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Short communication

Effects of terbogrel on platelet function and prostaglandin endoperoxide transfer

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Abstract

The present study describes the platelet-inhibitory effects of terbogrel (5-hexenoic acid, 6-[3-[[(cyanoamino)[(1,1-dimethylethyl)amino]methylene]amino]phenyl]-6-(3-pyridinyl)-, (ε)-), a novel combined thromboxane A_2 synthase inhibitor and thromboxane A_2 receptor antagonist. Terbogrel concentration-dependently inhibited collagen (0.6 μg/ml)- and U46619 (11α,9α-epoxymethano-15(*S*)-hydroxy-prosta-5*Z*,13*E*-dienoic acid) (1 μM)-induced aggregation and thromboxane synthesis of washed human platelets. In this system, terbogrel exhibited an equipotent (IC_{50} of about 10 nM) activity as thromboxane A_2 synthase inhibitor and thromboxane A_2 receptor antagonist. In addition, the compound favoured prostacyclin synthesis in cultured vascular smooth muscle cells by increasing the transfer of platelet-derived prostaglandin endoperoxides. Terbogrel appears to be a compound with an equipotent molar potency as thromboxane A_2 synthase inhibitor and receptor antagonist. © 1998 Elsevier Science B.V.

Keywords: Thromboxane A_2; Prostacyclin; Thromboxane A_2 synthase inhibition; Thromboxane A_2 receptor antagonism

1. Introduction

Antiplatelet therapy is useful in the secondary prevention of vascular complications of atherosclerotic diseases (Antiplatelet Trialists' Collaboration, 1994). The reference compound is acetylsalicylic acid that suppresses platelet-dependent thromboxane A_2 formation by nonselective inhibition of the constitutive cyclooxygenase (reviewed in the work of Schrör, 1997). In order to establish more specific therapeutic alternatives to acetylsalicylic acid, thromboxane A_2 receptor antagonists and thromboxane A_2 synthase inhibitors have been developed (reviewed in the work of Catella-Lawson and FitzGerald, 1997). In comparison to acetylsalicylic acid, thromboxane A_2 synthase inhibitors have the advantage of increasing the formation of vascular prostacyclin synthesis via prostaglandin endoperoxide transfer (Bunting et al., 1976; Marcus et al., 1980). However, the use of thromboxane A_2 synthase inhibitors is limited by platelet stimulatory actions of prostaglandin endoperoxides. Therefore, compounds combining thromboxane A_2 synthase inhibition with thromboxane A_2 receptor antagonism, such as ridogrel, have been developed (reviewed in the work of Vermylen and Deckmyn, 1992). In patients with myocardial infarction, ridogrel was not superior over acetylsalicylic acid (The RAPT Investigators, 1994). A potential explanation for this might be the insufficient potency of ridogrel as a thromboxane A_2 receptor antagonist (Catella-Lawson and FitzGerald, 1997).

In this study, we describe the effects of terbogrel (5-hexenoic acid, 6-[3-[[(cyanoamino)[(1,1-dimethylethyl) amino]methylene]amino]phenyl]-6-(3-pyridinyl)-, (ε)-), a novel combined thromboxane A_2 synthase inhibitor and thromboxane A_2 receptor antagonist on platelet function and prostaglandin endoperoxide transfer between platelets and smooth muscle cells.

2. Materials and methods

2.1. Preparation of washed human platelets

Washed human platelets were prepared as previously described (Weber et al., 1993). Briefly, fresh citrated blood was obtained from healthy volunteers and platelet-rich

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plasma was prepared by centrifugation at $250 \times g$ for 10 min at room temperature. The pH was adjusted to 6.5 with acidic citrate dextrose (Biostabil®, Biotest, Frankfurt, Germany). In some experiments, platelet-rich plasma was incubated for 15 min with acetylsalicylic acid (100 μM). The platelets were washed twice in a buffer (pH 6.5) containing (mM): 113 NaCl, 4 Na_2HPO_4, 24 NaH_2PO_4, 4 KH_2PO_4, 5 glucose, supplemented with 50 nM prostaglandin E_1. Washed platelets were resuspended in HEPES-buffered Tyrode solution (pH 7.4) of the following composition (mM): 134 NaCl, 12 $NaHCO_3$, 2.9 KCl, 0.36 NaH_2PO_4, 1 $MgCl_2$, 5 HEPES, 2 $CaCl_2$, 5 glucose.

2.2. Platelet aggregation and thromboxane formation

Platelet aggregation was measured as previously described (Weber et al., 1993). Briefly, 500 μl of washed platelet suspension were incubated in a two-channel aggregometer (Labor, Hamburg, Germany) for 2 min at 37°C. Platelets were stimulated by adding 10 μl of the respective agonist. In experiments with terbogrel, the compound was added to the platelets 10 min prior to stimulation with the respective agonists. Changes in light transmission were recorded during constant stirring of the samples (1200 rpm, 37°C). At the end of the aggregation period (5 min after addition of the agonist), platelet aggregates were sedimented by centrifugation at $10\,000 \times g$ for 2 min and platelet free supernatants were stored at -20°C until radioimmunoassay for thromboxane B_2 as previously described (Schrör and Seidel, 1988).

2.3. Transcellular prostaglandin endoperoxide metabolism

In order to study prostaglandin endoperoxide transfer from platelets to vascular cells, co-incubation experiments with washed human platelets and cultured bovine coronary artery smooth muscle cells were carried out. Smooth muscle cells were isolated using enzymatic digestion and were cultivated under standard conditions as previously described (Grosser et al., 1996). For the experiments, smooth muscle cells were serum-deprived for 24 h and pre-treated with acetylsalicylic acid (100 μM) for 15 min when indicated. Then, thrombin (0.1 U/ml)-stimulated washed platelets ($100\,000/\mu$l) were co-incubated with smooth muscle cells for 1 h at 37°C. 6-oxo-prostaglandin $F_{1\alpha}$, a stable degradation product of prostacyclin, was measured in cell-free supernatants by radioimmunoassay as previously described (Schrör and Seidel, 1988).

2.4. Substances and solutions

Acetylsalicylic acid (Aspirin®, Bayer, Leverkusen, Germany); acidic citrate dextrose (Biostabil®, Biotest); collagen (Hormon Chemie, München, Germany); thromboxane B_2 (Upjohn Diagnostics, Kalamazoo, MI, USA); [^{3}H]thromboxane B_2 (New England Nuclear, Dreieich, Germany). Terbogrel was kindly provided by Thomae (Biberach, Germany), α-thrombin by Dr. J. Stürzebecher (Erfurt, Germany) and U46619 (11α,9α-epoxymethano-15(S)-hydroxy-prosta-5Z,13E-dienoic acid) by Dr. R. Schmedemann (Upjohn, Heppenheim, Germany).

3. Results

3.1. Platelet aggregation and thromboxane formation

In order to confirm the specificity of terbogrel regarding thromboxane A_2-dependent platelet function, experiments were carried out in nontreated and in acetylsalicyclic acid (100 μM)-treated platelets. Acetylsalicylic acid did not impair thrombin (0.1 U/ml)-induced platelet aggregation despite of almost complete inhibition of thromboxane A_2 formation (120 ± 10 ng/ml vs. 2 ± 1 ng/ml, $n = 4$). This indicates that, under these conditions, thrombin-induced platelet activation was not dependent on endogenous thromboxane A_2 formation. Pretreatment of platelets with terbogrel (1 μM) also completely inhibited thrombin-induced thromboxane A_2 formation (2 ± 1 ng/ml) but did not result in any inhibition of platelet aggregation. These data indicate that terbogrel does not interfere with thromboxane A_2 independent platelet functions.

Platelet aggregation, induced by the stable thromboxane A_2 mimetic U46619 (1 μM), was not inhibited by acetylsalicylic acid, indicating that U46619-induced platelet activation was independent of endogenous thromboxane A_2. However, in contrast to thrombin, U46619-induced platelet

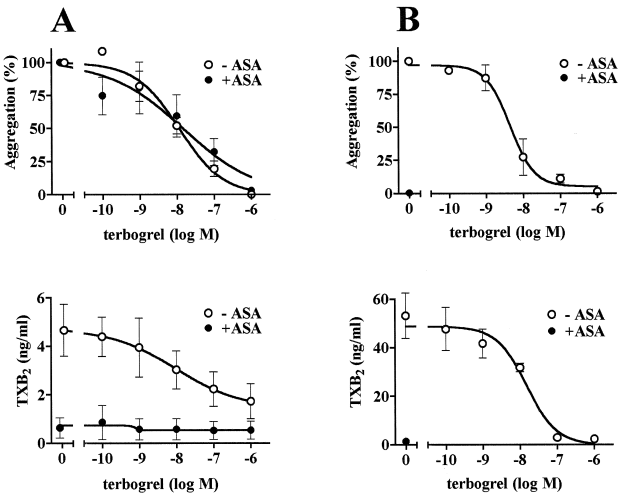

Fig. 1. Effects of terbogrel on aggregation (upper panels) and thromboxane B_2 (TXB_2) formation (lower panels) of washed human platelets stimulated with U46619 (1 μM) (A) or collagen (0.6 μg/ml) (B). Platelets were either untreated (− ASA) or pre-treated with acetylsalicylic acid (100 μM) (+ ASA). Data are mean ± S.E.M. of $n = 4$ independent experiments.

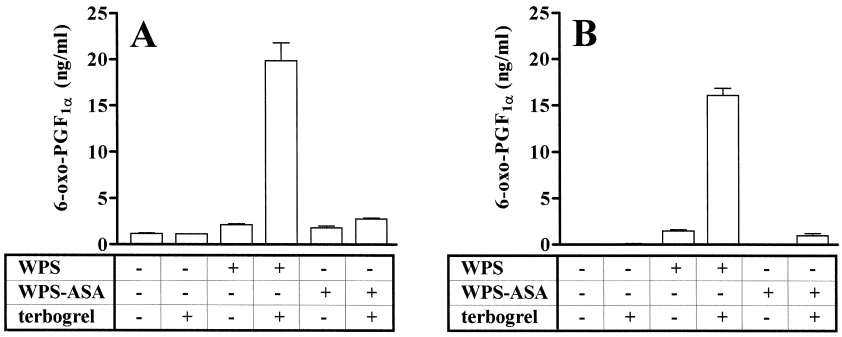

Fig. 2. Effects of terbogrel (1 μM) on 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) release from cultured vascular smooth muscle cells. Smooth muscle cells were either untreated (A) or pre-treated with acetylsalicylic acid (100 μM) (B). Measurements were performed either with smooth muscle cells alone, in co-incubation with untreated washed platelets (WPS), or with washed platelets pre-treated with acetylsalicylic acid (100 μM) (WPS-ASA). Data are mean ± S.E.M. of a typical experiment performed in triplicate wells. Similar results were obtained in $n = 3$ independent experiments.

aggregation was completely inhibited by terbogrel (1 μM), demonstrating the selective thromboxane A_2 receptor antagonistic activity of the compound. The IC_{50} for inhibition of U46619-induced platelet aggregation was about 10 nM. A similar IC_{50} was observed for the inhibition of U46619-induced thromboxane A_2 formation by terbogrel. The data are summarized in Fig. 1A.

In order to study platelet activation that is dependent on thromboxane A_2 synthase activity, the effects of terbogrel on collagen (0.6 μg/ml)-induced platelet aggregation and thromboxane A_2 formation were studied. At this concentration, collagen-induced aggregation was completely dependent on endogenous thromboxane A_2 formation, since no aggregation was observed in acetylsalicylic acid (100 μM)-treated platelets. In this system, terbogrel inhibited both, platelet aggregation and thromboxane A_2 formation with an IC_{50} of about 10 nM. The data are summarized in Fig. 1B.

3.2. Transcellular prostaglandin endoperoxide metabolism

Co-incubation of vascular smooth muscle cells with platelets only slightly (about two-fold) increased basal prostacyclin production. In contrast, a marked (about 20-fold) increase in prostacyclin production was seen when terbogrel (1 μM)-treated platelets were added. This effect was prevented by pre-treatment of platelets with acetylsalicylic acid (100 μM). These data are summarized in Fig. 2A. When smooth muscle cells were pre-treated with acetylsalicylic acid (100 μM), basal prostacyclin production was completely blocked. Interestingly, prostacyclin production was restored by addition of platelets. This effect was much more pronounced when terbogrel (1 μM)-treated platelets were co-incubated with smooth muscle cells. Some prostacyclin generation was observed when acetylsalicylic acid-treated platelets were added in the presence of terbogrel, probably indicating residual platelet cyclooxygenase activity. These data are summarized in Fig. 2B. Taken together, these findings indicate that platelet-derived prostaglandin endoperoxides are released from terbogrel-treated platelets that can be converted to prostacyclin by smooth muscle cells.

4. Discussion

In this study, we demonstrate that terbogrel, a novel combined thromboxane A_2 synthase inhibitor/thromboxane A_2 receptor antagonist, selectively inhibits thromboxane A_2-dependent platelet activation and exhibits an equipotent (IC_{50} of about 10 nM) activity as thromboxane A_2 synthase inhibitor and thromboxane A_2 receptor antagonist. In addition, terbogrel enhances prostacyclin synthesis in cultured vascular smooth muscle cells via transfer of platelet-derived prostaglandin endoperoxides.

The reference compound for platelet inhibition is acetylsalicylic acid (reviewed in the work of Schrör, 1997). Acetylsalicylic acid, at antiplatelet doses, nonselectively inhibits the constitutive fatty acid cyclooxygenase, resulting in a reduction of platelet thromboxane A_2 formation but also in an inhibition of vascular prostacyclin synthesis. Despite of a marked reduction in the antithrombotic dosage of acetylsalicylic acid, a full sparing of prostacyclin synthesis has not been attained (Patrono, 1989). A possible explanation is the finding that platelet prostaglandin endoperoxides can be utilized by cells of the vessel wall to form prostacyclin (Bunting et al., 1976; Marcus et al., 1980). Accordingly, platelet activation, as observed in atherosclerosis, is accompanied by increased vascular prostacyclin synthesis (FitzGerald et al., 1984). In fact, in patients with advanced atherosclerosis, a significant part of vascular prostacyclin synthesis capacity depends on transcellular prostaglandin endoperoxide transfer (Force et al., 1991). Thus, any antiplatelet dosage of acetylsalicylic acid might result in a reduction of prostacyclin production at the site of platelet–vessel wall interaction.

As a more specific alternative to acetylsalicylic acid, compounds that combine thromboxane A_2 synthase inhibition with thromboxane A_2 receptor antagonism, such as ridogrel, have been developed (reviewed in the work of

Vermylen and Deckmyn, 1992). These dual action compounds increase vascular prostacyclin formation and neutralize the proaggregatory action of prostaglandin endoperoxides. In the present study, we demonstrate that terbogrel exhibits equipotent (IC_{50} of about 10 nM) activity as thromboxane A_2 synthase inhibitor and thromboxane A_2 receptor antagonist in washed human platelets. Thus, both actions of the compound are detectable at low nanomolar concentrations and occur with the same potency. In addition, we have demonstrated that terbogrel favours the transfer of platelet-derived prostaglandin endoperoxides, resulting in an increased prostacyclin generation by vascular smooth muscle cells. This compound might be interesting for further in vivo investigations on platelet–vessel wall interactions in situations of endothelial injury.

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