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Evaluation of the effects of anti-thromboxane agents in platelet–vessel wall interaction

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Abstract

We evaluated the capacity of anti-aggregating agents to influence thromboxane A_2 and prostacyclin formation, arachidonic acid-endoperoxide redirection, platelet aggregation and vessel tone, in isolated rabbit aorta incubated with homologous platelets. Picotamide (*N*,*N*′ bis(3-pyridinylmethyl)-4-methoxy-isophthalamide), the only dual thromboxane A_2-synthase inhibitor/receptor antagonist in clinical use, inhibited arachidonic acid-induced platelet aggregation with low potency, increased 180-fold by aorta presence. It inhibited thromboxane A_2 formation in platelets and, in aorta presence, increased prostacyclin formation. Ozagrel (OKY-046, (*E*)-3-(4-(1-imidazolylmethyl)phenyl)-2-propenoic acid), a pure thromboxane A_2-synthase inhibitor, behaved similarly to picotamide, although the aorta caused a higher (600-fold) shift. The potency of the antagonist SQ 29,548 (1*S*-(1α,2β(5*Z*),3β,4α))-7-(3((2-((phenylamino)carbonyl)hydrazino)methyl)-7-oxabicyclo(2.2.1)hept-2-yl)-5-heptenoic acid) was unaffected by aorta. In coincubation experiments, arachidonic acid-challenge increased thromboxane A_2-dependent vessel tone; picotamide increased prostacyclin and reduced thromboxane A_2 formation and vasoconstriction. Ozagrel mimicked picotamide; aspirin (acetylsalicylic acid) reduced aorta contractility, thromboxane A_2 and prostacyclin formation. SQ 29,548 reduced vasoconstriction without affecting eicosanoids. We demonstrate the importance of redirection of eicosanoids in the mechanism of action of thromboxane A_2 inhibitors/antagonists within platelet–vascular wall interactions. These findings bear relevance in the development of novel anti-thrombotic drugs.

Keywords: Thromboxane A_2; Prostacyclin; Platelet; Vascular tissue; Picotamide; (Rabbit)

1. Introduction

Arachidonic acid metabolites play a fundamental role in the homeostasis of platelets and vasculature and in their interactions by exerting important and opposing actions on platelet aggregation and on vascular tone. In particular, thromboxane A_2, which is generated in platelets (Hamberg et al., 1975), acts as a very potent platelet aggregating agent and a potent vasoconstrictor (Whittle and Moncada, 1983); on the other hand, prostacyclin is formed by the vascular wall (Moncada et al., 1976), mainly by the endothelial cells, and is able to potently inhibit platelet aggregation and induce vasorelaxation (Whittle and Moncada, 1983). Furthermore, more recent studies have pointed out a significant stimulatory role of thromboxane in the proliferation of vascular smooth muscle cells (Sachinidis et al., 1995), and an opposed inhibitory role of prostacyclin (Jones et al., 1995), as well as new synthetic pathway of these eicosanoids via cyclooxygenase-2 in blood cells and in the arterial wall (Baker et al., 1999; Maclouf et al., 1998). This pathway cannot be controlled by a pure inhibitor of cyclooxygenase-1, such as aspirin (acetylsalicylic acid). Under the light of these findings, the interaction between platelets and the vascular wall, as well as the pharmacological interventions focused on eicosanoids synthesis and action, seems to deserve new investigations.

Blood cells and vessel wall cells cooperate in the biosynthesis of eicosanoids (Maclouf et al., 1998); for instance, the endoperoxides prostaglandin G_2 and prostaglandin H_2, precursors of both prostacyclin and thromboxane A_2, can be transferred from platelets to endothelial cells where they are converted into prostacyclin (Bunting et al., 1976; Marcus et al., 1980). Thus, the cellular cooperation, which occurs in vivo (Nowak and FitzGerald, 1989), but can also be observed under appropriate experimental conditions in vitro

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(Korbut et al., 1990), shifts the equilibrium between prostacyclin and thromboxane A_2 and therefore affects both platelet aggregation and vessel tone.

Anti-thromboxane A_2 agents, either inhibitors of the biosynthesis or receptor antagonists, have been proposed as anti-aggregating and anti-thrombotic drugs; the development of dual thromboxane A_2 synthase inhibitor/receptor antagonist was also suggested (Gresele et al., 1991). In vitro studies of anti-thromboxane A_2 agents, however, have been performed mainly in isolated systems, such as platelet-rich plasma or washed platelets, where the lack of cellular cooperation might bring about a misjudgment of the drug activity. Picotamide (*N*,*N*′ bis(3-pyridinylmethyl)-4-methoxy-isophthalamide) is the only dual thromboxane A_2 synthase inhibitor/receptor antagonist (Gresele et al., 1989) in clinical use (Modesti, 1995). The drug was recently shown to inhibit in vitro proliferation of arterial myocites stimulated by the combination of stable thromboxane A_2 analogue U46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostagladin $F_{2\alpha}$) and platelet-derived growth factor (Ratti et al., 1998). Furthermore, it slowed the evolution of early carotid lesions in a controlled study in diabetic patients (Cocozza et al., 1995).

On the basis of these considerations, we have compared the activity of picotamide with two drugs that represent useful pharmacological standards in their categories: SQ 29,548 (1*S*-(1α,2β(5*Z*),3β,4α))-7-(3((2-((phenylamino)carbonyl)hydrazino)methyl)-7-oxabicyclo(2.2.1)hept-2-yl)-5-heptenoic acid), which is a competitive thromboxane A_2 receptor antagonist (Darius et al., 1985), and ozagrel (OKY-046, (*E*)-3-(4-(1-imidazolylmethyl)phenyl)-2-propenoic acid hydrocloride monohydrate), which is a specific inhibitor of thromboxane A_2 synthase (Naito et al., 1983). We have used two novel experimental systems: inhibition of arachidonic acid-induced aggregation was evaluated in rabbit washed-platelets in the presence of a ring of rabbit aorta; conversely, inhibition of arachidonic acid-triggered increase in vascular tone was assessed in a segment of intact isolated rabbit aorta in the presence of homologous platelets. The data reported provide a unique set of information monitoring, within the same experimental setting, vascular tone and reactivity, platelet-derived thromboxane A_2 and vascular endothelial cell-derived prostacyclin formation, the biochemical interactions underlying the cell–cell cross-talk and the effect of different pharmacological treatments.

2. Materials and methods

2.1. Materials

Zoletil 20 (tiletamine and zolazepam) was from Virbac (Milan, Italy); norepinephrine bitartrate salt, acetylcholine chloride and arachidonic acid sodium salt were from Sigma (St. Louis, MO, USA). Arachidonic acid was dissolved in absolute ethanol and then diluted in ultrapure water (MilliQ) just before the experiment; norepinephrine and acetylcholine were dissolved in ultrapure water. SQ 29,548 (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in absolute ethanol; picotamide monohydrate (kindly provided by Novartis, Origgio, Italy) was dissolved as the dihydrochloride and then diluted in ultrapure water; ozagrel (kindly provided by Kissei Pharmaceutical, Nagano, Japan) was dissolved in Tyrode-G-Ca-HEPES (see below). Acetylsalicylic acid, EDTA, indomethacin and inorganic salts were from Sigma; gelatin powder was from Merck (Darmstad, Germany). Ultrapure water (MilliQ) was from Millipore (Bedford, Ma, USA). Thromboxane B_2 and 6-keto prostaglandin $F_{1\alpha}$ EIA kits were from Cayman Chemical.

2.2. Blood collection and aorta isolation

The use of experimental animals adhered to the European Community guidelines. New Zealand male rabbits (Harlan Italy, Milan, Italy) weighing 2–2.5 kg were anesthetized (1 ml kg^{-1} Zoletil 20), the left carotid was isolated and cannulated for blood collection. Eighty milliliters of blood was diluted with 2 ml of 200 mM EDTA and then the rabbits were sacrificed by complete bleeding. The aorta was carefully removed to avoid damage of the endothelial lining and cleared of fat and connective tissue. Part of the thoracic tract was cut into 2–3-mm-wide transverse rings to test endothelium integrity and addition during platelet aggregation experiments, while a 3–4-cm thoracic-abdominal segment was cut for contractility studies.

2.3. Washed platelet preparation

Rabbit blood, anti-coagulated with EDTA, was centrifuged at 380 × *g* for 20 min at 20 °C to obtain platelet-rich plasma, which was then centrifuged at 1800 × *g* for 20 min at 20 °C to precipitate platelets. The pellet was carefully resuspended in Tyrode-G-EGTA (2.5 mM KCl, 1 mM $MgCl_2$, 120 mM NaCl, 25 mM $NaHCO_3$, 5 mM glucose, 0.25% gelatin, 0.2 mM EGTA; pH 6.5) and centrifuged under the same conditions (Bossant et al., 1990). The platelet pellet was finally resuspended in Tyrode-G-Ca-HEPES (2.5 mM KCl, 1 mM $MgCl_2$, 120 mM NaCl, 25 mM $NaHCO_3$, 5 mM glucose, 0.25% gelatin, 0.9 mM $CaCl_2$, 4.2 mM HEPES; pH 7.4) and the platelet count was adjusted to 450,000 cells μl^{-1} with Tyrode-G-Ca-HEPES.

2.4. Platelet aggregation study

Platelet aggregation was studied using the Born turbidimetric technique in a dual channel Elvi 840 aggregometer (Elvi Logos, Milan, Italy). Base-line signal (10% light transmission) was set using Tyrode-G-Ca-HEPES containing 22,500 cells μl^{-1}. Before aggregation experiments, the aortic rings were preincubated in gassed (O_2 95%, CO_2 5%) Tyrode-Ca-HEPES (same composition of Tyrode-G-Ca-HEPES but without gelatin) containing 1 mM acetylsali-

cylic acid for 30 min at room temperature to inhibit endothelial cell cyclooxygenase activity non-reversibly and then washed twice in Tyrode-G-Ca-HEPES.

Aliquots (250 μl) of washed platelets, in the presence or absence of aortic ring, were preincubated for 2 min at 37 °C under stirring and further preincubated for 3 min with drugs or their vehicles before challenge with the indicated concentration of arachidonic acid. Six minutes after challenge, the aggregatory reaction was stopped by adding 10 μM indomethacin and 7.6 mM EDTA. The platelet suspension was then centrifuged at $11,600 \times g$ for 5 min at room temperature. The supernatant was divided into two parts and kept at −20 °C until enzyme immunoassay (EIA) of arachidonic acid metabolites. The extent of aggregation was quantified as the area under the aggregation curve from 0 to 6 min after arachidonic acid stimulation, and expressed as weight of paper of uniform density (mg).

2.5. Control of endothelium integrity

Four aortic rings were joined together by surgical silk to form a chain and placed in a glass organ bath containing buffer Tyrode-Ca-HEPES at 37 °C, constantly oxygenated with a mixture of 95% O_2 and 5% CO_2. After an equilibration period of 1 h, under a basal tension of 2 g, changes in isometric contraction were monitored by a force transducer (mod. 7004 Basile, Varese, Italy) connected to a pen recorder (Gemini 7070 Basile). Responses to an endothelium-dependent vasodilating agent such as acetylcholine (3 μM) were tested following the enhancement of vascular tone with a submaximal (1 μM) concentration of norepinephrine, in order to verify the endothelium integrity. Vessels that gave a relaxation lower than 50% were not used.

2.6. Rabbit aorta contractility

By means of a silk surgical suture tied to its abdominal end, a 3–4-cm aortic segment was reversed inside-out, in order to achieve optimal exposure of the endothelial lining to the incubation medium in the isolated organ chamber.

The vascular segment, with the endothelial lining exposed, was suspended in a plastic organ chamber to a isotonic force transducer (mod. 7006, Basile) that transmits the vessel stretching due to vasoconstriction. A 2-g tension was given and vessel equilibrated for 2 h in an oxygenated buffer (Tyrode-Ca-HEPES). Norepinephrine (0.1 μM) and acetylcholine (3 μM) were administered to test contractility and the presence of a functionally intact endothelium, respectively; the entire chamber volume was then substituted with the washed platelet suspension (2.5 ml, 450,000 cells μl^{-1}), pretreated for 15 min with the drugs under test or with vehicle. Arachidonic acid (12 μM) was added 15 min later to stimulate thromboxane formation from platelets that caused vascular contraction (30 min total platelet-drug incubation). Vessel response is expressed as percent of norepinephrine-induced contraction. Aliquots of the incubation suspension (200 μl) were collected both before and 15 min after arachidonic acid-challenge for EIA quantification.

2.7. Enzyme immunoassay

The stable metabolites of thromboxane A_2 and prostacyclin (thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$, respectively) were evaluated by selective EIA (Pradelles et al., 1985), carried out directly on aliquots of the incubation media, diluted in EIA buffer (0.1 M phosphate buffer pH 7.4 containing 0.1% w v^{-1} BSA, 0.01% w v^{-1} NaN_2, 0.4 M NaCl, 1 mM EDTA). The assay was performed in a 96-microwell plate coated with an anti-rabbit immunoglobuline G (anti-rabbit IgG). The amount of eicosanoids was measured spectrophotometrically at 412 nm (Autoreader SPECTRA, Cayman). Results were analyzed and referred to a standard curve (thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ 1.35–0.01 pmol ml^{-1}).

2.8. Data analysis

Data are expressed as mean ± S.E.M.. The concentration–response curves of platelet aggregation were analysed and drawn by means of the computer programme ALLFIT (De Lean et al., 1978) and evaluation of the statistical significance of the parameter difference was based on the F test for the extra sum of square principle (Draper and Smith, 1966). Statistical evaluation of the data was carried out by analysis of variance (one ANOVA or ANOVA repeated measure with one grouping factor, as indicated); $P < 0.05$ was considered statistically significant.

3. Results

3.1. Platelet response

Arachidonic acid caused rabbit platelet aggregation in a concentration-dependent manner, when the area under the aggregation curve (from 0 to 6 min) was assessed (Fig. 1A,C), while the maximal extent of aggregation was not greatly affected by increasing concentrations of arachidonic acid (Fig. 1A). The concentration-dependency was maintained also in the presence of an aortic ring, although the response was less sustained and therefore the area under the curve was significantly lower (Fig. 1B,C). Arachidonic acid showed a similar potency in both experimental situations with EC_{50} values of 0.6 ± 0.1 and 1.0 ± 0.1 μM, with and without an aortic ring, respectively, while the upper plateaus of the concentration–response curves were significantly different ($P < 0.05$).

Arachidonic acid-induced aggregation was accompanied by synthesis of thromboxane A_2 (assessed as thromboxane B_2) both in the presence and in the absence of an aortic ring (Fig. 1D). The concentration-dependent thromboxane B_2 formation was reduced by the presence of the vascular tissue

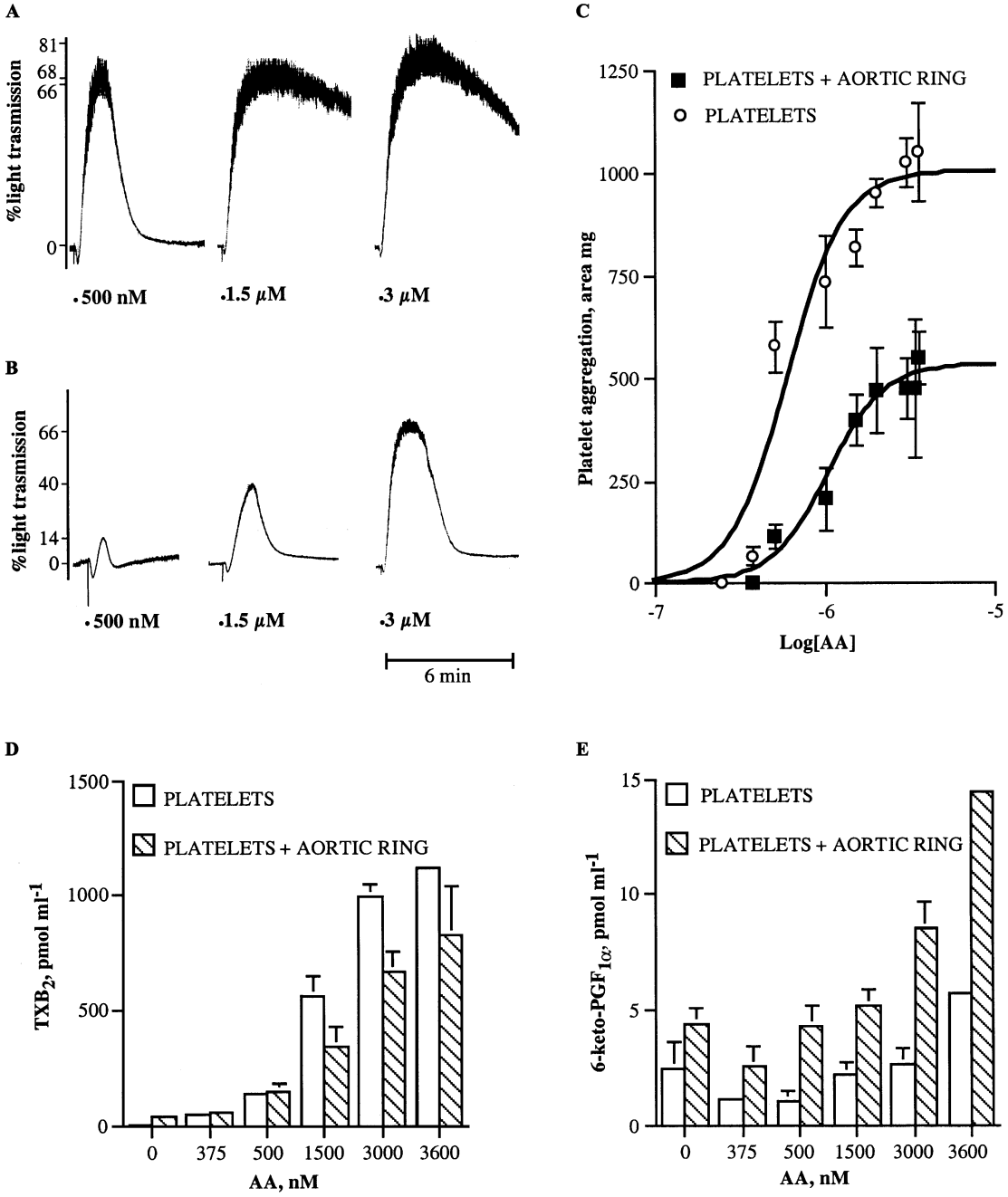

Fig. 1. Panel A: Representative tracing of platelet aggregation after challenge with 500 nM–3 μM arachidonic acid (AA) in platelets alone or Panel B: in the presence of the aortic ring (preincubated with 1 mM acetylsalicylic acid, ASA, for 30 min). Panel C: concentration–response curves of arachidonic acid-induced aggregation in the absence (open circle) or in the presence (closed square) of aortic ring. Aggregation is assessed as the area under the aggregation curve in the first 6 min after arachidonic acid stimulation and expressed as mg of paper (see Methods). The difference between the upper plateaus of the two curves was statistically significant ($P<0.05$). Panel D–E: concentration–response curves for arachidonic acid-induced thromboxane B_2 (D) and 6-keto-prostaglandin $F_{1\alpha}$ (E) formation in rabbit platelets in the absence or in the presence of aortic ring. Both thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ production was statistically different in the presence of aortic ring vs. its absence (two-way ANOVA followed by Bonferroni's test, $P<0.01$). Thromboxane B_2 formation in the presence or in the absence of aortic ring and 6-keto-prostaglandin $F_{1\alpha}$ formation in the presence of aortic ring were concentration-dependent (one-way ANOVA followed by Bonferroni's test, $P<0.01$). Data are expressed as mean ± S.E.M.; $n=3–15$.

($P<0.01$) and was coupled to a concentration-dependent increase in prostacyclin formation (assessed as 6-keto-prostaglandin $F_{1\alpha}$, Fig. 1E, $P<0.01$ vs. platelets alone).

Anti-thromboxane drugs were evaluated in platelets stimulated with a sub-maximal aggregating concentration of arachidonic acid (1.5–3 μM). Fig. 2A shows that picotamide concentration-dependently inhibited platelet aggregation, being much more potent in the presence ($IC_{50}=10.6\pm1.9$ μM, $IC_{25}=1.7\pm0.2$ μM) than in the absence ($IC_{25}=305\pm166$ μM) of the aortic ring. The range

of picotamide concentrations was restricted by the acidity of the solution medium (>0.06% HCl), and therefore as 300 μM was the highest concentration tested in platelets alone, a complete concentration–response curve was not possible. As expected, picotamide also inhibited thromboxane B_2 formation, but this was not greatly affected by the presence of the vascular tissue (Fig. 2B). Conversely, picotamide was able to stimulate 6-keto-prostaglandin $F_{1\alpha}$ formation in a concentration-dependent way ($P<0.01$) only when the vascular ring was present, but not when it was absent.

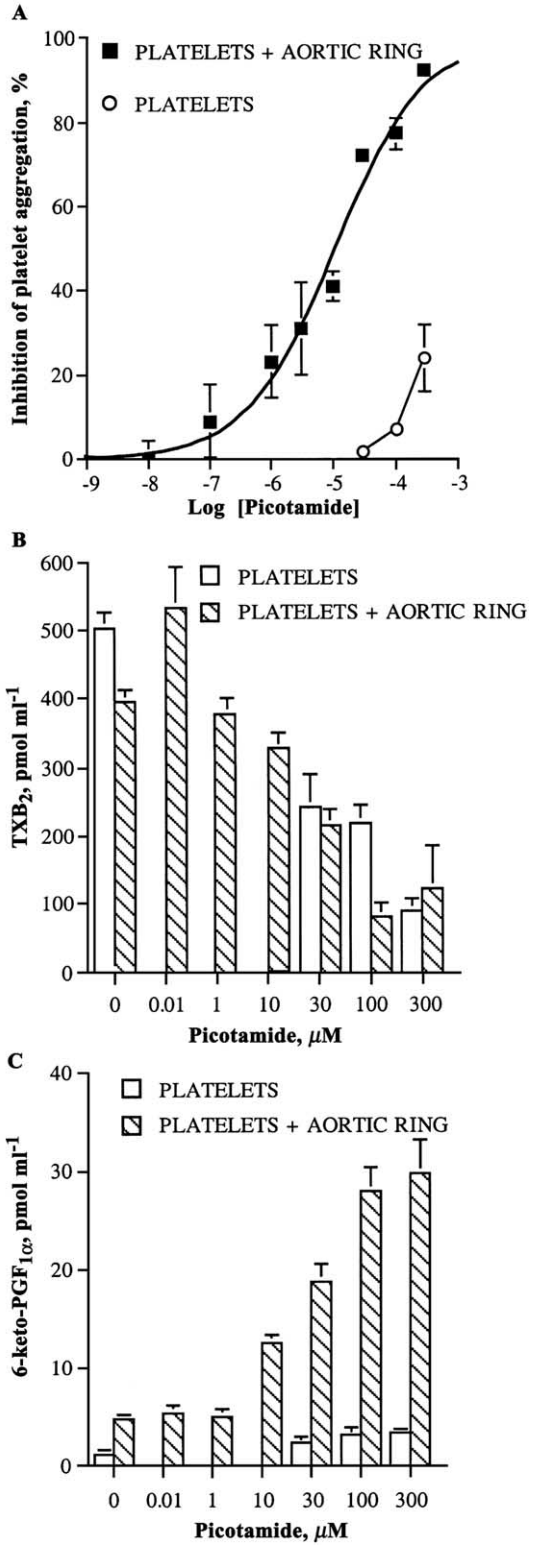

Fig. 2. Panel A: Concentration–response curves of picotamide-induced inhibition of platelet aggregation after challenge with sub-maximal arachidonic acid (AA) concentration (1.5–3 μM) in the absence (open circle) or in the presence (closed square) of aortic ring. The difference between the IC_{25} of the two curves was statistically significant ($P<0.05$). Panel B–C: effect of picotamide (3 min) on thromboxane B_2 (B) and 6-keto-prostaglandin $F_{1\alpha}$ (C) formation in rabbit platelets after challenge with sub-maximal arachidonic acid concentration (1.5–3 μM) in the absence or in the presence of aortic ring (preincubated with 1 mM acetylsalicylic acid, ASA, for 30 min). 6-Keto-prostaglandin $F_{1\alpha}$ production was statistically different in the presence of aortic ring vs. its absence (two-way ANOVA followed by Bonferroni's test, $P<0.01$). Thromboxane B_2 formation in the presence or in the absence of aortic ring and 6-keto-prostaglandin $F_{1\alpha}$ formation in the presence of aortic ring were concentration-dependent (one-way ANOVA followed by Bonferroni's test, $P<0.01$). Data are expressed as mean ± S.E.M.; $n=3–6$.

Ozagrel behaved similarly to picotamide in inhibiting platelet aggregation with and without the aortic ring (Fig. 3A), but the separation of the curves was even higher ($IC_{50}=2.5\pm0.5$ μM and 1.5 ± 0.1 mM, respectively). Thromboxane B_2 formation was obviously inhibited (Fig. 3B), and, as for picotamide, 6-keto-prostaglandin $F_{1\alpha}$ formation was stimulated in a concentration-dependent way ($EC_{50}=5.5\pm0.3$ μM, $P<0.01$).

SQ 29,548 inhibited rabbit platelet aggregation completely both in the absence and in the presence of the aortic ring, with the same potency ($IC_{50}=103\pm17$ and 63 ± 16 nM, respectively, Fig. 4). As expected, neither thromboxane B_2 nor 6-keto-prostaglandin $F_{1\alpha}$ synthesis was affected by SQ 29,548 (data not shown).

3.2. Vascular response

The contractile effect of arachidonic acid was tested in the aortic segment with the entire endothelial lining intact, as demonstrated by vascular relaxation ($-70\%\pm4$ vs. norepinephrine contraction) after muscarinic challenge (1 μM acetylcholine, $n=9$).

When vessels were incubated with the washed platelet suspension (450×10^6 cells ml^{-1}) and challenged with 12 μM arachidonic acid, a strong contraction occurred. The incubation of washed platelets and vessel with 300 μM picotamide (30 min) caused a significant reduction of arachidonic acid-induced contraction (-40.6%, $P<0.01$); when platelets were pretreated with aspirin (acetylsalicylic acid, 1 mM), the vascular contraction was reduced as well (-83.0%, $P<0.01$) (Fig. 5). When washed platelets were incubated with either SQ 29,548 (1 μM) or ozagrel (3 mM), a significant decrease in vessel constriction was observed (-65.8% and -73.9%, respectively, $P<0.01$).

Thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ were assessed by EIA in supernatants from platelet suspension

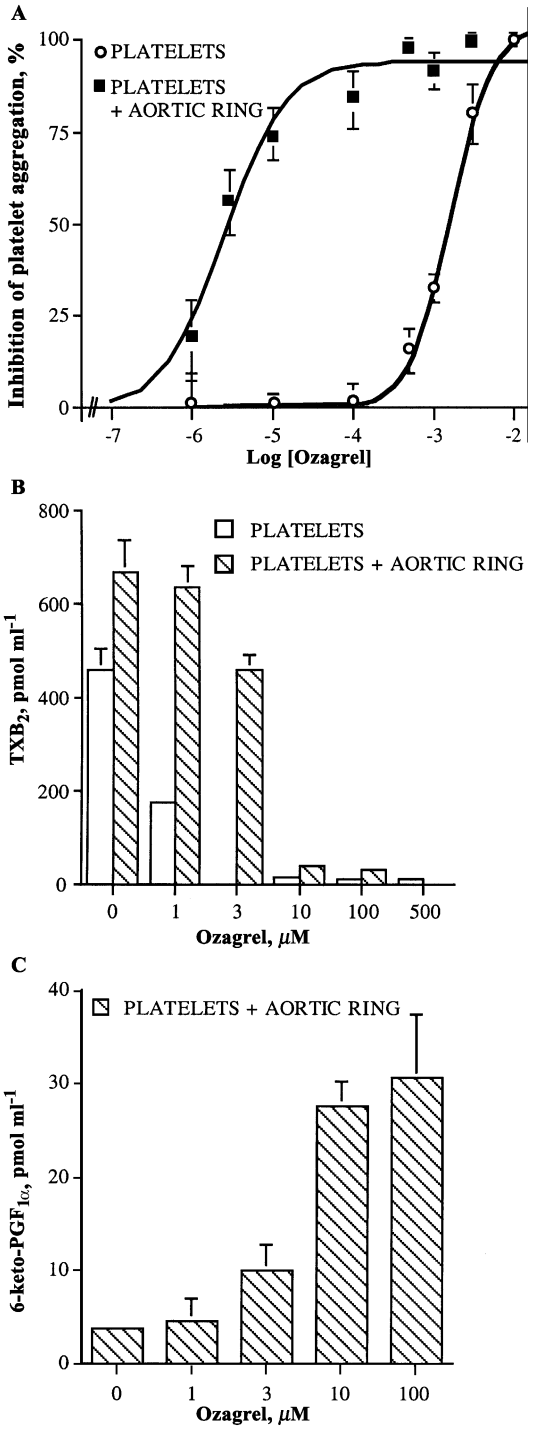

Fig. 3. Panel A: Concentration–response curves of ozagrel-induced inhibition of platelet aggregation after challenge with sub-maximal arachidonic acid concentration (AA, 1.5–3 μM) in the absence (open circle) or in the presence (closed square) of aortic ring. The difference between the IC_{50} of the two curves was statistically significant (ALLFIT, $P<0.01$). Panel B–C: effect of ozagrel (3 min) on thromboxane B_2 (B) and 6-keto-prostaglandin $F_{1\alpha}$ (C) formation in rabbit platelets after challenge with sub-maximal arachidonic acid concentration in the absence or in the presence of aortic ring (preincubated with 1 mM acetylsalicylic acid, ASA, for 30 min). Thromboxane B_2 formation in the presence or in the absence of aortic ring and 6-keto-prostaglandin $F_{1\alpha}$ formation in the presence of aortic ring was concentration-dependent (one-way ANOVA followed by Bonferroni's test, $P<0.01$). Data are expressed as mean ± S.E.M.; $n=3$–4.

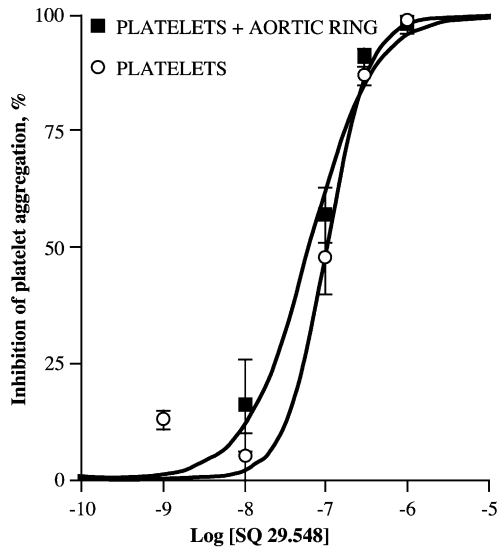

Fig. 4. Concentration–response curves of SQ 29,548-induced inhibition of platelet aggregation after challenge with sub-maximal arachidonic acid concentration (AA, 1.5–3 μM) in the absence (open circle) or in the presence (closed square) of aortic ring. The effect of SQ 29,548 was concentration-dependent ($P<0.05$). Data are expressed as mean ± S.E.M.; $n=3$.

incubated with the vessel. Washed platelets-vessel preparations produced thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ when challenged with 12 μM arachidonic acid (Fig. 6). Picotamide (300 μM) caused a significant decrease (−84.8%, $P<0.01$) of thromboxane B_2 synthesis, with a concomitant increase in 6-keto-prostaglandin $F_{1\alpha}$ level (3.48-fold, $P<0.01$). Incubation with 1 mM acetylsalicylic acid ablated the formation of both metabolites (−99.3%, $P<0.01$ for thromboxane B_2 and −81.4%, $P<0.05$ for 6-keto-prostaglandin $F_{1\alpha}$). Ozagrel (3 mM) incubation with

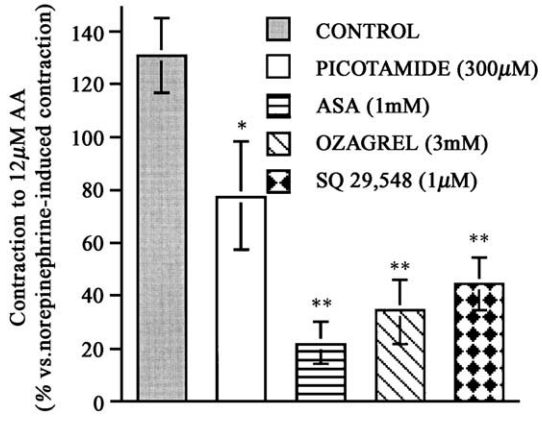

Fig. 5. Contraction in response to 12 μM arachidonic acid (AA) of rabbit aorta incubated with homologous washed platelets ($n=19$) and 300 μM picotamide ($n=9$), 1 mM acetylsalicylic acid (ASA, $n=6$), 3 mM ozagrel ($n=4$), 1 μM SQ 29,548 ($n=6$). All the drugs inhibited significantly the rabbit aorta contraction (ANOVA repeated measure with one grouping factor followed by single contrast, $*P<0.05$, $**P<0.01$). Each column represents the mean value of n experiments ± S.E.M.

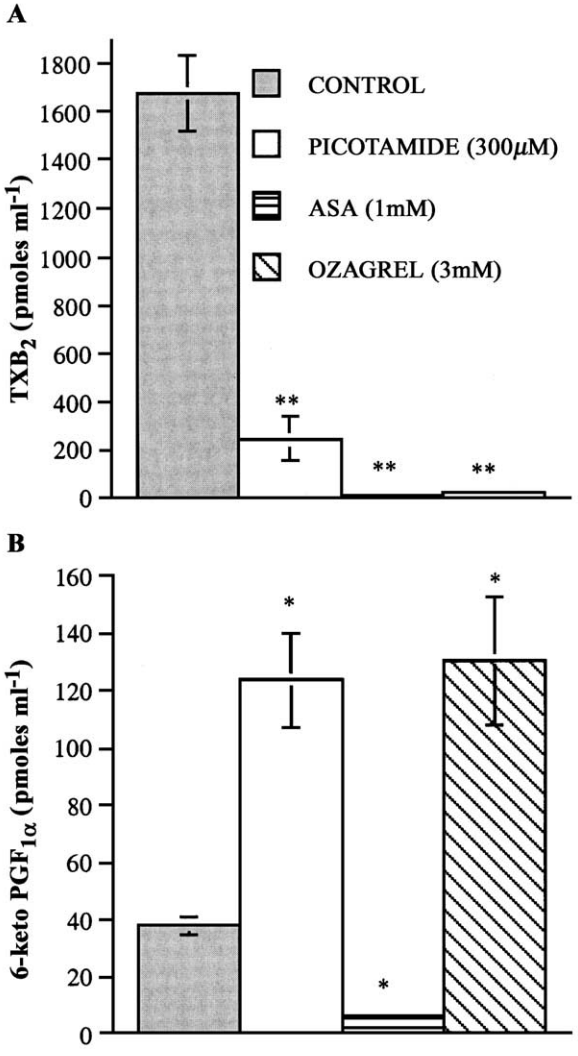

Fig. 6. Thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ production by rabbit aorta incubated with homologous washed platelets and stimulated with 12 μM arachidonic acid (AA). Panel A: effect of 300 μM picotamide ($n=5$), 1 mM acetylsalicylic acid (ASA, $n=5$), 3 mM ozagrel ($n=3$). All the drugs significantly inhibited thromboxane B_2 formation (ANOVA repeated measure with one grouping factor followed by single contrast, $**P<0.01$). Panel B: 6-keto-prostaglandin $F_{1\alpha}$ formation: effect of 300 μM picotamide ($n=3$), 1 mM acetylsalicylic acid ($n=4$), 3 mM ozagrel ($n=3$). Picotamide and ozagrel enhanced, while acetylsalicylic acid inhibited 6-keto-prostaglandin $F_{1\alpha}$ formation significantly (ANOVA repeated measure with one grouping factor followed by single contrast, $*P<0.05$). Each column represents the mean value of n experiments ± S.E.M.

washed platelets caused a marked thromboxane B_2 drop (-98.8%, $P<0.01$) and 6-keto-prostaglandin $F_{1\alpha}$ increase (4.10-fold, $P<0.05$) (Fig. 6). SQ 29,548 did not affect either thromboxane B_2 or 6-keto-prostaglandin $F_{1\alpha}$ formation (data not shown).

4. Discussion

The importance of platelet activation stems not only from the ability of platelet aggregates to start thrombus formation, but also from the complex interaction of platelets and vascular wall. On one hand, platelet-derived metabolites such as thromboxane A_2 can trigger vasospasms (Kalsner, 1995), on the other hand, endothelium can release mediators which control platelet activation and vascular reactivity, such as prostacyclin (Kita et al., 1997; Radomski et al., 1987; Vanhoutte and Mombouli, 1996).

Because thromboxane A_2 and prostacyclin have opposite effects on platelets, the pharmacological modulation of their ratio, rather than the inhibition of cyclooxygenase, may have important therapeutic implications. Thus, inhibition of thromboxane A_2-synthase has been considered an interesting approach to the pharmacological control of thrombotic disorders, as it abolishes the formation of prothrombotic thromboxane A_2, but not that of anti-thrombotic prostacyclin. However, this approach has not yielded satisfactory results, mainly because the precursor endoperoxides prostaglandin G_2 and prostaglandin H_2, which mimic thromboxane A_2 effects, accumulate when their metabolism to thromboxane is inhibited (Gresele et al., 1991). For this reason, thromboxane A_2 receptor antagonists, such as SQ 29,548, and dual antagonist/inhibitors, such as picotamide, have been proposed and assessed both in vitro and in vivo.

The method we have set up allows the evaluation of the anti-thromboxane A_2 drugs in two related models (both involving the coincubation of washed platelets and aorta from the same rabbit), where the interaction of platelets and vascular wall is taken into account, from both the functional and the biosynthetic point of view. In the contractility studies, the use of an entire vessel segment instead of joined rings was found to be a good tool to avoid excessive platelets activation/thromboxane A_2 production due to vascular cross-section. We assessed platelet aggregation, vascular contraction and formation of thromboxane A_2 and prostacyclin. The presence of functional endothelium was routinely checked in our vascular preparations. A similar method of platelet aggregation had been previously described (Terashita et al., 1996), but it had the drawback that platelets and aorta were from different species (rabbit and rat); furthermore, only a single thromboxane A_2-synthase inhibitor was tested and vascular response was not evaluated.

We demonstrate here that the potency of thromboxane A_2-synthase inhibitors and dual inhibitor/antagonist on platelet aggregation is greatly increased by the presence of vascular wall.

Arachidonic acid was chosen as a trigger for both platelet aggregation and vascular contraction for two reasons: (1) arachidonic acid-induced platelet aggregation is exquisitely thromboxane-dependent (contrary to thrombin, which only partially depends on thromboxane A_2); (2) at variance with other stimuli (e.g. collagen), arachidonic acid is fully active also on washed platelets. The second point is important inasmuch as the evaluation of vascular tone, which we conducted in parallel with platelet aggregation, could not

be performed in the presence of platelet-rich plasma; indeed, platelet-rich plasma oxygenation generated an abundant foam which disturbed the recording of smooth muscle tone and contractility. Analysis of the curve for arachidonic acid on platelet aggregation revealed that, with increasing concentrations of the stimulus, the extent of the response was not greatly increased, while the aggregation was more slowly reversible; therefore, the area under the aggregation curve, rather than the height of the peak, was chosen to quantify the response in all the experiments.

The decrease in arachidonic acid efficacy observed in the presence of the aortic ring can be explained by the diminished thromboxane A_2 and augmented prostacyclin formation, brought about by the redirection of the substrate (arachidonic acid and/or endoperoxides) from the platelets to the endothelial lining of the vascular tissue.

The evaluation of the pure thromboxane A_2 antagonist SQ 29,548 indicated that, as expected (Darius et al., 1985; Ogletree et al., 1985), it is active on platelet aggregation, but its curve is unaffected by the presence of the aortic ring. Its effect on the aortic segment confirms that arachidonic acid-induced contraction depends on thromboxane A_2, at least partially, in agreement with the results obtained with acetylsalicylic acid.

More interestingly, the anti-aggregating behaviour of both the thromboxane A_2-synthase inhibitor (ozagrel) and of the dual inhibitor/antagonist (picotamide) is markedly affected by the presence of the aortic ring: the potency of both compounds was significantly higher when the vascular tissue was present. The expected inhibition of thromboxane A_2 formation was accompanied by a concentration-dependent increase in prostacyclin formation only when the aortic ring was present. This phenomenon can be explained by eicosanoid transcellular metabolism (Marcus et al., 1980): the inhibition of thromboxane A_2-synthase brings about the accumulation in platelets of the prostaglandin endoperoxides, which, in turn, can be taken up by the endothelial cells and metabolized to prostacyclin.

The shift of the curves for inhibition of aggregation was more marked for ozagrel (600-fold) than for picotamide (180-fold, based on IC_{25} comparison). It could be hypothesized that the shift-difference is due to the different mechanism of action of the two compounds: the anti-aggregatory effect of picotamide is the result of thromboxane A_2 receptor antagonism and thromboxane A_2-synthase inhibition, but only the latter is influenced by the presence of vascular tissue (as confirmed by the results obtained with SQ 29,548). On the contrary, the whole effect of ozagrel, pure thromboxane A_2-synthase inhibitor, is affected by the coincubation of platelets with the aortic ring.

It might be interesting to point out that although picotamide is approximately equipotent with ozagrel in inhibiting platelet aggregation (based on IC_{25} comparison), the two compounds have a different profile when the inhibition of thromboxane formation is considered: ozagrel almost completely suppresses thromboxane A_2 synthase activity at concentrations (10–100 μM) at which it does not elicit any effect on platelet aggregation, while this discrepancy does not occur with picotamide. This apparent paradox might be explained considering that, when thromboxane A_2 formation is inhibited, prostaglandin endoperoxides accumulate and trigger platelet aggregation by binding to the thromboxane TP receptor (Hamberg et al., 1974); at variance with ozagrel, picotamide, which also acts as a TP receptor antagonist, is able to block the pro-aggregating effect both of the residual thromboxane A_2 and of the endoperoxides.

Interestingly, anti-thromboxane drugs are able to reduce the vasospasm that might occur upon platelet activation. Indeed, the contraction of rabbit aorta induced by arachidonic acid in the presence of homologous platelets is almost completely dependent on contractile eicosanoids, among which thromboxane A_2 is the most potent, as demonstrated by the effect of acetylsalicylic acid; in this experimental model, picotamide, ozagrel and SQ 29,548 all inhibit contraction at least partially at the concentrations tested. In the case of picotamide and ozagrel, diminished vascular contraction could be due to the expected inhibition of thromboxane A_2, which is accompanied by a marked increase in the formation of prostacyclin through the mechanism of transcellular metabolism, as previously discussed.

In conclusion, we have demonstrated that our experimental approach, being closer to a physiological condition than isolated platelets, allows a more correct evaluation of the in vitro potency of inhibitors of thromboxane A_2 synthase and dual inhibitors/antagonists. Indeed, our results explain why anti-aggregating drugs, such as picotamide and ozagrel, which were shown to be potent in vivo, did not display a comparable potency when tested in vitro on isolated platelets (Gresele et al., 1991). Our investigation also points out the importance of redirection of eicosanoids substrates and their transcellular metabolism in the complex interactions between platelets and vascular wall. These findings might bear relevance for the development of novel anti-thrombotic drugs.

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