The hepoxilin analog PBT-3 inhibits heparin-activated platelet aggregation evoked by ADP

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Abstract We have previously shown that PBT-3, a stable synthetic analog of hepoxilins, inhibits the aggregation of human platelets in vitro evoked by collagen through inhibition of thromboxane A_2 formation and action on the TP receptor. We now show that PBT-3 is capable of potently inhibiting the second phase of aggregation evoked by ADP in both washed human platelets and platelet-rich plasma (PRP), a phase associated with thromboxane formation. Aspirin blocks this second phase as well; so does the thromboxane receptor antagonist SQ 29,548. When ADP-evoked aggregation in PRP is activated by heparin through an enhancement of thromboxane formation, PBT-3, aspirin as well as SQ 29,548 block this activation through different mechanisms. These data confirm the inhibitory action of PBT-3 on aggregation of human platelets through inhibition of both thromboxane formation and blockade of thromboxane receptor action and suggest that this family of compounds may be useful in the treatment of thrombotic disorders in combination with heparin. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Hepoxilin; PBT-3; Heparin; Aggregation; ADP

1. Introduction

The coagulation system can be modulated by a variety of factors which are released in vivo and either cause or inhibit the aggregation of platelets [1]. One of these pathways is the thromboxane pathway, mediated via the conversion of arachidonic acid into thromboxane A_2, a potent thrombotic and vasoconstrictor metabolite [2,3]. Aspirin, an inhibitor of the formation of thromboxane and other prostanoids, is a first line of clinical defense as an anticoagulant but it is not without serious side effects [4–10]. Even heparin, another potent anticoagulant used clinically to prevent coagulation in individuals prone to clotting, can sensitize platelets to form thromboxane A_2 and evokes enhanced aggregability of platelets [11]. We report herein that PBT-3, a compound belonging to a family of hepoxilin analogs, synthesized through modification of natural hepoxilins [12], antagonizes the in vitro aggregation of preparations of washed human platelets and human platelet-rich plasma (PRP) evoked by ADP. Most importantly, we demonstrate that the heparin-evoked activation of platelets can be blocked by PBT-3.

2. Materials and methods

2.1. Materials

PBT-3 was prepared as reported previously [12]. SQ 29,548 was purchased from the Cayman Chemical Co., Ann Arbor, MI, USA. Aspirin was from Sigma, St. Louis, MO, USA.

2.2. Preparation of human platelets

Healthy human subjects who had not taken non-steroidal anti-inflammatory agents for at least 2 weeks were used. Blood was drawn into plastic syringes containing citric acid-sodium citrate–dextrose (9:1, v/v). It was immediately centrifuged at 23°C at $200\times g$ for 15 min. The PRP was either used as such or it was transferred into fresh plastic tubes and centrifuged at $400\times g$ for 5 min. The supernatant was discarded and the platelet pellet was resuspended in fresh medium (referred to as washed platelets) containing NaCl (137 mM), KCl (1 mM), NaH_2PO_4 (0.4 mM), glucose (5.5 mM), HEPES (20 mM) and $CaCl_2$ (1 mM), pH 7.4, and allowed to stand at room temperature for 30 min. The platelet count was adjusted to 350×10^6 cells with medium or platelet-poor plasma (PPP) to 0.5 ml/assay/cuvette for each measurement.

2.3. Measurement of platelet aggregation

Appropriate calibration of the platelet aggregometer (PAP-4C) for 0% and 100% transmission was carried out with a sample of platelet suspension and cell-free medium or PRP and PPP respectively. 0.5 ml of platelet suspension (PRP or washed platelets) was added to siliconized glass tubes (four samples at a time) and heated with magnetic stirring (900 rpm) to 37°C for 1 min in the aggregometer. Either vehicle alone (ethanol, 1 μl) or PBT-3, aspirin or SQ 29,548 at various concentrations in ethanol (1 μl) was added, followed by ADP 2 min later. The response was recorded for the next 5 min. In experiments where PRP was activated with heparin (2.5 μl of Hepalean (heparin sodium 1000 U/ml), Organon Teknika, Toronto, ON, Canada), heparin was incubated with PRP for 5 min at 37°C, then PBT-3 or aspirin or SQ 29,548 was added and 1 min later, ADP was added. Control studies in the absence of the three drugs were carried out at the same time in which 1 μl of ethanol only was added.

Test compounds were prepared in glass-distilled ethanol (100%) at the concentrations tested so that the drugs were added in 1 μl to the 0.5 ml cuvette containing 350×10^6 platelets.

2.4. Statistical analysis

Values stated are the mean ± S.D. of the number of observations (n) indicated. Analysis of statistical significance was performed using Student's t-test involving the Macintosh Statview software program.

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Abbreviations: PBT, hepoxilin cyclopropane analogs; PBT-3, 10(*S*)-hydroxy-11,12-cyclopropyl-eicosa-5*Z*,8*Z*,14*Z*-trienoic acid methyl ester; I-BOP, 5-heptenoic acid, 7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-,[15-[1α,2α(*Z*),3β(1*E*,3*S**),4α]]; U46619, 5-heptenoic acid, 7-[6-(3-hydroxy-1-octenyl)-2-oxabicyclo-[2.2.1]-hept-5yl]-[1*R*-[1α,4α,5β(*Z*),6α(1*E*,3*S**)]-9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$; SQ 29,548, [1*S*-[1a,2a(*Z*),3a,4a]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]-hept-2-yl]-5-heptenoic acid

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3. Results and discussion

We report herein that a stable analog of hepoxilins, PBT-3, antagonizes the aggregation of human platelets evoked by ADP. Only the second phase of aggregation, caused by thromboxane A_2 generation, is blocked. The same second phase is blocked by aspirin, due to inhibition of thromboxane generation, and by SQ 29,548, a selective antagonist of the thromboxane receptor in platelets [13]. We have demonstrated these effects in both washed human platelets and in PRP.

The aggregatory effects of ADP and the inhibition of this effect by PBT-3 are shown in washed human platelets (Fig. 1A). PBT-3 has been shown to act both as a thromboxane synthase inhibitor [14] and as a thromboxane receptor antagonist in platelets, with the latter action more effective than the former [15]. Fig. 1B shows results of a similar study but using PRP instead of washed platelets. In PRP unlike in washed platelets, platelets are activated by heparin [11], and this activation is associated with thromboxane formation. The heparin-evoked sensitization of platelets in PRP is blocked by all three compounds. Note that *only the second* phase (thromboxane-mediated) is inhibited. This is clearly shown in Fig. 1B. Aspirin's effects are mediated via inhibition of thromboxane generation, those of SQ 29,548 through inhibition of thromboxane action at the platelet thromboxane receptor. Fig. 2 shows dose–response curves for all three compounds describing their inhibition of the heparin-evoked sensitization of PRP to ADP. This data shows that PBT-3 is quite potent, with an $IC_{50} = 1.81 \times 10^{-6}$ M, while that of aspirin is 3.3×10^{-5} M, but that of the selective thromboxane receptor antagonist SQ 29,548 is 4.41×10^{-9} M. The enhanced activity of SQ 29,548 over PBT-3 may be due to decreased sequestration of the former compound to protein. We have shown in a recent study [15] that PBT-3 antagonized the aggregation in washed human platelets caused by two thromboxane receptor agonists, I-BOP and U46619, and that PBT-3 antagonized the binding of ^{125}I-BOP in platelet membranes with an $IC_{50} = 8 \times 10^{-9}$ M, just 16-fold less active than I-BOP itself, and only two-fold less active than U46619.

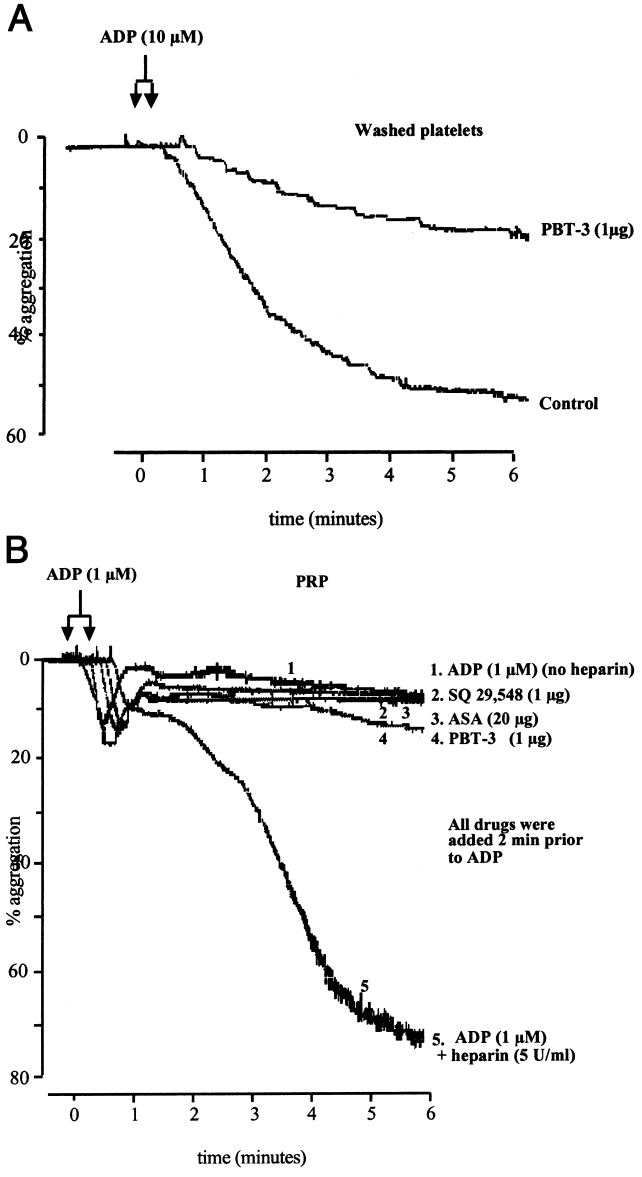

Fig. 1. Aggregation curves showing the effects of ADP on (A) washed human platelets and (B) PRP pretreated with heparin. The stimulatory effects by heparin (2.5 U) of ADP-evoked aggregation (compare lanes 1 – no heparin, and 5 – containing heparin) of human PRP and the inhibitory actions of PBT-3, aspirin and SQ 29,548 on blocking the ADP/heparin effect are shown. All drugs were added at a dose of 1 μg (in 1 μl ethanol) to the cuvettes containing 350×10^6 platelets in 0.5 ml with the exception of aspirin which was not active at 1 μg/ml, and therefore was added at 20 μg/ml as shown. All drugs were added 1 min prior to the addition of ADP which was made within the time period shown by arrows.

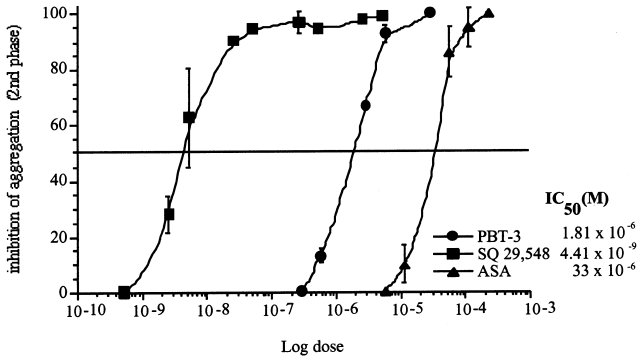

Fig. 2. Dose–response curves showing inhibition by PBT-3, SQ 29,548 and aspirin of the ADP-evoked aggregation in heparin-stimulated PRP. IC_{50} values are shown.

In summary, the present results demonstrate that PBT-3 is capable of inhibiting the thromboxane-mediated activation of platelets sensitized by heparin. The mechanism whereby this takes place may be through the previously demonstrated actions of PBT-3 in blocking thromboxane formation and the thromboxane receptor. Thus, PBT-3 should be considered and further explored as a new anti-aggregatory drug which may amplify the actions of heparin as first line anticoagulant defense by diminishing heparin's known side effects of thrombocytopenia.

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