45$; EV-077, 100 nM: 8 ± 1%, $n = 50$; $P < 0.001$). Arachidonic acid did not induce appreciable platelet aggregation; therefore, the inhibitory effect of EV-077 was not tested.

Effect of EV-077 on platelet aggregation induced by arachidonic acid in hirudin anticoagulated whole blood and PRP measured by impedance aggregometry (Multiplate)

Arachidonic acid induced substantial platelet aggregation in hirudin whole blood despite the aspirin treatment. There was no statistically significant correlation between arachidonic acid-induced platelet aggregation and serum glucose, HbA1c or CRP (data not shown). Exposure of blood samples to EV-077 significantly inhibited this arachidonic

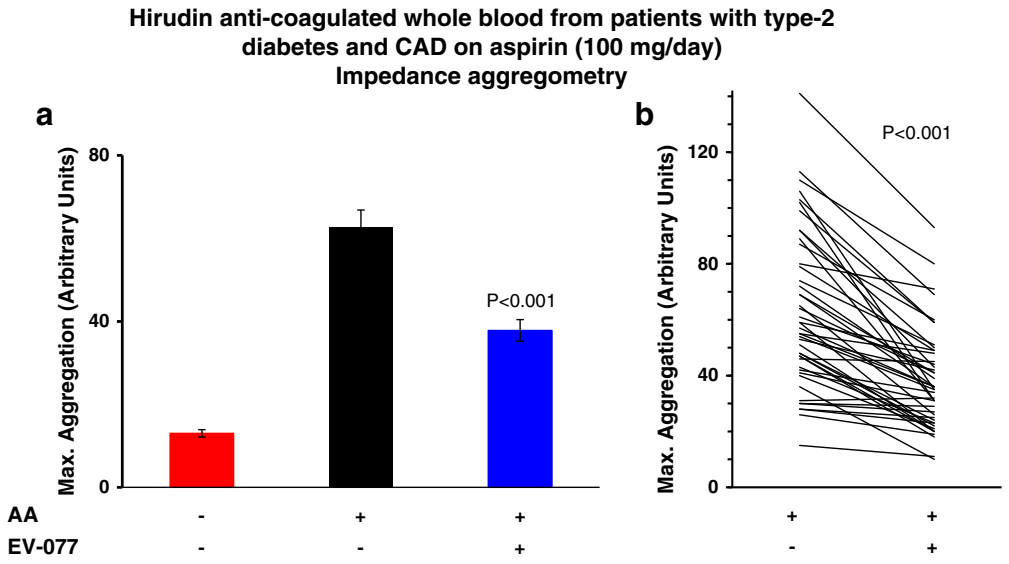

Fig. 1. Platelet aggregation in hirudin-anticoagulated whole blood taken from patients with type-2 diabetes and CAD on chronic treatment with aspirin 100 mg daily, 2 h after the last dose of aspirin. Platelet aggregation was induced by arachidonic acid (1 mmol/l), and measured by impedance aggregometry (Multiplate). Maximal aggregation: arbitrary units. No agonist (N = 32), arachidonic acid (N = 46), arachidonic acid plus EV-077 (N = 46). A. Mean ± SEM. B. Values from individual blood samples without or with treatment with EV-077 (100 nmol/l).

acid-induced platelet aggregation (P<0.001) (Fig. 1). The additional inhibitory effect of EV-077 on top of aspirin on the arachidonic acid-induced platelet aggregation varied and was lower in blood samples with relative high inhibition of platelet aggregation by aspirin alone (maximal aggregation <50 arbitrary units). Arachidonic acid-induced platelet aggregation in hirudin PRP was virtually absent.

Effect of EV-077 on arachidonic acid-induced TxB_2 generation in hirudin-anticoagulated PRP and whole blood

Arachidonic acid-induced TxB_2 production in whole blood from diabetic patients treated with aspirin was significantly decreased by 100 nM EV-077 (P<0.05) (Table 2). TxB_2 production induced by arachidonic acid in PRP was virtually absent, and, as a consequence, the effect of EV-077 was not tested (Table 2).

Effect of EV-077 on 8-iso-PGE_2 induced umbilical artery contraction

The isoprostane 8-iso-PGE_2 evoked concentration-dependent contraction of the human umbilical artery, yielding a pEC_{50} value of 7.33 ± 0.05 (EC_{50}: 47 nmol/l). The maximal response was 92.34 ± 1.97% of that induced by 5-HT (10 μM; 40.2 ± 4.6 mN).

EV-077 (1, 3, 10 and 30 nmol/l) caused a rightward shift in the 8-iso-PGE_2 concentration-response curve (Fig. 2). The pEC_{50} values in the presence of all EV-077 concentrations were significantly different from the control (P<0.01). None of the concentrations tested altered the maximal response to 8-iso-PGE_2, indicating that EV-077 acted as a competitive antagonist.

Analysis of the data obtained by Schild regression showed that the slope was not significantly different from unity (Fig. 3); the calculated pK_B was 8.87 ± 0.10, corresponding to a K_B of 1.3 nmol/l.

Lack of effect of EV-077 on the resting umbilical artery tension

U-46619 induced concentration-dependent contraction in human umbilical cord artery rings with an EC_{50} value of 31.2 ± 9.5 nmol/l and a maximal response of 95.25 ± 6.80% (95% CI: = 81.60%–108.90%) of 5-HT 10 μM. In contrast, EV-077 (0.1 nmol/l - 100 μmol/l) failed to evoke any contractile response.

Table 2
Effect of EV-077 on 1 mmol/l arachidonic acid- induced TxB_2 generation in whole blood and PRP from patients with type-2 diabetes and CAD on chronic aspirin (100 mg/day) treatment 2 hours after the last intake of aspirin.

	TxB_2 (ng/ml)		
	Control (n = 32)	EV-077 (100 nmol/l) (n = 32)	*P*
PRP	8 ± 14	ND	ND
Whole blood	154 ± 76	115 ± 81	0.05*

* 1-tailed *T*-test. ND: not determined.

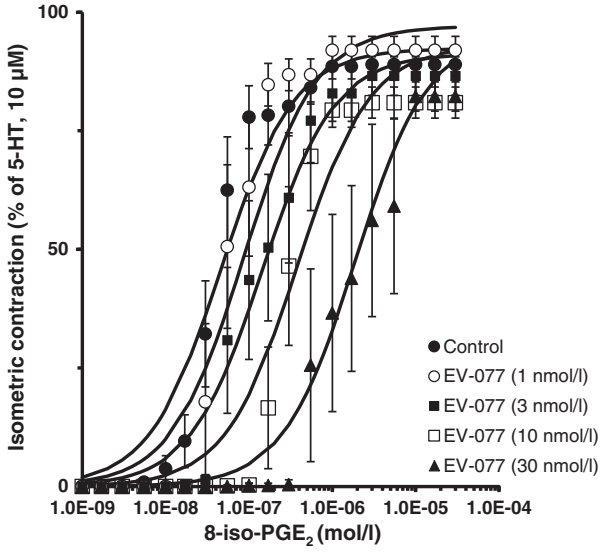

Fig. 2. Effect of EV-077 on the contraction induced by 8-iso-PGE_2 in human umbilical artery rings. Concentration-response curves. Means and SEM. The lines were constructed using the calculated EC_{50} and maximal contraction.

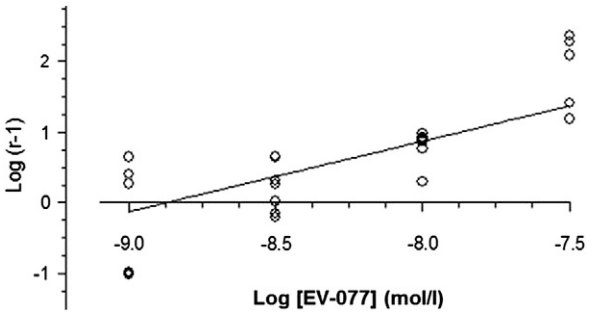

Fig. 3. Effect of EV-077 on the contraction induced by 8-iso-PGE_2 in human umbilical artery rings. Schild plot for EV-077 *vs.* 8-iso-PGE_2.

Discussion

The main finding of this study is the significant reduction of arachidonic acid-induced platelet aggregation by EV-077 in whole blood from patients with type-2 diabetes and CAD on chronic aspirin therapy. The potential mechanism of this reduction could be due to inhibition of the effects of isoprostanes and TxA_2 formed by the patient's leukocytes, since EV-077 is both an inhibitor of TS and an antagonist of the TP receptor for isoprostanes and TxA_2 (Fig. 4). The potential role of leukocytes in the metabolism of arachidonic acid to active TP receptor agonists is compatible with our demonstration that arachidonic acid failed to induce platelet aggregation in PRP, while the role of EV-077 as inhibitor of TP receptor activation is also demonstrated by our observations that it inhibited U-46619-induced platelet aggregation and 8-iso-PGE_2-mediated TP receptor contraction of the human umbilical artery.

The extent of the additional EV-077 inhibition of arachidonic acid-induced platelet aggregation on top of aspirin varied to some extent. Lower additional EV-077 inhibition was more frequently observed in patients who displayed strong inhibition of platelet aggregation; this suggests that EV-077 could be complementary in patients in whom aspirin is unable to completely inhibit the production of TP agonists.

The significant reduction in TxB_2 caused by EV-077 is likely mediated by its inhibition of TS in leukocytes, in which prostanoid biosynthesis is mediated by COX-2, which is not inhibited by the low dose aspirin (100 mg) administered to the diabetic patients.

It has previously been shown that urinary levels of TxB_2, isoprostanes, and HETEs are increased in diabetic patients [10,38–40]. The isoprostanes are potent TP receptor agonists and have recently been identified as a potential cause of the platelet aggregation after aspirin treatment in diabetes patients [23,24]. Furthermore, there is

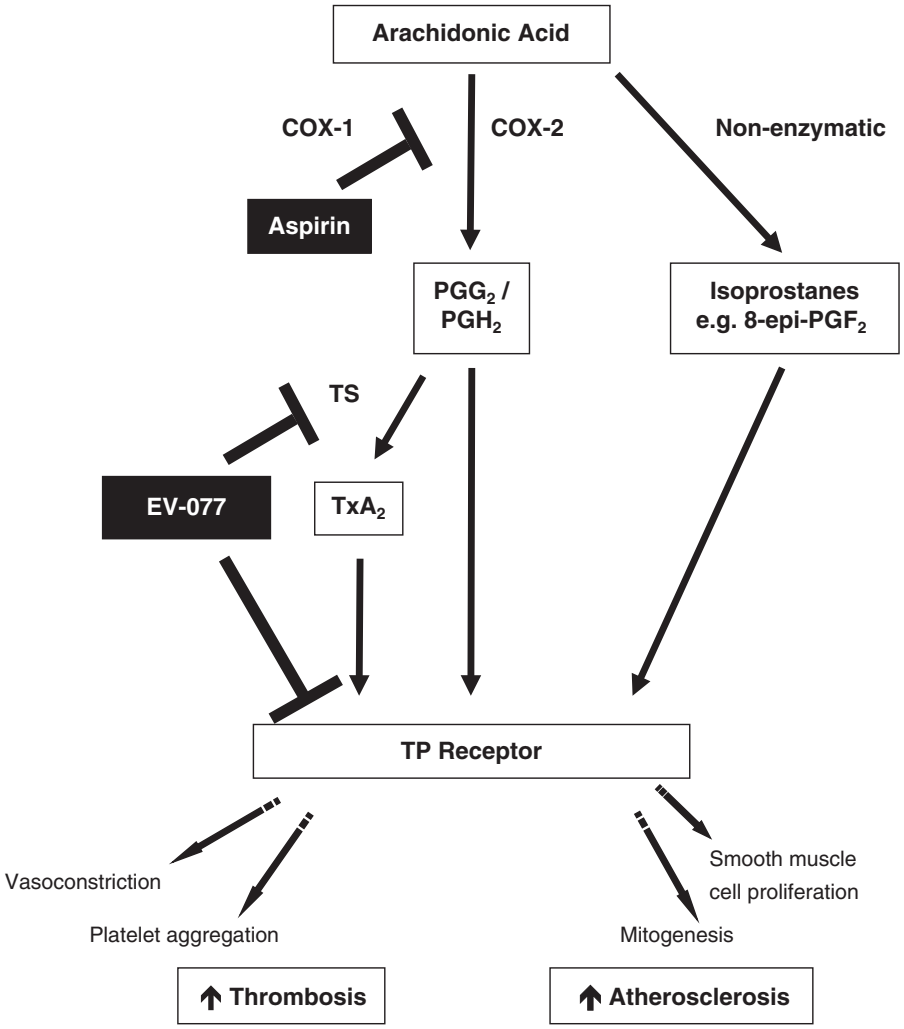

Fig. 4. Arachidonic acid cascade of metabolites, which interact with the TP receptor on platelets, monocytes/macrophages, endothelial cells and vascular smooth muscle cells. Pathological consequences of these interactions are indicated. The effect of TxA_2 on platelets (*via* the TP receptor) can be mimicked *in vitro* by the stable analogue U-46619. Thromboxane A_2 synthase (TS), thromboxane receptor (TP).

reason to anticipate that vascular inflammation and oxidative stress may contribute to increased platelet activation and aggregation in patients with diabetes through the corresponding increased formation of prostanoids, isoprostanes and HETEs [1,3,4,6,7,12]. The biological action of prostanoids and isoprostanes interacting with TP receptor is inhibited by EV-077, whereas its potential inhibition of HETEs has not yet been investigated.

The demonstration that arachidonic acid-induced platelet aggregation in whole blood of diabetic patients is not completely inhibited by aspirin is not new [41]. This residual platelet aggregation in whole blood may be due to the production and translocation of eicosanoids from other blood cells, particularly prostanoids and isoprostanes, which can by-pass COX inhibition at this relatively low dose of daily 100 mg aspirin and subsequently activate the TP receptor and the platelet aggregation. This observation suggests that inhibition of these TP receptor agonists by EV-077 may have a clinical benefit in patients with type 2 diabetes.

EV-077 was also shown to be a potent antagonist of isoprostane contraction of human umbilical arteries. The present results demonstrate that EV-077 produced a dose-dependent rightward displacement of 8-iso-PGE_2 concentration-response curves without affecting the maximal response. 8-iso-PGE_2 has previously been shown to induce vasoconstriction of the human umbilical artery by activation of the TP receptor since a selective TP receptor antagonist caused a parallel rightward shift in the concentration-response curve to 8-iso-PGE_2 in a similar manner as to the selective TP reference agonist U-46619 [5]. Consequently, the present results show that EV-077 is a potent, competitive, and reversible antagonist of the TP receptor in the human umbilical artery that completely inhibits the effect of isoprostanes.

Conclusions

Patients with type-2 diabetes and CAD were found to still have significant arachidonic acid-induced platelet aggregation in whole blood that had not been blocked by the aspirin treatment. The inhibitory effect of EV-077 *in vitro* on arachidonic acid-induced platelet aggregation on top of aspirin therapy in whole blood from these diabetics suggests that EV-077 may potentiate the effects of aspirin *in vivo*. A clinical study with patients suffering from type-2 diabetes is necessary to substantiate this effect of EV-077. Such a study may further show additional beneficial effects of EV-077 on various diabetic vascular complications where prostanoids and isoprostanes are involved [42,43].

Conflict of Interest Statement

Evolva provided financial support for the study. K.S. Sakariassen is a member of the cardiovascular advisory board of Evolva. P. Alberts, J.-P. Meyer, and A. Santana Sorensen are employees of Evolva.

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