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ANTITHROMBOTIC ACTIVITY OF LOSARTAN IN TWO KIDNEY, ONE CLIP HYPERTENSIVE RATS. A STUDY ON THE MECHANISM OF ACTION

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The potential antithrombotic action of losartan, an AT_1 receptor antagonist, administered to two-kidney, one-clip rats (2K1C) in an experimental model of venous thrombosis was evaluated. The involvement of nitric oxide (NO) in this effect was also studied. Venous stasis was induced by ligation of the vena cava. Losartan after single dose (10 mg/kg, p.o.) significantly reduced the venous thrombus growth. The antithrombotic action of losartan in 2K1C rats was abolished by N^G-nitro-L-arginine methyl ester (L-NAME, 30 mg/kg s.c.) and restored by L-arginine (1000 mg/kg s.c.). Platelet adhesion to fibrillar collagen significantly decreased after administration of losartan. No changes in primary hemostasis and platelet aggregation were observed. Moreover, coagulation parameters such as activated partial thromboplastin time, prothrombin time and euglobulin clot lysis time were found unchanged after losartan administration either in systemic circulation or at the place of thrombus formation. Our results indicate that antithrombotic activity of losartan in 2K1C rats is NO — dependent; observed inhibition of platelet adhesion could also play a role in this phenomenon.

Key words: *losartan, renal hypertensive rats, venous thrombosis, nitric oxide.*

INTRODUCTION

It is known that angiotensin II (Ang II), the main active substance of the renin-angiotensin system (RAS), is an important etiologic factor responsible for hypertension development and congestive heart failure, conditions in which the risk of thrombotic diseases significantly increases. A potential link between the RAS and thrombosis has recently been widely confirmed, since Ang II induced the production and secretion of plasminogen activator inhibitor -1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2) in endothelial cells and smooth muscle cells (1, 2). Moreover, the infusion of Ang II promoted a rapid and dose

dependent increase in PAI-1 in humans (3). It is also known than Ang II (both circulating and released from activated blood platelets), when binding to its membrane receptor, enhanced the aggregating response of thrombocytes (4) - cells which play a role both in arterial and venous thrombosis.

On the other hand, pharmacological interruption of the RAS with angiotensin converting enzyme inhibitors (ACE-Is) reduced the development of atherosclerosis in experimental animals (5) and has been shown to reduce the incidence of recurrent myocardial infarction in selected populations of humans (6). Recent randomised, clinical trials indicate that treatment with losartan, AT_1 receptor antagonist, was also associated with lower mortality in patients with heart failure (7). Although the action of losartan is mainly attributed to the blockade of AT_1 receptors, the role of nitric oxide (NO), prostacyclin (PGI_2) and lately also kinins (6, 7, 10) in the mechanism of action of this drug has been suggested. Indeed, in our earlier study the NO — dependent mechanism of the antithrombotic action of losartan was demonstrated in spontaneously hypertensive rats (SHR) (11). Since SHR were generally considered to be a model exhibiting normal plasma renin activity (PRA) (12) we were interested in examining the potential antithrombotic effect of losartan in a renin - dependent model of hypertension. For this purpose we used two kidney, one clip (2K1C) renal hypertensive rats at a time when they had elevated PRA.

The aim of the present study was to investigate the influence of losartan on venous thrombus formation, in particular its effect on NO release, and its possible influence on platelet function as well as on coagulation and fibrinolysis systems. It is the first study to elucidate the mechanism of the antithrombotic effect the of AT_1 receptor antagonist in a model of renal hypertension.

MATERIAL AND METHODS

Animals and induction or renovascular hypertension

Male Wistar rats (150—170 g) were used in the experiments. Two kidney, one clip model of hypertension was induced by partial, standardised clipping of the left renal artery under pentobarbital anaesthesia (40 mg/kg, i.p.) and the rats were then left untouched for the next 6 weeks. A sham operated group (SO) of rats received similar surgical intervention except for the clipping of the artery.

Experimental protocol

2K1C and SO rats were treated per os with a single dose of losartan (10 mg/kg) 2 hours before venous thrombosis induction. In another set of experiments, rats with established hypertension were randomly divided into groups that received losartan with or without simultaneous

administration of the following drugs: (1) the NO synthase inhibitor — N^G-nitro-L-arginine methyl ester (L-NAME) (30 mg/kg, s.c.), (2) L-arginine (1000 mg/kg s.c.). Losartan was administered — 2 hours, L-NAME — 20 min and L-arginine — 5 min before venous thrombosis induction.

Blood pressure measurement

Indirect systolic blood pressure (SBP) was measured in conscious rats by a tail-cuff method (13). Each value was the average of three consecutive readings.

Plasma renin activity assay

PRA was determined by radioimmunoassay using a Immunotech radioimunnoasay kit (Prague, Czech Republic). PRA was calculated as the amount of angiotensin I (Ang I) generated from endogenous substrate per ml plasma during incubation of the sample for 1 hour at 37°C.

Experimental venous thrombosis model

The stasis induced venous thrombosis model was induced according to Reyers *et al.* (14). Briefly, the animals were anaesthetized with pentobarbital (40 mg/kg, i.p.), the abdomen was opened and, after careful dissection, a ligature (with cotton thread) was placed around the vena cava just below the renal vein. Thereafter, the abdominal incision was closed. 2 h later the animals were reanaesthetized and their abdominal wall reopened. The inferior vena cava was isolated, then cut longitudinally and the formed thrombus removed. 24 h later the dry weight of the thrombus was recorded. The results were expressed as the incidence of thrombosis (%) and the thrombus weight (mg). The average thrombus weight of all the animals included in the test was calculated in each group.

Ex vivo platelet aggregation

The assay for blood platelet aggregation was carried out according to Born and Cross (15). Blood for platelet aggregation studies was drawn 2 h after venous thrombosis induction, by cardiac venopuncture in 3.13 % trisodium citrate (9:1). Platelet rich plasma (PRP) was obtained by centrifugation at 490 G for 3 min at room temperature. After PRP was removed and pooled, the residual blood sample was centrifuged for 20 min to obtain platelet poor plasma (PPP). The PRP platelet count was adjusted to $250 \times 10^3/\mu l$ with autologous PPP. Platelet aggregation was induced by ADP. A threshold-aggregating concentration (TAC) defined as the lowest concentration of aggregating agent which induced irreversible platelet aggregation was observed 3 min after ADP addition.

Platelet adhesion to fibrillar collagen

Blood was taken from the heart 2 hours after oral losartan administration at the dose of 10 mg/kg. Platelet adhesion was carried out as described (16). Platelet samples (1.5×10^8 platelets in 0.25 ml) were incubated in an Elvi aggregometer at 37°C and stirred at 900 r.p.m. with EDTA (5mM) to prevent platelet aggregation. After 5 min collagen (50 $\mu g\ ml^{-1}$) was added and the samples incubated for a further 10 min. Platelets were counted before and 15 min after adding the collagen using a light microscope and the difference in platelet count was taken as an index of their adhesion to collagen.

Hemostasis analyses

Blood samples were taken 2 hours after venous thrombosis induction: 1. from the site of the thrombus formation by a puncture of the vena cava just below the ligature to evaluate local changes in coagulation and fibrinolysis; 2. from the heart to assess haemostatic changes in systemic circulation. Blood was mixed with 3.13% sodium citrate in a volume ratio 9:1, centrifuged for 20 min at 490 G and 4°C. Activated partial thromboplastin time (aPTT), prothrombin time (PT), and euglobulin clot lysis time (ECLT) were determined by routine laboratory assays. "Template" bleeding time (BT) was measured just before venous thrombosis induction by longitudinal incision of a rat tail, according to Dejana *et al.* (17).

Chemicals and drugs

Losartan (DuP 753, kindly provided by DuPont Merck Pharmaceutical Co., USA), L-NAME (N^G-nitro-L-arginine methyl ester, RBI, USA), pentobarbital (Vetbutal, Polfa, Poland), L-arginine (Serva, Germany), ADP (adenosine diphosphate, Sigma Chemical Co., USA), collagen (Chronolog, USA) and trisodium citrate (Polish Chemical Reagents, Poland) were used in the experiments.

Statistical analyses

Multiple group comparisons were performed by one-way analysis of variance (ANOVA), and, when significant intergroup differences occurred, were assessed by a Student-Neuman-Keuls test. Incidence of venous thrombosis was evaluated by Fisher's exact test. Student's unpaired t - test was also used to determine the significance between means for two groups. A value of $p<0.05$ was considered as statistically significant.

RESULTS

Characteristics of 2K1C and sham operated rats. (Table 1)

Table 1. Characteristics of 2K1C and SO rats.

	2K1C	SO
Number of rats	13	10
Body weight (g)	352	364
Left kidney weight (g)	0.45 ± 0.09	0.98 ± 0.08
Right kidney weight (g)	1.61 ± 0.05 *a,d*	0.98 ± 0.07
Plasma renin activity (ng/ml/h)	10.65 ± 3.3 *b*	3.52 ± 1.8
Basal systolic blood pressure (mmHg)	158 ± 4 *c*	137 ± 4

a — $p < 0.001$ vs contralateral kidney, *b* — $p < 0.01$ vs sham operated, *c* — $p < 0.001$ vs sham operated, *d* — $p < 0.01$ vs sham operated.

The 2K1C rats had significantly higher SBP (158 ± 4 mmHg vs 137 ± 4 mmHg; $p<0.001$) and plasma renin activity (10.65 ± 3.3 ng/ml/h vs 3.52 ± 1.8 ng/ml/h; $p<0.01$) compared with sham operated controls. Left and

right kidney weights were similar in sham controls; however in 2K1C rats the weight of the clipped (left) kidney was significantly smaller (0.45 ± 0.09 g; $p < 0.001$) than those of the right kidney (1.61 ± 0.05 g) and both kidneys in the sham operated group.

Blood pressure. (*Table 2*)

Table 2. Systolic blood pressure and venous thrombus growth in 2K1C and SO rats.

	n	SBP	Incidence of thrombosis (%)	Thrombus weight (mg)
SO	14	127 ± 4	65	0.82 ± 0.46
SO + Los	14	120 ± 5	58	1.18 ± 0.54
2K1C	26	160 ± 4	85	3.25 ± 0.62 *c*
2K1C + Los	23	137 ± 4 *a*	24 *e*	1.39 ± 0.29 *d*
2K1C + L-NAME	20	175 ± 5 *b*	65	3.01 ± 0.90
2K1C + Los + L-NAME	24	165 ± 4	79	2.26 ± 0.73 *f*
2K1C + Los + L-NAME + L-Arg	17	130 ± 8	20	1.53 ± 0.40 *g*

a — $p < 0.001$ vs 2K1C, *b* — $p < 0.01$ vs 2K1C, *c* — $p < 0.01$ vs SO, *d* — $p < 0.01$ vs 2K1C, *e* — $p < 0.01$ vs 2K1C, *f* — $p < 0.05$ vs 2K1C + Los, *g* — $p < 0.05$ vs 2K1C + Los + L-NAME, *h* — $p < 0.05$ vs 2K1C + Los, i $p < 0.05$ vs 2K1C + Los + L-NAME.

Losartan induced a significant decrease of systolic blood pressure in 2K1C rats (from 160 ± 4 mmHg to 137 ± 4 mmHg; $p < 0.001$) but did not change the SBP in sham operated rats (127 ± 4 mmHg vs 120 ± 5 mmHg; ns). The administration of L-NAME to 2K1C rats caused an increase of SBP (from 160 ± 4 mmHg to 175 ± 5 mmHg; $p < 0.01$). SBP during the concurrent administration of losartan and the NO synthase inhibitor to the 2K1C was similar to that observed in untreated 2K1C rats (165 ± 4 mmHg vs 160 ± 4 mmHg; ns). In rats pre-treated with losartan, L-arginine and L-NAME the value of SBP was similar to that observed after losartan administration (130 ± 8 mmHg vs 137 ± 4 mmHg; ns).

Venous thrombosis development. (*Table 2*)

Renal hypertensive rats appeared to be more sensitive than sham operated rats to our model of stasis induced venous thrombosis since the thrombus weight estimated in the first group was significantly increased, if compared to that of sham operated rats (3.25 ± 0.62 mg vs 0.82 ± 0.46 mg; $p < 0.01$). The incidence of thrombosis was also slightly higher in 2K1C rats but it did not reach significance (85% vs 65%). A marked decrease in the thrombus weight was observed after losartan administration (3.25 ± 0.62 mg vs 1.39 ± 0.29 mg;

$p<0.01$) with any influences of the drug on thrombus weight in normotensive rats (0.82 ± 0.46 mg vs 1.18 ± 0.54 mg; ns). Losartan induced a significant fall in venous thrombosis incidence (85% to 24 %; $p<0.01$) in renal hypertensive rats, while failing to exert this effect in sham operated rats (65% vs 58%; ns). L-NAME, by itself, did not change the thrombus weight (3.01 ± 0.90 mg vs 3.25 ± 0.62 mg; ns). Pre-treatment with NO synthase inhibitor almost completely abolished the thrombus weight reduction produced by losartan (2.26 ± 0.33 mg vs 1.39 ± 0.29 mg; $p<0.05$). After concomitant administration of losartan, L-NAME and L-arginine, the thrombus weight was significantly reduced in comparison with values obtained in 2K1C + Los + L-NAME group (1.53 ± 0.40 mg vs 2.26 ± 0.33 mg; $p<0.05$). The reduction of the incidence of venous thrombosis observed in 2K1C group treated with losartan (24%) was abolished by L-NAME (79%; $p<0.05$) and restored by L-arginine (20%; $p<0.05$) administration.

Hematological parameters. (Table 3)

Table 3. Influence of losartan on some hematological parameters, platelet aggregation, platelet adhesion to fibrillar collagen and "template" bleeding time in 2K1C rats.

		local changes			*systemic changes*	
	n	2K1C	2K1C + Los	n	2K1C	2K1C + Los
aPTT (sec)	5	25.0 ± 1.7	30.3 ± 0.6	11	24.5 ± 1.7	27.8 ± 1.3
PT (sec)	5	28.1 ± 1.1	27.7 ± 2.8	12	29.5 ± 1.0	27.7 ± 1.2
ECLT (min)	5	283 ± 43	262 ± 52	12	165 ± 32	386 ± 148
TAC (μM)		—	—	5	3.9 ± 2.2	2.9 ± 1.0
Platelet adhesion (%)		—	—	7	31.5 ± 1.1	24.9 ± 1.0 *a*
Bleeding time (sec)				13	128 ± 13	133 ± 15

a — $p < 0.001$ vs 2K1C.

After losartan administration there were no differences in aPTT, PT, and ECLT, either in systemic or local plasma. BT and platelet aggregation were also not influenced by losartan. When platelets were incubated with collagen (50 μg ml^{-1}) 31.5 ± 1.1% of the platelets adhered to collagen. Single losartan administration significantly decreased platelet adhesion to fibrillar collagen to 24.9 ± 1.0 % ($p<0.001$) when compared to control animals 31.5 ± 1.1%.

DISCUSSION

In this study a well established model of venous thrombosis in rats was used. The mechanisms of the thrombus formation in this experimental model are similar to those of venous thrombosis in humans. The formation of

thrombus in these conditions has been shown to depend on platelet function and coagulation activation and could be inhibited by both antiplatelet agents and anticoagulants (18, 19). Our results showed that losartan, an AT_1 receptor antagonist, after acute administration to 2K1C rats, reduced the venous thrombus growth, estimated as the decrease of thrombus weight and incidence of thrombosis. Its antithrombotic activity was accompanied by inhibition of platelet adhesion, but was related neither to activation of the fibrinolytic system nor to inhibition of coagulation. Since the antithrombotic effect of losartan was inhibited by the nitric oxide synthase inhibitor — L-NAME, we suggest that losartan reduced thrombus formation by NO release from vascular endothelium and/or platelets.

The mechanism by which losartan may activate the release of NO appears to be a complex issue. It has been reported that Ang II was able to stimulate NO synthesis *via* AT_2 receptors both *in vitro* (20) and *in vivo* (21). Moreover, it was shown that acute losartan administration increased nitric oxide-dependent aortic cGMP production in stroke — prone, spontaneously hypertensive rats (10). On the other hand, losartan administration results in an elevation of angiotensin -[1—7] (Ang -[1—7]) (22), a potent stimulator of nitric oxide release in the vascular bed (23). Thus there are at least two mechanisms which might be responsible for excessive nitric oxide production after losartan treatment. Firstly, after AT_1 receptor blockade, Ang II binds to AT_2 receptors leading to the stimulation of NO synthesis. Secondly, by the above mentioned elevation of Ang -[1—7] production. Therefore, nitric oxide elevation may play a role in the antithrombotic activity of losartan, as was also demonstrated by its hypotensive effect (8).

Besides endothelial cells, NO synthesis could be also stimulated in platelets. This pathway, playing a role in the modulation of platelet aggregation, is also involved in the regulation of platelet-vessel wall interactions (24). NO has been shown to reduce the expression of platelet surface glycoproteins GPIIb/IIIa, which was crucial for platelet adhesion and aggregation (25). On the other hand, it was reported that platelet adhesion to collagen was increased following L-NMMA infusion, suggesting that inhibition of NO production potentiated *ex vivo* platelet adhesion to collagen (26). In our study, single losartan administration resulted in a 20% inhibition of platelet adhesion to fibrillar collagen with simultaneous reduction of thrombus weight and incidence of thrombosis by 57% and 70% respectively. Thus, besides the influence on platelet adhesion some other mechanism(s) could be involved in the antithrombotic action of the studied drug.

Although Ang II had no direct effect on platelet aggregation, it significantly potentiated platelet responsiveness to other agonists (4). It was also shown that losartan inhibited Ang II binding to its receptor on human platelets (27). Moreover, recent observation demonstrates that losartan is a weak, competi-

tive antagonist of humans (28) and spontaneously hypertensive rats (29) platelet TXA_2/PGH_2 receptors. In the present experiments we have not observed the influence of losartan on ADP induced platelet aggregation in 2K1C rats. This is in agreement with our previous study on normotensive and spontaneously hypertensive rats (11, 30). However, it should be consider, that the potential changes in platelet function at the place of thrombus formation may not be found in the systemic circulation. Moreover, a previous report by Hornstra *et al.* (31) showed no relationship between *in vivo* and *in vitro* measurements of platelet function in animal studies. Standard aggregation techniques are very convenient in assessing platelet function, but the interaction of the thrombocytes with the vessel wall components under dynamic shear conditions cannot be examined in an agregometer. It is also interesting to note that the NO donors which demonstrated antithrombotic effects *in vivo* were unable to inhibit platelet aggregation *in vitro* (32).

Adhesion of neutrophils to the vascular endothelium represents the first step of venous thrombus growth (33). It has been shown that infusions of L-NAME promoted leukocyte adhesion in post capillary venules in cats (34). Moreover, it was reported that endothelium — derived NO attenuates neutrophil adhesion in *ex vivo* superfusion experiments (35). We cannot exclude that in our study the influence of losartan on neutrophil — vascular wall interaction was NO-mediated.

Clinical and experimental observations suggested the role of the fibrinolysis and coagulation pathways in the antithrombotic activity of losartan (3, 36). On the other hand, some activity of NO in biological systems is possibly due to inhibition of the release of plasminogen activator inhibitor (37). Thus, it is tempting to speculate that losartan can affect the plasma fibrinolytic system by interfering with NO release. For these purposes, we have evaluated some of the parameters of coagulation and fibrinolysis. However, we have not found any changes in activated partial thromboplastin time, prothrombin time and euglobulin clot lysis time either in systemic circulation or at place of thrombus formation. This is in agreement with the *in vivo* study of patients with essential hypertension (38) and our study in SHR (11) in which no beneficial effect of losartan on the plasma fibrinolytic balance was observed.

In our study no changes in bleeding time were detected. There are data reporting that NO is involved in bleeding tendencies *in vivo* by inhibiting platelet adhesion but not platelet aggregation (39). In view of our data concerning the inhibition of platelet adhesion, the prolongation of BT after losartan treatment was expected. However, no bleeding prolongation was recorded after losartan administration. The explanation of this discrepancy might be due to fact that bleeding time was correlated with a strong inhibition of platelet adhesion (40). We observed only a moderate decrease in platelet adhesion, that could be not sufficient to result in bleeding time prolongation.

Moreover, the lack of influence of losartan on BT time is consistent with our previous research both in normotensive and hypertensive rats (11,41).

Acute losartan administration normalized the SBP in 2K1C rats. Its antihypertensive activities in this and other hypertensive models have been widely reported (42). It is documented that losartan has a minimal influence on blood pressure in normotensive rats (42), which has been also confirmed in our study. In the present investigation, pre-treatment with L-NAME has suppressed hypotensive response to losartan. Therefore, it may be submitted that the hypotensive action of losartan is mediated partially by endothelial NO in 2K1C hypertensive rats. Previously, such action of losartan was reported by Cachofeiro (8) and our group in spontaneously hypertensive rats (11). One may suggest that the antithrombotic activity of losartan is a result of blood pressure reduction. Since L-NAME when administered alone enhanced SBP (*Table 2*), but did not affect venous thrombus formation, the contribution of the losartan dependent hypotensive component in our study should be excluded. Moreover, in this examination the 57% inhibition of thrombus weight after acute losartan administration in 2K1C rats was observed at a time, when plasma renin activity was high. This is comparable with figures observed in our previous study with SHR rats (55%) (11), in a model generally accepted to have low or normal PRA (12). Thus, we suggest that the mechanism of antithrombotic potency of losartan in our experimental condition does not depend on renin-angiotensin system activation.

In summary, we have demonstrated that acute losartan administration reduced thrombus growth in 2K1C rats by NO-related mechanism(s). For an answer to the question whether losartan may be potentially useful in preventing venous thrombotic complications in patients with renal and essential hypertension, further intensive experimental and clinical studies are needed.

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