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LOSARTAN INHIBITS THE ADHESION OF RAT PLATELETS TO FIBRILLAR COLLAGEN — A POTENTIAL ROLE OF NITRIC OXIDE AND PROSTANOIDS

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The aim of the study was to evaluate the effect of losartan on rat platelet adhesion to fibrillar collagen. Washed platelets were counted before and after 15 minutes incubation with collagen (50 µg/ml) and the percentage of adhering platelets was calculated as the index of their adhesion. When the platelets were incubated with collagen $40.8 \pm 0.3\%$ of the platelets adhered. Losartan produced a dose dependent decrease in a number of adhering platelets both when the drug was administered to the animals *ex vivo* at doses of 3, 10 and 30 mg/kg ($p < 0.01 - 0.001$) or was added to the preparation of washed platelets *in vitro* in concentrations of $10^{-8} - 10^{-5}$ M ($p < 0.01 - 0.001$). In the next step of the study we assessed the influence of L-NAME (10 mg/kg *ex vivo*, 30 µM *in vitro*) and indomethacin (2.5 mg/kg *ex vivo*, 30 µM *in vitro*) on the antiadhesive effect of losartan (10 mg/kg *ex vivo*, 10^{-6} M *in vitro*). Blockade of nitric oxide synthase with L-NAME partially reversed the antiadhesive effect of losartan both *ex vivo* and *in vitro*. Indomethacin diminished the inhibitory effect of losartan on platelet adhesion when administered *ex vivo*, but it failed to modify this parameter when added to the suspension of platelets *in vitro*. In conclusion, losartan reduces platelet adhesion to fibrillar collagen in a dose-dependent manner. The observed action of losartan seems to be mediated mainly by endothelium- and platelet-derived nitric oxide.

K e y   w o r d s: *losartan, platelets, adhesion, AT_1* — receptor, rats, nitric oxide, prostacyclin.

INTRODUCTION

Thrombosis is a clue pathogenetic factor in acute coronary syndromes, including unstable angina, myocardial infarction and sudden death (1). The first step of a haemostatic plug formation, particularly in arteries, is an adhesion of circulating thrombocytes to the subendothelial matrix of a damaged vessel wall. Adhered platelets recruit additional platelets within a developing aggregate (2), which becomes a thrombus by the incorporation of fibrin (3). Among the components of the vessel wall collagen is considered to be the most important element involved in this process since it is

a unique ligand for platelet adhesion causing also platelet activation and aggregate formation (4).

In our previous experiments we have proved an antithrombotic action of the AT_1 receptor antagonist, losartan, in experimental arterial thrombosis in rats (5). Several studies have demonstrated antiplatelet effect of losartan as assessed by the inhibition of platelet aggregation (6—9). Losartan has been also shown to enhance nitric oxide and prostacyclin synthesis (10, 11), which are both potent inhibitors of platelet function. However, as far as we know, there are no data concerning the influence of this drug on platelet adhesion. Taking the above into account, the aim of the present study was to determine the effect of losartan on rat platelet adhesion to fibrillar collagen and to assess the role of nitric oxide and prostanoids in this process. The experiments were conducted *ex vivo* to evaluate the potential role of endothelial mediators in the action of losartan and *in vitro* to assess a direct influence of the examined drug on platelets.

MATERIALS AND METHODS

Animals

Male Wistar rats 220—340 g in weight were used throughout the study. The animals were housed in a room with a 12-h light/dark cycle, in group cages as appropriate, were given tap water and fed a standard rat chow. 24 hours before the experiments the animals were deprived of food but had free access to water.

Procedures involving the animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and Guidelines for the Use of Animals in Biomedical Research (Thromb Haemost 1987; 58: 1078—84).

Drugs

Losartan (DuP 753, DuPont Merck Pharmaceutical Co., USA), indomethacin (RBI,USA), L-NAME (N^G — nitro-L-arginine methyl ester, RBI, USA), collagen (Chronolog, USA), trisodium citrate, citric acid, glucose, sodium chloride, potassium chloride, magnesium chloride, sodium bicarbonate (all from Polish Chemical Reagents, Poland), HEPES (N-(2-hydroxyethyl) piperazine-N'-(2-ethanosulfonic acid), Sigma, USA), apyrase (Sigma, USA) and pentobarbital (Vetbutal, Polfa, Poland) were used in the study.

Drugs administration

In *ex vivo* experiments losartan (LOS) was diluted in saline and administered intraperitoneally (i.p.) 1 hour before the blood collection at doses of 3, 10 and 30 mg/kg. Control animals received saline (VEH) instead. To evaluate the role of nitric oxide and prostacyclin in the antiadhesive effect of losartan, N^G — nitro-L-arginine methyl ester (L-NAME, 10 mg/kg, i.p.) or indomethacin (INDO, 2.5 mg/kg, i.p.) were co-administered in a separate injection with the middle dose of losartan (10 mg/kg, i.p.). In all cases the total volume of administered drug solutions amounted to 10 ml/kg.

In *in vitro* experiments losartan was diluted in saline and added to the washed platelets in concentrations of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M at the beginning of the 5 minutes preincubation

time. In the control group VEH was added in the same volume and manner. In this part of the experiments L-NAME or INDO were added in a concentrations of 30 μM together with losartan in a dose of 10^{-6} M. The volume of the added solutions did not exceed 10% of the washed platelets sample volume.

Preparation of washed platelets

The animals were anaesthetised with pentobarbital (45 mg/kg i.p.) and blood samples were taken from the heart on anticoagulant (170 mM trisodium citrate, 130 mM citric acid and 101 mM glucose) in a volume ratio 9:1. Platelet washing was carried out as described with slight modifications (12). In brief, platelet rich plasma (PRP) was obtained by a centrifugation of the blood at 180 g for 20 min at room temperature. PRP was then centrifuged at 400 g for 15 min and obtained platelets were washed with calcium — free Tyrode's buffer (137 mM NaCl, 2.6 mM KCl, 12 mM $NaHCO_3$, 0.9 mM $MgCl_2$, 5.5 mM glucose, albumin 0.35%, apyrase 0.5 U/ml, pH 6.5) by a centrifugation at 400 g for 15 min. The washed platelets were finally suspended in a calcium — free Tyrod — HEPES buffer (137 mM NaCl, 2.6 mM KCl, 5 mM HEPES, 0.9 mM $MgCl_2$, 5.5 mM glucose, albumin 0.35%, pH 7.4). The final concentration of platelets was 3×10^5 platelets/μl.

Platelet adhesion to fibrillar collagen

Platelet adhesion was carried out according to Mant (13). 250 μL washed platelet samples were incubated in an Elvi aggregometer at 37°C and stirred at 900 rpm with EDTA (5 mM) to prevent platelet aggregation. After 5 min preincubation time collagen (50 μg/ml) was added and platelets were further incubated for 15 min. Samples of the suspension were obtained before and 15 min after adding the collagen and platelets were counted in a haemocytometer after dilution with Unopette system. Index of adhering platelets was calculated using a formula ((platelet count before adding the collagen -platelet count after adding the collagen)/platelet count before adding the collagen) $\times 100\%$.

Statistical analysis

The data are shown as mean ± SEM. The index of adhesion was compared between groups by means of the Mann — Whitney U test. The p values less than 0.05 were considered statistically significant.

RESULTS

When the platelets were incubated with collagen, $40.8 \pm 0.3\%$ of the platelets adhered (n = 20). In *ex vivo* experiments pretreatment with losartan resulted in a dose — dependent reduction of this parameter in comparison with control animals (*Fig. 1 a*). Significant inhibition of adhesion was observed with all the doses used with a maximum effect at 30 mg/kg ($23.9 \pm 1.1\%$, $p < 0.01$ vs VEH). Similar dose-dependent action on the number of adhering platelets was observed with all concentrations of losartan *in vitro* (*Fig. 1 b*), with a maximum reduction of the adhesion index to 17.1 ± 0.9 % ($p < 0.01$ vs VEH) by the concentration of 10^{-5} M.
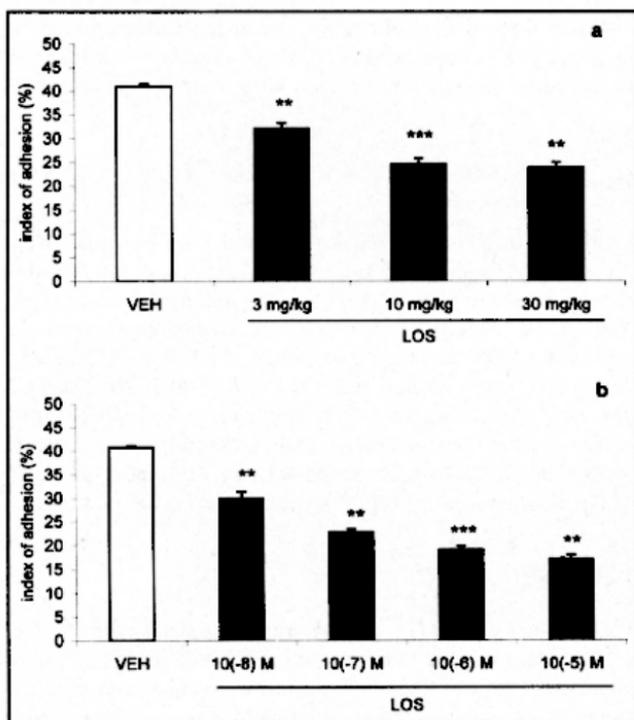
Fig. 1. The influence of increasing doses of losartan (LOS) on platelet adhesion *ex vivo* (a) and *in vitro* (b) in comparison with saline (VEH). The columns represent mean index of adhesion ± SEM (n = 4—6 in each experimental group). ** $p < 0.01$, *** $p < 0.001$ vs VEH.

Fig. 2. The influence of L-NAME on the antiadhesive effect of losartan (LOS) *ex vivo* (a) and *in vitro* (b). The columns represent mean index of adhesion ± SEM (n = 4—6 in each experimental group). * $p < 0.05$, *** $p < 0.001$.
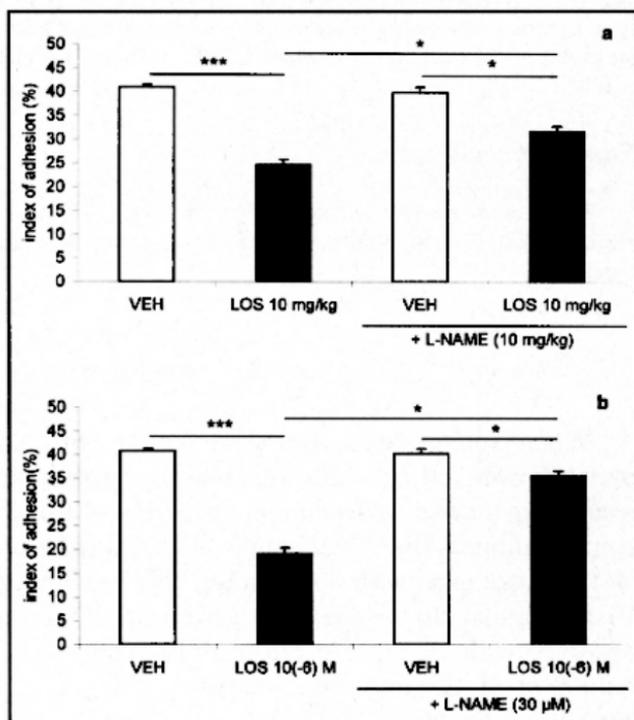
In the next step of the study we determined if nitric oxide synthase or cyclooxygenase blockade could influence the antiadhesive effect of losartan. In the following experiments doses of 10 mg/kg (*ex vivo*) and 10^{-6}M (*in vitro*) of losartan were used (reducing the adhesion index to $24.6 \pm 1.2\%$, $p < 0.001$ vs VEH and $19.1 \pm 0.7\%$, $p < 0.001$ vs VEH, respectively). L-NAME and indomethacin in the doses used did not modify the number of adhering platelets when given alone either *ex vivo* or *in* vitro. The blockade of nitric oxide synthesis with L-NAME partially reversed the antiadhesive effect of losartan both *ex vivo* ($31.8 \pm 1.0\%$, $p < 0.05$ vs LOS, $p < 0.05$ vs L-NAME) (*Fig. 2a*) and *in vitro* ($35.6 \pm 0.5\%$, $p < 0.05$ vs LOS, $p < 0.05$ vs L-NAME) (*Fig. 2b*). The antiadhesive effect of losartan in *ex vivo* experiments also was partially diminished by pretreatment with indomethacin ($36.7 \pm 1.2\%$, $p < 0.05$ vs LOS, $p < 0.05$ vs INDO) (*Fig. 3a*). However, when indomethacin was added to the washed platelets *in vitro*, no influence on the antiadhesive action of losartan could be observed ($19.8 \pm 1.0\%$, ns vs LOS, $p < 0.01$ vs INDO) (*Fig. 3b*).
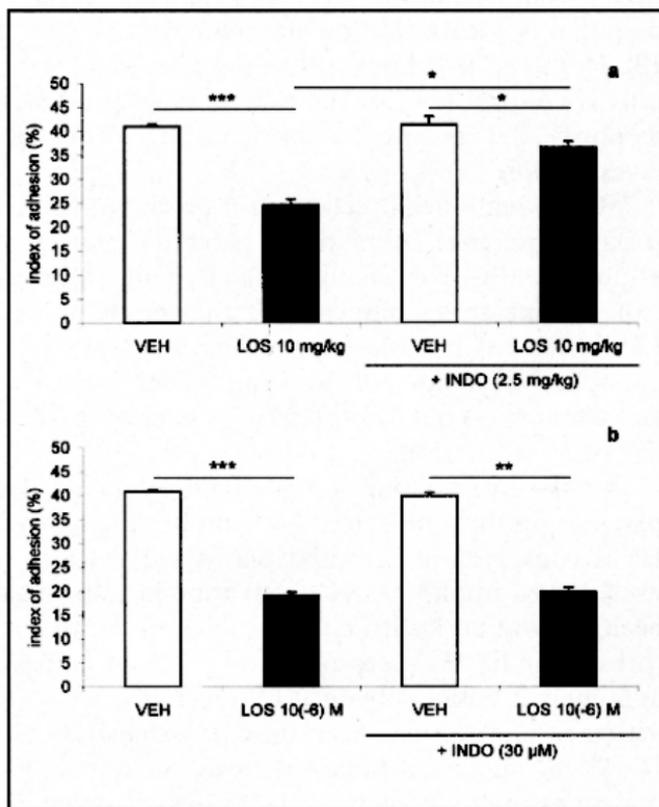

Fig. 3. The influence of indomethacin (INDO) on the antiadhesive effect of losartan (LOS) *ex vivo* (a) and *in vitro* (b). The columns represent mean index of adhesion $\pm$ SEM (n = 4—6 in each experimental group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

To evaluate a potential antiplatelet action of losartan we used a well established model of platelet adhesion to fibrillar collagen (13, 14). Our results show that AT_1 angiotensin II receptor antagonist, losartan, is able to suppress platelet adhesion to fibrillar collagen both in *ex vivo* experiments and when added to the suspension of washed platelets *in vitro*.

The mechanism by which losartan inhibits platelet adhesion appears to be a complex issue, since platelet — vessel wall interaction can be modified both by endothelium and platelet — related mechanisms. It has been reported that losartan increases a release of nitric oxide (10, 15, 16) and is a potent stimulus of prostacyclin synthesis (11). Both the endothelium — derived autacoids are known to act synergistically to diminish platelet aggregation (17). Nitric oxide is also a potent inhibitor of platelet adhesion to collagen and extracellular matrix (14). In our study we observed that blockade of nitric oxide synthase partially reversed the decrease in a number of adhering platelets caused by losartan, pointing to the role of endothelium — derived nitric oxide in the observed action. Surprisingly, the blockade of prostacyclin synthesis also diminished the effect of losartan in similar degree as L-NAME administration, despite it is known that prostacyclin exerts only a weak antiadhesive effect (14, 18). However, it is known that the release of both autacoids from endothelial cells is coupled (19), so the inhibition of prostacyclin synthesis could in turn diminish the release of nitric oxide. This possibility demands further investigation.

Beside endothelial cells, nitric oxide is synthesized also by thrombocytes (17). Its presence in platelets plays an important role in an intra-platelet negative feedback mechanism which modulates their response after stimulation (20). In our study blockade of this mechanism *in vitro* by an addition of L-NAME to platelet suspension resulted in a partial reversal of the antiadhesive action of losartan. Therefore, we conclude that not only endothelium — but also platelet — derived nitric oxide can be responsible for the observed action.

Unlike nitric oxide, prostacyclin synthesis pathways are not present in platelets. In thrombocytes, the main product of cyclooxygenase, prostaglandin H_2 is converted to thromboxane A_2 (21), which is able to activate platelets itself and to amplify platelet activation in response to other agonists (22). It has been shown that losartan is a weak competitive antagonist of thromboxane A_2 /prostaglandin H_2 receptor (TP) in human platelets (23). The hypothesis that antagonistic activity against TP receptors could contribute to the antiplatelet action of losartan has been recently extensively tested in rats (6) and humans (7—9). In all these studies aggregation of platelets stimulated with a stable thromboxane A_2 mimetic, U46619, was strongly diminished by losartan, while

other AT_1 receptor antagonists, devoid of TP receptor antagonistic properties, exerted a much weaker effect or were ineffective. Therefore, blockade of TP receptors by losartan seems to play a key role in its antiaggregatory action. The question if it is also important for its antiadhesive effect remains to be elucidated. Although thromboxane A_2 is primarily involved in the mechanisms of platelet aggregation, some data indicate that it can also promote platelet adhesion (24, 25). Therefore, a significance of TP receptor blockade in the mechanism of not only antiaggregatory but also the antiadhesive action of losartan should be taken into account. To evaluate the role of thromboxane A_2 in the direct action of losartan on thrombocytes, we blocked cyclooxygenase in platelets by an addition of indomethacin to the preparation of platelets *in vitro*. Concomitant addition of losartan and indomethacin did not modify the number of adhering platelets when compared to losartan alone, thus pointing to a conclusion that TP receptor blockade plays a minor role in the observed antiadhesive effect of losartan. Moreover, our preliminary results with other AT_1 receptor antagonists, valsartan and an active metabolite of losartan — EXP3174, show that both substances suppress platelet adhesion to fibrillar collagen *in vitro* despite they are devoid of antagonistic properties against TP receptor (data not published). Thus, another mechanism than TP receptor blockade should be taken under consideration in the antiadhesive effect of losartan. In humans, it has been found that thrombocytes contain AT_1 receptors (26, 27) and that stimulation of platelets with angiotensin II potentiates their activation by other substances (28, 29). However, as far as we know there is no evidence for a presence of AT_1 receptors on rat thrombocytes. Therefore, the involvement of AT_1 receptor in the mechanism of antiadhesive action of losartan demands further investigation.

In summary, we demonstrated that AT_1 receptor antagonist, losartan, reduced the adhesion of rat platelets to fibrillar collagen in a dose-dependent manner when administered to animals in *ex vivo* experiments as well as when added to the suspension of washed platelets *in vitro*. This action was partially reversed by the inhibition of nitric oxide synthase both *ex vivo* and *in vitro*. The antiadhesive effect of losartan was also diminished by an inhibition of cyclooxygenase *ex vivo*, but not *in vitro*. Thus, we conclude that losartan action was mainly associated with increased release of endothelium- and platelet- derived nitric oxide and partially with a stimulation of prostacyclin synthesis.

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