Various AT₁ receptor antagonists including losartan are known to inhibit human platelet activation by antagonising TXA₂/PGH₂ receptors (TP receptors). Presently, we check a hypothesis that losartan, an imidazole derivative in contrast with valsartan, a non-imidazole compound, may inhibit human platelet activation also through inhibition of TXA₂ synthesis. Inhibitory action of losartan (2-n-butyl-4-chloro-5-hydroxymethyl-1-β(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl] imidazole), its active metabolite EXP 3174 (2-n-butyl-4-chloro-1-β(2-(1H-tetrazol-5-yl) biphenyl-4-yl) methyl]imidazole-5-carboxylic acid) and valsartan ((S)-N-valeryl-N-[(β2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]valine), on collagen-induced platelet aggregation and TXA₂ generation was compared to effects achieved by each compound on U46619-induced aggregation in aspirinized platelets. Losartan and aspirin inhibited collagen-induced platelet aggregation with approximately the same potency, whereas EXP 3174 and valsartan showed much weaker antiplatelet effects. Interestingly, losartan, EXP 3174 and valsartan displayed similar potencies as inhibitors of U46619-induced aggregation in aspirinized platelets as in collagen-induced aggregation in non-aspirinized platelets. None of the above three AT₁ antagonists, up to a concentration of 300 μM, did influence collagen-induced TXA₂ synthesis in human platelets. In conclusion, antiplatelet effects of AT₁ antagonists, irrespective of the presence or absence of non-condensed imidazole in their chemical structure, involve antagonism of TP receptors but not inhibition of TXA₂ synthesis in platelets.

Key words: platelets, AT₁ antagonists, TP receptors, TXA₂ synthase, imidazole.

INTRODUCTION

There is abundant experimental and clinical evidence for thromboxane A₂ (TXA₂) and its precursor prostaglandin H₂ (PGH₂) being involved in pathophysiology of occlusive vascular events (1, 2). Pharmacological
prevention of their effects is beneficial in management of thrombotic disorders (3). Most commonly it is achieved by platelet COX inhibition by aspirin (4), although TXA\textsubscript{2} synthase inhibition combined with TXA\textsubscript{2}/PGH\textsubscript{2} (TP) receptor antagonism have been suggested as an alternative antithrombotic strategy (5).

Interestingly, it was demonstrated that various nonpeptide AT\textsubscript{1} — receptor antagonists exerted an antiplatelet effect, related to a blockade of TP receptor (6—10). It was also pointed out that losartan and irbesartan were significantly stronger inhibitors of platelet aggregation than valsartan, candesartan or telmisartan (10). Noteworthy, losartan and irbesartan, but not candesartan or telmisartan contain a non-condensed imidazole ring in their molecules, while valsartan is a non-imidazole compound. It is well known that various imidazole derivatives are able to suppress enzymatic conversion of PGH\textsubscript{2} to TXA\textsubscript{2} (11). That is why we suggest that imidazole type AT\textsubscript{1} antagonist may have a dual mechanism of action, i.e. consisting of blockade of TP receptor and imidazole-dependent inhibition of TXA\textsubscript{2} synthesis. For testing this hypothesis we chose losartan, its active metabolite — EXP 3174 and valsartan. We compared their inhibitory potencies on collagen-induced and U46619-induced platelet aggregation, without or with pretreatment of platelets with aspirin, respectively.

**MATERIALS AND METHODS**

**Preparation of platelet-rich plasma (PRP)**

Citrated blood (1/10 volume of trisodium citrate 3.14 %) was taken from healthy human volunteers in University Hospital Blood Bank Center. Volunteer donors had not ingested any drugs for preceding 2 weeks. For preparation of PRP blood was centrifuged for 20 min at 200 × g at the room temperature. PPP was obtained by centrifugation of remaining blood for 5 min at 2000 × g.

**Platelet aggregation**

Platelet aggregation was studied in PRP in a model of dual channel Chronolog aggregometer using a method of Born (12). Baseline on the aggregometer was set using PRP whereas PPP was used to set full transmittance. PRP (500 μl) was equilibrated at 37°C for 3 min with continued stirring at 1100 rev/min and then stimulated with collagen or TP receptor agonist — U46619. In the beginning of each experiment submaximal concentrations for collagen and U46619 were determined. They were in the range of 0.3 μg/ml—0.8 μg/ml and in the range of 0.01—0.03 μg/ml for collagen and U46619 — induced aggregation, respectively. Antiplatelet effects of AT\textsubscript{1} antagonists were studied using submaximal concentration of aggregating compounds.
Losartan, EXP 3174, valsartan or aspirin were added to PRP 2 min before platelets were stimulated with collagen. PRP for U46619 — induced aggregation was added with aspirin (300 μM) 5 min prior to stimulation with U46619 and then with a AT<sub>1</sub> antagonist 2 min before stimulation with U46619.

**Measurement of Thromboxane B<sub>2</sub>**

TXB<sub>2</sub> measurements were performed in the samples taken from aggregation experiments. Following measurements of peak aggregation (8 minutes after addition of collagen) samples were treated with indomethacin (5 μM) to prevent any further TXB<sub>2</sub> formation and placed on ice (2—4°C). Samples were then transferred to Eppendorff tubes, centrifuged at 300 × g for 3 min at the room temperature to obtain plasma and stored frozen at −20°C until assayed.

TXB<sub>2</sub> levels were determined in duplicates in aliquots of diluted plasma using commercially available ELISA kit (Cayman Chemical Company, USA). TXB<sub>2</sub> levels were expressed in ng/ml.

**Reagents and drugs**

Collagen was purchased from Chrono-log Corporation (USA), Aspirin from Bayer (Germany), U46619 from Cayman Chemical Company (USA). Losartan and EXP 3174 was obtained from DuPont Merck Pharmaceutical Co. (USA), valsartan from Novartis Pharma AG (Switzerland).

**Statistical evaluation**

All values are expressed as mean ± S.E.M. To determine the statistical significance of the data unpaired Student’s t test was used. P < 0.05 was considered statistically significant.

**RESULTS**

Losartan, EXP 3174, and valsartan inhibited collagen-induced platelet aggregation and U46619-induced platelet aggregation in a concentration-dependent manner (Fig. 1A); the latter in the presence of aspirin.

Collagen-induced aggregation was inhibited by losartan and aspirin with a similar potency (IC<sub>50</sub> of 36.1 μM and 47.6 μM, respectively), while EXP 3174, and valsartan, at the highest concentration used of 300 μM inhibited platelet aggregation only by 33.2 ± 7% and 23.1 ± 6%, respectively (Fig. 1A, B).

A similar profile of antiplatelet potencies was found for three AT<sub>1</sub> receptor antagonists in aspirinized platelets, which were aggregated with U46619 (Fig. 1A). Moreover, losartan, EXP 3174 and valsartan did not influence collagen-induced TXA<sub>2</sub> production in platelets (Fig. 2).
Fig. 1. A) Comparison of antiplatelet effects of losartan, EXP 3174 and valsartan on collagen-induced and U46619-induced aggregation in human platelets. The effects of AT\(_1\) antagonists on aggregation induced by U46619 were studied in platelets pretreated with aspirin. Both collagen and U46619 were used at submaximal concentrations (see methods). Values are means±SEM for n = 6–8 experiments.

B) Inhibition of collagen-induced aggregation of human platelets by aspirin. Platelets were aggregated with submaximal concentration of collagen. Values are means±SEM for n = 5–6 experiments.
We studied a hypothesis that a stronger antiplatelet effect of losartan in comparison with valsartan (10) might be related to its dual mechanisms of action i.e. blocking of TP receptor and inhibiting of TXA$_2$ synthesis in platelets. We thought that the latter effect of losartan might be associated with the presence of an imidazole ring in its chemical structure.

What we showed in human platelet-rich plasma is as follows; Losartan, but not EXP 3174 or valsartan, was equally potent as aspirin in inhibiting collagen-induced platelet aggregation. Each of three AT$_1$ antagonists displayed similar inhibitory effects on collagen-induced aggregation and on U46619-induced aggregation of aspirinized platelets. Neither of three AT$_1$ antagonists, up to concentration of 300 $\mu$M, did influence the collagen-induced TXA$_2$ synthesis. These results suggest that antiplatelet actions of losartan, EXP 3174 and valsartan depend on TP receptor antagonism but not on TXA$_2$ synthesis inhibition in platelets.

Nearly 10 years ago it was found that losartan had been a competitive antagonist of TP receptor in human platelets (6). Involvement of TP receptors in the antiplatelet action of losartan as well as of other non-peptide AT$_1$ antagonists was studied in rat platelets (7) as well as in human platelets (8—10). Interestingly, it has been recently shown that imidazole-type AT$_1$ receptor
antagonists, such as losartan and irbesartan, are stronger anti-platelets agents against aggregation induced by ADP or TP receptor agonist (U46619) than valsartan, candesartan or telmisartan which do not contain a non-condensed imidazole ring in their structure (10). Presently, in order to test a possible contribution of TXA2 synthase inhibition to the antiplatelet action of imidazole-type AT1 antagonists, we compare the inhibitory effect of three AT1 antagonists (two imidazole-type AT1 antagonists such as losartan, EXP 3174 and one non-imidazole compound such as valsartan) on platelet aggregation induced by submaximal concentration of collagen with that evoked by U46619 in presence of aspirin. The collagen induced platelet aggregation is dependent on TXA2 synthesis in platelets and on TP receptor stimulation by released TXA2, whereas U46619-induced aggregation of aspirinized platelets is a TP receptor-dependent but TXA2 synthesis-independent response. This pharmacological approach allows us to exclude the possibility that TXA2 synthase inhibition contributes to antiplatelet effects of imidazole-type AT1 antagonists. This conclusion is supported by the lack of the effect of AT1 antagonists on collagen-induced TXA2 synthesis.

Moncada et al. (11) demonstrated that imidazole derivatives had inhibited TXA2 synthesis. However, these authors also pointed out that the potency of TXA2 synthase inhibition of imidazole derivatives was crucially dependent on the substituent in the imidazole ring. Imidazole or 1-methylimidazole were potent inhibitors with IC50 of 15 µM and 22 µM, respectively, whereas 1-phenylimidazole derivatives had IC50 > 500 µM. Losartan and EXP 3174 are nearly identical imidazole-type of AT1 antagonist compounds which have the same methyl-biphenyl-tetrazol substituent in position 1 of the imidazole ring (13). It may well be that such a bulky substituent in position 1 of imidazole in the molecule of losartan and EXP 3174 was responsible for the lack of the effect of these compounds on TXA2 synthesis in human platelets, even when they were used at as high concentrations as 300 µM.

Interestingly, it seems that the minor change in position 5 of imidazole which differentiates losartan and EXP 3174 (methylhydroxyl and carboxyl group, respectively) is responsible for different affinity of these compounds towards AT1 and TP receptors. It is known that EXP 3174 has 10—15 times higher affinity towards AT1 receptors then losartan (13). Our data suggest that losartan is a more potent antagonist of TP receptors on human platelets in comparison with EXP 3174.

It was demonstrated that human platelets contained AT1 receptors (14, 15) and some (16,17) but not all (10) authors were able to show that angiotensin II would amplify aggregation induced by other agonists. Thus, antiaggregatory effects of losartan, EXP 3174 and valsartan could also involve a blockade of platelet AT1 receptors. However, losartan selectively inhibits the cellular response to angiotensin II in human platelets with an IC50 of around $5 \times 10^{-8}$
M (18), while antiplatelet effect of losartan in the present study was observed with much higher concentration of this drug. Moreover, EXP 3174 is approximately 10—15 fold more potent antagonist of AT$_1$ receptors than losartan (13), whereas it displayed weaker antiaggregatory effect. These data indicate that antiplatelet action of AT$_1$ antagonists is not related to blockade of AT$_1$ receptors in platelets as suggested also by Monton et al. (10).

In summary, our results demonstrate that losartan is endowed with a more potent antiplatelet action than EXP 3174 and valsartan. This particular property of losartan is related to its more potent TP receptor antagonism but not to imidazole ring-dependent TXA$_2$ synthase inhibition, or to AT$_1$ receptor antagonism.

The present data do provide evidence that TP antagonism-dependent antiplatelet effect of losartan was comparable in potency to COX-1 inhibition-dependent antiplatelet effect of aspirin. The efficacy of aspirin in antithrombotic treatment and prophylaxis is well-documented (4). So, these findings suggest that antiplatelet action of losartan may be also of therapeutic importance.

In Evaluation of Losartan in the Eldery (ELITE) study, the largest clinical study of an AT$_1$ receptor antagonist in patients with heart failure, an unexpected reduction in mortality due to sudden cardiac death was observed among patients randomised to losartan (19). It is well known that sudden cardiac death in the most of cases is related to acute mural thrombosis in coronary vessel (20). Thus, it may well be that the antiplatelet effect of losartan contributed to the reduction in the occurrence of sudden cardiac death observed in ELITE study (19).

Acknowledgements: The authors would like to thank Dr J. Michalska for her helpful comments on chemical structures of AT$_1$ antagonists and Mrs Lena Stelmach for her excellent technical assistance. We acknowledge the generous gifts of losartan and EXP3174 from DuPont Merck Pharmaceutical Co., USA and Merck, Sharp & Dohme, Poland, and of valsartan, from Novartis Pharma AG, Switzerland.

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Received: October 3, 2000
Accepted: October 18, 2000

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