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Study of the mechanisms of aldosterone prothrombotic effect in rats

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Abstract

Introduction: We investigated the role of primary haemostasis, fibrinolysis, nitric oxide (NO) and oxidative stress as well as mineralocorticoid receptors (MR) in acute aldosterone prothrombotic action.

Materials and methods: Venous thrombosis was induced by stasis in Wistar rats. Aldosterone (ALDO; 10, 30, 100 μg/kg/h) was infused for 1 h. Eplerenone (EPL; 100 mg/kg, p.o.), a selective MR antagonist, was administered before ALDO infusion. Bleeding time (BT) and platelet adhesion to collagen were evaluated. The expression of nitric oxide synthase (NOS), NADPH oxidase, superoxide dismutase (SOD) and plasminogen activator inhibitor (PAI-1) was measured. NO, malonyl dialdehyde (MDA) and hydrogen peroxide (H_2O_2) plasma levels were assayed.

Results: Significant enhancement of venous thrombosis was observed after ALDO infusion. ALDO shortened BT and increased platelet adhesion. Marked increases were observed in PAI-1, NADPH oxidase and SOD mRNA levels. MDA and H_2O_2 levels were augmented in ALDO-treated groups, and NOS expression and NO level were decreased. EPL reduced ALDO effects on thrombus formation, primary haemostasis, PAI-1 expression and MDA level.

Conclusion: Short-term ALDO infusion enhances experimental venous thrombosis in the mechanism involving primary haemostasis, fibrinolysis, NO and oxidative stress-dependent pathways. The MR antagonist only partially diminished the ALDO effects, suggesting the involvement of additional mechanisms.

Keywords

Aldosterone, eplerenone, haemostasis, rat, thrombosis

Introduction

There is evidence indicating that aldosterone (ALDO) has a circadian rhythm, and that the major peak of ALDO occurs during the morning hours.[1] The morning surge of blood pressure along with some haemostatic changes (for example, alterations in endothelial activity, changes in fibrinolysis, an increase in platelet aggregability) and diurnal variations of the renin–angiotensin–aldosterone system (RAAS), with an increased level of ALDO suggest a correlation with circadian variations in the time of onset of thrombotic disorders and acute cardiovascular events.[2-4] Moreover, it has been established that the level of ALDO rises significantly in the plasma during an operation and in the post-operative period in patients undergoing intra-abdominal surgery. This short-lasting 'hyperaldosteronism' may be related to electrolyte disturbances during the operative period, as well as to changes in RAAS function.[5,6] Thus, it could be suggested that thrombotic complications during surgery may be a consequence of hyperaldosteronism connected with surgical intervention.

Some recent studies have provided evidence for a link between ALDO and haemostatic disturbances, although they mostly pertain to conditions with chronically elevated ALDO levels.[7] It has been demonstrated in a model of cardiovascular disease that chronic administration of ALDO potentiates thrombotic microangiopathy lesions in stroke-prone, spontaneously hypertensive rats.[8] It was also shown that

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chronic ALDO treatment results in enhanced thrombosis following arterial injury in atherosclerotic mice, and this effect was blocked with spironolactone.[9]

We attempted to find out whether a short-lasting increase in ALDO level causes thrombotic complications in normotensive rats. These animals were chosen to distinguish the effects of ALDO from hypercoagulable disorders related to hypertension and atherosclerosis. Our preliminary study showed that acute ALDO infusion at a dose of 30 μg/kg/h (ALDO 30) enhanced thrombosis in the mechanism involving the activation of coagulation, expressed as an increase in tissue factor (TF) plasma level, and fibrinolysis impairment, which resulted in a reduced tissue plasminogen activator (t-PA) level as well as increased thrombin activatable fibrinolysis inhibitor (TAFI) and plasminogen activator inhibitor (PAI-1) plasma concentrations.[10]

It could also be suggested that ALDO may influence primary haemostasis by altering platelet function, since the existence of a mineralocorticoid receptor (MR) in human platelets has been recently reported.[11] Moreover, an MR antagonist, eplerenone (EPL), reduced platelet activation in patients with heart failure by a mechanism partially dependent on increased nitric oxide (NO) bioavailability.[12] It has also been shown that acute ALDO infusion attenuates NO-dependent vasodilatation to acetylcholine in healthy volunteers, providing evidence for an ALDO-induced endothelial dysfunction.[13]

Thus, our aim was to extend our study and investigate:

- The effect of acute ALDO infusion on primary haemostasis.
- The role of NO and oxidative stress in the mechanism of the ALDO prothrombotic action.
- The role of MR in the mechanism of the ALDO prothrombotic effect.

Materials and methods

Animals

Normotensive male Wistar rats weighing 300–350 g were used in this study. The animals were housed in a room with a 12-h light/dark cycle, and were given tap water and fed a standard rat chow. For 24 h before the experiment, the animals were fasted but allowed free access to water. The 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 the Guidelines for the Use of Animals in Biomedical Research.[14]

Chemicals

The following reagents were used in the study: collagen (Chronolog, USA), EPL (Pfizer, Poland), heparin (Polfa, Poland), pentobarbital sodium (Biowet, Poland), Oligotex Kit (Qiagen, USA), qPCR™ Mastermix, SYBR Green I (Eurogentec Seraing, Belgium), TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, USA), and Trizol (Invitrogen Life Technologies, USA). Bovine albumin, aldosterone, apyrase and HEPES (N-(2-hydroxyethyl) piperazine-N'-(2-ethanosulfonic acid) were delivered by Sigma-Aldrich (Poland). EDTA, ethanol, glucose, gummi arabici, magnesium chloride, natrium chloride, potassium chloride, sodium bicarbonate and trisodium citrate were provided by Polish Chemicals Reagents (Poland).

Experimental protocol

All investigations were carried out at the same time of the day (9 AM) to minimise any effect of diurnal variation in the haemostasis. ALDO was dissolved in 0.4% ethanol. ALDO (10, 30 and 100 μg/kg/h) or vehicle (VEH; 0.4% ethanol; 2 ml/kg/h) was infused into the femoral vein 5 min before the induction of venous thrombosis continuously for 1 h and 5 min (Constant-Rate Infusion Pump, Kwapisz, Poland). EPL, a selective MR antagonist, was administered (p.o., 5% aqueous gummi arabici solution) at a dose of 100 mg/kg 30 min before ALDO (30 μg/kg/h) or VEH infusion. The venous thrombosis was performed by ligation of the vena cava following pentobarbiturate anaesthesia (45 mg/kg i.p.), as previously described.[10,15] After 1 h, bleeding time (BT) was measured and blood was drawn from the right ventricle of the heart prior to thrombus removal. For further investigations, whole blood, plasma and aortas were collected and deep-frozen (-70°C).

Experimental procedure

Aldosterone and potassium ions. The ALDO serum level was determined by radioimmunoassay (Aldosterone Coat-A-Count RIA Kit; DPC, Poland). The potassium ion (K^+) concentration was measured in urine samples from the urinary bladder (Bayer Diagnostic 348 Analyzer).

Primary haemostasis

Template bleeding time. Template BT was measured according to Dejana *et al*.[16] Briefly, at the end of the experiment, a standardised device was applied longitudinally on the dorsal part of the rat tail 9 cm from the tip, taking care to avoid large veins. Then the tail was placed into a cylinder with isotonic saline solution at 37°C. BT was measured in seconds from the time when the tail was cut until bleeding stopped (no rebleeding within 30 s).

Platelet adhesion to fibrillar collagen ex vivo and in vitro. After BT measurement, blood samples were taken into plastic syringes containing anticoagulant (170 mM trisodium citrate, 130 mM citric acid and 101 mM glucose) in a volume ratio of 9:1. Platelet rich plasma (PRP) was obtained by centrifugation of the blood at 180 *g* for 20 min at room

temperature. PRP was then centrifuged at 400 *g* for 15 min and the 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, 0.35% albumin, 0.5 U/ml apyrase, pH 6.5) by centrifugation at 400 *g* for 15 min. The washed platelets were finally suspended in calcium-free Tyrode-HEPES buffer (137 mM NaCl, 2.6 mM KCl, 5 mM HEPES, 0.9 mM $MgCl_2$, 5.5 mM glucose, 0.35% albumin, pH 7.4). The final concentration of platelets was 3×10^5 platelets/µl.

Platelet adhesion was carried out according to Mant.[17] Briefly, 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 the platelets were incubated for 15 min. The platelets were counted optically before and 15 min after collagen addition in a Bürker chamber after dilution with the Unpette system. The platelet adhesion index was calculated using formula: [(platelet count before adding the collagen - platelet count after adding the collagen) / platelet count before adding the collagen] × 100%.

In the in vitro experiments, ALDO (10^{-8}–10^{-5} M) was added to the washed platelets at the beginning of the 5-min preincubation time. In the control group, VEH was added in the same volume and manner. Platelet adhesion was then carried out as described above.

Haemodynamic parameters. The haemodynamic parameters were measured continuously throughout the study by an invasive method. The right carotid artery was isolated, a flow probe was gently placed and carotid blood flow (CBF) was continuously measured using the Ultrasonic Doppler Flowmeter (Transonic Systems Inc., Ithaca, USA). The systolic and diastolic blood pressure (SBP and DBP) were measured from the left carotid artery via a transducer (Harvard Apparatus, Germany). Heart rate (HR) was measured with ECG electrodes.

PAI-1 mRNA levels. PAI-1 mRNA levels were measured in the whole blood using the real-time PCR technique.

Nitric oxide bioavailability. The NO plasma level was measured colorimetrically as a nitrite/nitrate (NO_2/NO_3) concentration with the Correlate Assay Nitric Oxide NO-2/NO-3 Assay Kit (Assay Designs, USA). To determine a direct influence of ALDO on NO synthase (NOS) activity, mRNA amounts of NOS were measured in the aorta using the real-time PCR technique.

Oxidative stress. The markers of oxidative stress – hydrogen peroxide (H_2O_2) and malonyl dialdehyde (MDA) levels – were assayed in plasma with commercially available kits (Hydrogen Peroxide Colorimetric Detection Kit; Assay Designs, USA and MDA Adducts ELISA Kit; Cell Biolabs, USA). To evaluate the influence of ALDO on oxidative stress in the aorta, amounts of mRNA of NADPH oxidase and superoxide dismutase (SOD) were measured in rat aortas using the real-time PCR technique.

Real-time quantitative RT-PCR. NADPH oxidase, SOD, NOS, PAI-1 and β-actin expressions were quantified by real-time PCR using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA USA). Total RNA (1 µg) was extracted from the cells (whole blood/aorta) using Trizol reagent, and was processed directly to cDNA synthesis using the TaqMan Reverse Transcription Reagents kit. Briefly, 2.5, 2.0, 1.5, 1.0, 0.5 and 0.25 µl of synthesised cDNA were amplified in triplicate for both β-actin and each of the target genes to create a standard curve. Likewise, 2 µl of cDNA was amplified in triplicate in all the isolated samples for each primer/probe combination and β-actin. Each sample was supplemented with both respective 0.3 µM forward and reverse primers, a fluorescent probe, and made up to 50 µl using qPCR™Mastermix for SYBR Green I. Each of the following PCR primers was designed using PrimerExpress software (Applied Biosystem): 5' CATCCA GCTGTACCT CAGTC 3', and 5' GAAAGACTCTTTATTGTATTG 3', 5' GGAAACGCT GGAAGTCGTTTG 3' and 5' CTCACTA CAGGTACTTTAAAG 3', 5' CATCGGCGTGC TGCGG GATCAG 3' and 5' GGGCTGTTGGTGTCTGAGCCGG 3' 5' TGCTGGT GAATGCCCTCTACT 3' and 5' CGGT CATTCCCAGGTTCTCTA 3' 5'GTGGGGC GCCCCA GGCACCA 3' and 5' CTCCTTAATGTCACGCACGAT TTC 3' specific for mRNA of NADPH oxidase, SOD, NOS, PAI-1 and β-actin, respectively. β-actin was used as an active and endogenous reference to correct for differences in the amount of total RNA added to the reaction and to compensate for different levels of inhibition during reverse transcription of RNA and during PCR. Each target probe was amplified in a separate 96-well plate. All samples were incubated at 50°C for 2 min and at 95°C for 10 min, and then cycled at 95°C for 30 s, 56°C for 1 min and 72°C for 1 min for 40 cycles. SYBR Green I fluorescence emission data were captured and mRNA levels were quantified using the critical threshold (C_t) value. Controls without reverse transcriptase and with no template cDNA were performed with each assay. To compensate for variations in input RNA amounts, and efficiency of reverse transcription, β-actin mRNA was quantified and the results were normalised to these values. Relative gene expression levels were obtained using the $\Delta\,\Delta C_t$ method.[18]

Statistical analysis

Data are expressed as mean ± SEM and %. The results were compared between the groups by means of the Mann–Whitney *U* test. The incidence of thrombosis was compared between the groups using the exact Fisher test. *p*-values of less than 0.05 were considered statistically significant.

Table 1. Aldosterone serum levels, thrombus weight and incidences of thrombosis

	VEH	ALDO 10	ALDO 30	ALDO 100	ALDO 30 + EPL	EPL
ALDO level [pg/ml]	1015 ± 135	2156 ± 97**	2664 ± 45***	2929 ± 30***	2759 ± 36*** †††	1273 ± 145
Thrombus weight [mg]	0.41 ± 0.09	0.61 ± 0.23	0.86 ± 0.08**	0.68 ± 0.23*	0.67 ± 0.14* # ††	0.16 ± 0.09*
Incidences of thrombosis [%]	80	72	100	67	90	37

ALDO infusion at doses of 10, 30 and 100 μg/kg/h resulted in a significant, dose-dependent increase in the serum hormone level. ALDO at doses of 30 and 100 μg/kg/h significantly increased thrombus mass and incidences of thrombosis. Co-administration of EPL significantly diminished ALDO-induced thrombosis augmentation. When administered alone, EPL decreased thrombus mass and reduced the incidence of thrombosis in comparison to VEH.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. VEH; #$p < 0.05$ vs. ALDO 30; ††$p < 0.01$, †††$p < 0.001$ vs. EPL; $n = 8–12$

ALDO, aldosterone; EPL, eplerenone; VEH, vehicle

Results

Aldosterone serum levels and potassium urine concentrations

Acute ALDO infusion caused significant dose-dependent rises in serum concentration of hormone in comparison with the VEH group (table 1). Nevertheless, our VEH rats expressed increased basal concentrations of ALDO (1015 ± 135 pg/ml), as compared with non-operated rats (120 ± 30 pg/ml, $p < 0.001$) or animals with abdominal surgery (sham-operated, without venous stasis) (670 ± 48 pg/ml, $p < 0.01$), since the VEH rats were infused with saline, and additionally had concomitant stasis (local hypoxia and endothelium injury). Intra-abdominal surgery in normotensive rats (sham-operated), but without venous thrombosis induction, enhanced ALDO level in comparison with non-operated animals ($p < 0.001$). Increases in ALDO levels were paralleled by a dose-dependent increase in K^+ extraction. Significant rises in urinary potassium concentrations for ALDO 10 (103.5 ± 2 mM, $p < 0.01$), ALDO 30 (135.2 ± 4.3 mM, $p < 0.001$) and ALDO 100 (163.5 ± 4.4 mM, $p < 0.001$) were noted in comparison with VEH (56.9 ± 2.8 mM). Co-administration of EPL and ALDO 30 reduced this effect (77.3 ± 4, $p < 0.01$).

Venous thrombosis

In our earlier study, a 1-h infusion of ALDO at a dose of 30 μg/kg/h resulted in a significant rise in venous thrombus weight.[10] To obtain further confirmation for ALDO-induced thrombosis in normotensive rats, we performed a study with an acute ALDO infusion also at doses of 10 and 100 μg/kg/h. In this study, we found that doses of 30 and 100 μg/kg/h increased thrombus mass over around 52% and 40%, respectively, in comparison with the VEH group. Co-administration of EPL reduced thrombus mass (a decrease of 22% versus ALDO 30). The incidence of thrombosis showed the same pattern, with the highest frequency of thrombosis observed after ALDO 30. When administered alone, EPL significantly decreased thrombus mass and reduced the incidence of thrombosis in comparison with VEH (table 1). To investigate the mechanism by which ALDO promotes thrombosis, we used ALDO at a dose of 30 μg/kg/h (ALDO 30), because only this dose caused 100% of thrombosis in our study.

Primary haemostasis

To evaluate the influence of ALDO 30 on primary haemostasis BT was measured. A 1-h ALDO 30 infusion shortened BT in comparison with VEH ($p < 0.05$) (figure 1). Co-administration of EPL reversed the ALDO-induced effect. Moreover, EPL administered alone slightly lengthened BT in comparison with VEH.

In the next step of the primary haemostasis examination, platelet adhesion to fibrillar collagen was measured. Ex vivo platelet adhesion was higher in rats infused with ALDO 30 in comparison with VEH ($p < 0.01$). Co-administration of EPL reduced ALDO-increased platelet adhesion ($p < 0.01$). EPL given alone reduced platelet adhesion ($p < 0.05$ vs.

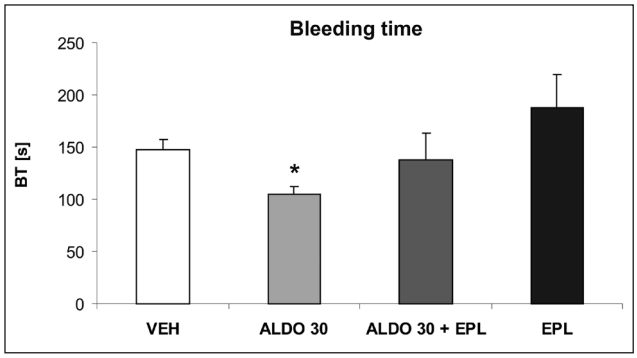

Figure 1. The effects of ALDO and MR blockade on bleeding time (BT).

A significant shortening in BT was observed after a 1-h ALDO 30 infusion. Co-administration of EPL reversed this effect. When administered alone, EPL slightly lengthened BT.

*$p < 0.05$ vs. VEH, $n = 6$

ALDO, aldosterone; BT, bleeding time; EPL, eplerenone; MR, mineralocorticoid receptor; VEH, vehicle

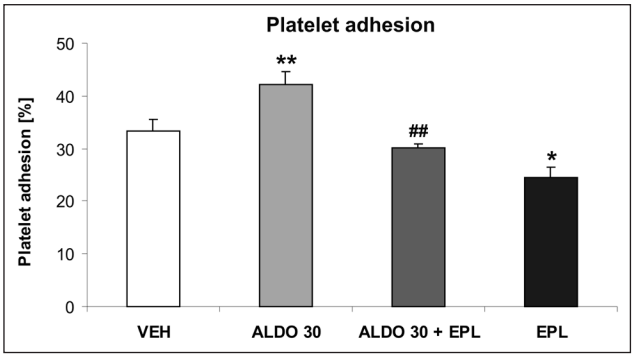

Figure 2. The effects of ALDO and MR blockade on platelet adhesion ex vivo.
ALDO 30 infusion significantly increased platelet adhesion. Co-administration of EPL reduced the effect of ALDO. When given alone, EPL considerably reduced platelet adhesion.
$^{*}p < 0.05$, $^{**}p < 0.01$ vs. VEH; $^{\#\#}p < 0.01$ vs. ALDO 30, $n = 6$
ALDO, aldosterone; EPL, eplerenone; MR, mineralocorticoid receptor; VEH, vehicle

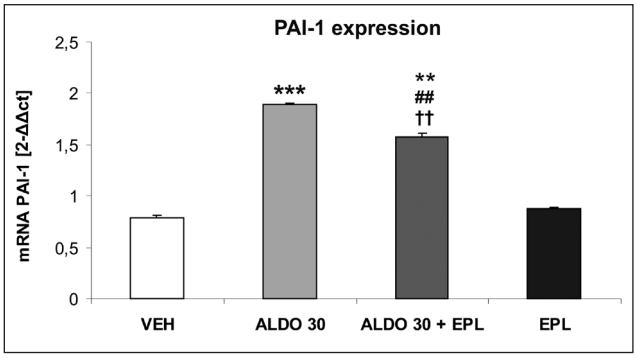

Figure 3. The effects of ALDO and MR blockade on PAI-1 expression in rat aorta.
A 1-h ALDO 30 infusion caused a significant rise in the PAI-1 mRNA level. EPL co-administered with ALDO 30 reduced the rise in PAI-1 expression. EPL given alone did not alter PAI-1 expression in comparison to VEH.
$^{**}p < 0.01$, $^{***}p < 0.001$ vs. VEH; $^{\#\#}p < 0.01$ vs. ALDO 30; $^{\dagger\dagger}p < 0.01$ vs. EPL, $n = 8–12$
ALDO, aldosterone; EPL, eplerenone; MR, mineralocorticoid receptor; PAI-1, plasminogen activator inhibitor; VEH, vehicle

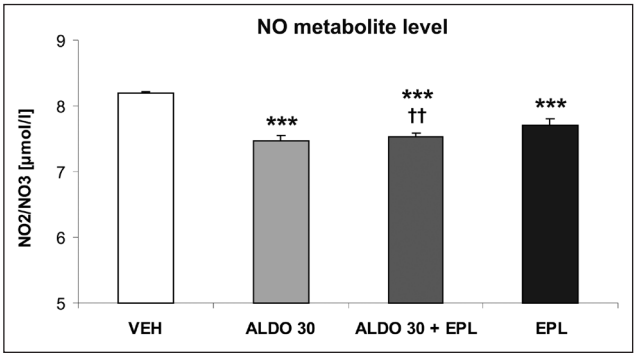

Figure 4. The effects of ALDO and MR blockade on plasma NO metabolite concentration.
A significant decrease in plasma NO_2/NO_3 level was noted in rats infused with ALDO 30. This effect was not influenced by MR blockade. When administered alone, EPL significantly reduced the plasma NO metabolite level.
$^{***}p < 0.001$ vs. VEH; $^{\dagger\dagger}p < 0.01$ vs. EPL, $n = 8–12$
ALDO, aldosterone; EPL, eplerenone; MR, mineralocorticoid receptor; NO, nitric oxide; VEH, vehicle

VEH) (figure 2). In an in vitro study, ALDO (10^{-8}–10^{-5} M) did not change platelet adhesion in comparison to the control group (data not shown).

Haemodynamic parameters

To investigate if ALDO-induced shortening of BT is haemodynamic dependent, we measured CBF, SBP, DBP, and HR during a 1-h ALDO 30 infusion. We observed that the initial values of CBF were similar in all groups and averaged similar levels at the end of the study. The initial rates of SBP and DBP were also similar in VEH, ALDO 30, and ALDO 30 with EPL groups, and were unaffected throughout the study. However, when administered alone, EPL caused a temporary (from the 15th to the 35th minute) fall in DBP ($p < 0.01$ vs. VEH) (data not shown). The initial and final values of HR were comparable in all groups.

PAI-1 expression

In our previous study, the increase in thrombus mass was paralleled by a marked increase in PAI-1 plasma level in the group treated with ALDO at a dose of 30 µg/kg/h.[10] We subsequently attempted to test whether changes in PAI-1 plasma concentrations were supported by changes in PAI-1 expression in the blood. A 1-h ALDO 30 infusion increased PAI-1 mRNA level ($p < 0.001$ vs. VEH). EPL co-administered with ALDO 30 reduced this effect ($p < 0.01$). EPL given alone did not alter PAI-1 expression in comparison with VEH (figure 3).

Nitric oxide bioavailability

To establish the influence of acute ALDO infusion on NO activity, plasma NO metabolite levels were measured. ALDO 30 decreased plasma NO_2/NO_3 level as compared with VEH ($p < 0.001$). This effect was not influenced by EPL. However, EPL administered alone reduced NO metabolite level in comparison with VEH ($p < 0.001$) (figure 4).

The fall in plasma NO metabolite concentration in the ALDO 30-treated group was paralleled by a decrease in aorta NOS mRNA level. EPL did not reverse the effect of ALDO 30, although when administered alone, it reduced the basal mRNA NOS level ($p < 0.05$) (figure 5).

Oxidative stress

To determine whether acute ALDO infusion changes the oxidative stress parameters, plasma levels of H_2O_2 and

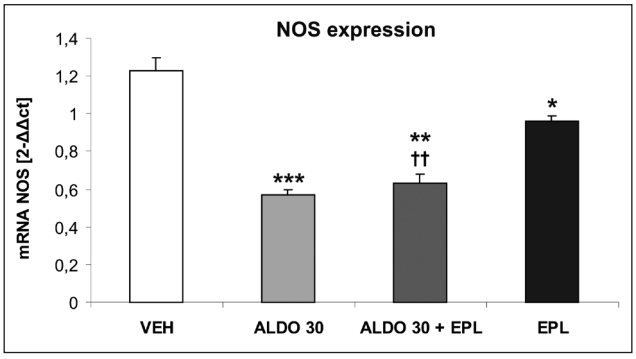

Figure 5. The effects of ALDO and MR blockade on NOS expression in rat aorta.
A marked fall in the aorta NOS mRNA level was observed in the ALDO 30-treated group. EPL did not reverse the effect of ALDO 30, although when administered alone, it reduced the basal mRNA NOS level.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. VEH; $^{\dagger\dagger}p < 0.01$ vs. EPL, $n = 8–12$
ALDO, aldosterone; EPL, eplerenone; MR, mineralocorticoid receptor; NOS, nitric oxide synthase; VEH, vehicle

MDA were assayed. Moreover, expressions of NADPH oxidase and SOD were measured in the aorta (table 2).

ALDO 30 infusion increased H_2O_2 plasma level ($p < 0.001$ vs. VEH). EPL did not reverse the effect of ALDO 30, although when given alone it increased the basal H_2O_2 plasma level ($p < 0.001$) (table 2).

A marked rise in MDA plasma concentration was observed after ALDO 30 infusion. EPL reduced MDA level in comparison with ALDO 30 ($p < 0.01$). A single administration of EPL increased the basal MDA level ($p < 0.001$) (table 2).

Following-up changes in oxidative stress plasma parameters, significant alterations in oxidative stress enzyme expression were observed. Infusion of ALDO 30 increased aortic expression of NADPH oxidase. Co-administration of EPL did not change the effect of ALDO. A single administration of EPL increased the basal NADPH oxidase mRNA level (table 2).

Simultaneously to the rise in NADPH oxidase expression, a considerable increase was observed in the aortic expression of SOD after ALDO 30. This effect was not changed by co-administration of EPL. Given alone, EPL increased the SOD mRNA basal level (table 2).

Discussion

In the present study, acute ALDO infusion was found to increase experimental venous thrombosis in the mechanism involving primary haemostasis, fibrinolysis, nitric oxide- and oxidative stress-dependent pathways. Our study is the first to indicate a direct prothrombotic effect of the acute ALDO action and its influence on haemostasis in normotensive rats. We also showed that MR blockade with its selective antagonist, eplerenone, partially yet significantly reduced ALDO-induced venous thrombosis augmentation.

The prothrombotic effect observed here developed within a two- to threefold increase in ALDO serum levels. The achieved ALDO serum concentrations were supraphysiological, which is in agreement with the finding that the level of a hormone may reach from 3–20 times the normal level during a circadian rhythm, hypertension or heart failure.[1,19,20] The local production of ALDO in the rat heart and local physiological ALDO levels were estimated to be approximately 20-fold higher in the heart tissue than in plasma.[21] Thus, tissue levels rather than plasma levels might be responsible for cardiovascular ALDO actions. Consequently, high plasma levels following ALDO infusion might be necessary to reach adequate pathological tissue levels to produce the effects shown here. We observed that the rises in ALDO serum levels were paralleled by increases in urinary K^+. This may also indicate that thrombosis development may increase according to ALDO-dependent K^+ raised excretion. A direct correlation of ALDO levels with mortality in patients with severe heart failure was shown in the CONSENSUS trial.[22] Moreover, the UK-HEART trial showed that the risk of sudden cardiac death in patients with chronic heart failure strongly correlates with a reduction in serum K^+ levels.[23] It was also shown that acute elevation of K^+ plasma concentration inhibits experimental thrombosis and decreases the sensitivity of human platelet aggregation to thrombin ex vivo.[24,25] Thus, we cannot exclude that ALDO-dependent electrolyte changes may participate in thrombosis development.

Table 2. The effects of ALDO and MR blockade on oxidative stress parameters

	VEH	ALDO 30	ALDO 30 + EPL	EPL
H_2O_2 [ng/ml]	130.9 ± 2.3	432.1 ± 9.2***	472.9 ± 3.4***†	427.7 ± 18.3***
MDA [pmol/mg]	0.26 ± 0.01	0.87 ± 0.02***	0.61 ± 0.01$^{***\#\#\dagger\dagger}$	0.41 ± 0.02***
NADPH oxidase mRNA[$2^{-\Delta\Delta ct}$]	1.34 ± 0.02	2.11 ± 0.08***	2.11 ± 0.05**†	1.95 ± 0.02**
SOD mRNA [$2^{-\Delta\Delta ct}$]	1.17 ± 0.01	1.57 ± 0.03***	1.54 ± 0.03**††	1.33 ± 0.02**

ALDO 30 infusion resulted in significant rises in H_2O_2 and MDA plasma levels, as well as in increased expression of SOD and NADPH oxidase. EPL, co-administered with ALDO 30, only reduced MDA level. When given alone, EPL increased the basal levels of the oxidative stress parameters.
$^{**}p < 0.01$, $^{***}p < 0.001$ vs. VEH; $^{\#\#}p < 0.01$ vs. ALDO 30; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ vs. EPL; $n = 8–12$
ALDO, aldosterone; EPL, eplerenone; MDA, malonyl dialdehyde; SOD, superoxide dismutase; VEH, vehicle

The interaction of platelets with subendothelial structures is thought to be of fundamental importance in thrombus formation. Thus, we investigated the influence of ALDO on primary haemostasis. BT has been extensively used to evaluate primary haemostasis in which platelets and vascular tone are the main factors.[26] We showed that a 1-h ALDO infusion at a dose of 30 μg/kg/h significantly reduced BT. We also observed that the ALDO infusion caused a 27% increase in platelet adhesion. In an in vitro study, preincubation with ALDO (10^{-8}–10^{-5} M) did not change platelet adhesion, indicating an indirect proadhesive action of ALDO. Since BT is also determined by actual pressure in the vessel, we also determined whether changes in blood pressure contribute to BT shortening. ALDO infusion did not influence CBF, SBP, DBP and HR. This is in agreement with the findings of other groups that neither acute 4-h ALDO infusion at a dose of 12 pmol/min/kg nor ALDO bolus injection at a dose of 100 μg changed basal BP and HR in healthy volunteers.[13,27] Thus, the effect of ALDO on BT appears to be dissociated from the vascular effects. Furthermore, the lack of changes in haemodynamic parameters during ALDO infusion indicates that the observed ALDO-induced thrombosis is not haemodynamic dependent. We suggest that the effect of ALDO on BT is MR dependent, since there was a tendency for BT prolongation in EPL-treated rats. Also, the proadhesive action of ALDO was mediated via MR, since EPL co-administration significantly reduced ALDO-induced platelet adhesion. Thus, influence on primary haemostasis appears to be one of the mechanisms of the acute ALDO prothrombotic action in normotensive rats.

When administered alone, EPL markedly diminished platelet adhesion and caused slight BT prolongation. Furthermore, EPL significantly, although temporarily, diminished DBP in normotensive rats. Thus, we postulate that EPL-induced changes in primary haemostasis result from the drug's influence on platelet and haemodynamic components. These are the first data showing the positive effect of EPL on platelet adhesion in rats.

We have previously shown that ALDO-induced thrombosis involved the activation of coagulation, expressed as an increase in TF plasma level, and fibrinolysis impairment, which resulted in a decrease in t-PA plasma level, and increases in TAFI and PAI-1 plasma concentrations. These haemostasis changes were partially MR dependent.[10] Consistently, a significant rise in mRNA PAI-1 level after ALDO infusion was noted in the present study. Co-administration of EPL significantly yet partially reduced PAI-1 expression. Therefore, we determined that one of the mechanisms by which ALDO impairs fibrinolysis is inhibition of PAI-1 synthesis, which is MR dependent. Recently, it was demonstrated that chronic EPL administration resulted in a decrease in myocardial mRNA expression of PAI-1 in rats with myocardial infarction.[28] Moreover, Brown *et al*. showed that chronic MR blockade with spironolactone significantly decreased PAI-1 mRNA expression in rat kidneys following radiation injury.[29] The ALDO-up-regulated PAI-1 expression was previously shown in cultured renal mesangial cells and rat cardiomyocytes.[30,31] We conclude that the increase in PAI-1 synthesis is one of the mechanisms of the acute ALDO prothrombotic action in rats.

We propose that the possible mechanisms of ALDO-augmented thrombosis could also be related to endothelial dysfunction caused by decreased NO bioavailability and increased oxidative stress.[32,33] A direct involvement of reactive oxygen species (ROS) and NO in thrombus formation in vivo has been reported. It has been shown that platelet recruitment to a growing venous thrombus was decreased after intravenous administration of SOD and catalase in rats, whereas it was enhanced by inhibition of NOS.[34] Thus, we investigated the effect of acute ALDO infusion on NO bioavailability in rats undergoing thrombosis. A marked decrease in plasma NO metabolites level was observed after ALDO infusion. The mechanism of decreased NO level may be related to reduced NO synthesis, since we observed reduced NOS expression in the ALDO group. There has been one report strongly supporting the harmful effect of acute ALDO infusion on endothelial function in vivo. Farquharson and Struthers demonstrated that 4-h ALDO infusion resulted in endothelial, NO-dependent vasodilator dysfunction in volunteers.[13] Although the pattern of ALDO-induced NO bioavailability changes in our and other studies is similar, comparison of the results seems to be difficult due to differences in experimental protocols (species, ALDO doses, time of administration, assayed parameters). So far, a more direct ALDO effect on NO synthesis has been shown mainly in in vitro studies. Nagata *et al*. described diminished eNOS expression in human umbilical vein endothelial cells after 16-h incubation with ALDO.[35] It has also been proved that ALDO inhibits NO synthesis under cytokine-stimulated conditions in cultured rat vascular smooth muscle cells.[36]

A few data have demonstrated that PAI-1 expression could be regulated by NO. It has been shown that NOS inhibition leads to increased PAI-1 expression in endothelial cells, whereas the NO precursor, L-arginine, reverses this effect.[37] Thus, the ALDO-induced fibrinolytic imbalance in our model could be at least partially related to reduced NO bioavailability. Notably, the lack of ALDO influence on platelet adhesion in vitro may suggest the indirect mechanism of ALDO-induced platelet activation associated with a decrease in NO synthesis and release from endothelial cells. It has been shown that NOS inhibitors intensified platelet adhesion leading to shortened BT in healthy humans.[38]

Bearing in mind the close relationship of NO and oxidative stress, the expressions of NADPH oxidase and SOD in rat aortas, as well as plasma H_2O_2 and MDA levels were measured after ALDO infusion. We found the rises in enzyme mRNA levels were accompanied by a notable augmentation in the plasma concentration of oxidative stress parameters. The expressions of enzymes were increased

and H_2O_2 plasma level was elevated after ALDO infusion. A significant rise was also observed in the level of MDA. A similar pattern of changes was shown by Miyata *et al*., who found that 3-h incubation of rat mesangial cells with ALDO stimulated NADPH oxidase activation.[39] Furthermore, chronic ALDO administration increased oxidative stress in rats, expressed as levels of plasma 8-isoprostane and thiobarbituric acid-reactive substances, as well as NADPH expression in endothelial cells.[40,41] These changes were observed even when the ALDO level was far lower (approximately fivefold) than the one we obtained. We suggest that short-term ALDO infusion increases oxidative stress with a simultaneous decrease in NO bioavailability in rats, leading to thrombosis augmentation. Similarly, Yao *et al*., showed that catalase and SOD prevented platelet-dependent cyclic flow variations, suggesting that both O_2^- and H_2O_2 enhance thrombus formation in vivo.[42]

The role of MR in ALDO-induced oxidative stress and the decrease in NO bioavailability is not fully evident. In our study, EPL markedly abrogated the effect of ALDO on the level of MDA, the product of membrane phospholipid peroxidation, while MR blockade did not have an influence on the expression of NADPH oxidase, SOD and NOS, or H_2O_2 and NO metabolite levels. It has been proven that EPL may change NO bioactivity by mechanisms not related to MR antagonism. Mutoh *et al*. showed MR-independent down-regulation of endothelial caveolin-1 (which plays a role as a negative regulator of eNOS) and increased eNOS phosphorylation by EPL.[43] In contrast, chronic EPL administration normalised O_2^- generation in intact aortas and reduced NADPH oxidase activity to basal levels in rabbits with atherosclerosis, which was associated with improvements in relaxations to acetylcholine.[44] Farquharson and Struthers have proved that spironolactone therapy in patients with chronic heart failure improves NO-dependent endothelial vasodilator dysfunction.[45] We also showed that spironolactone improved fibrinolysis and NO bioavailability as well as reduced oxidative stress in renal hypertensive rats (2K1C), while it was not effective or even impaired fibrinolysis and increased oxidative stress in normotensive rats.[46] Also in the present study, EPL had similar effects – though less pronounced – to ALDO on fibrinolysis and oxidative stress when administered alone. Correspondingly, Ma *et al*. also noticed spironolactone-improved fibrinolysis in hypertensive patients, whereas drug therapy was neutral in normotensive subjects.[47] The differential effects of MR antagonists in normotensive and hypertensive subjects may reflect the relative activity of the RAAS in these two groups. The antithrombotic effect of EPL in normotensive rats demonstrated here, involving primary haemostasis, seems not to be related to RAAS inhibition.

We suggest that the endogenous ALDO level increase could be responsible for the observed acute EPL action, since an additional increase in the endogenous ALDO serum level after a single EPL administration was observed. According to EPL pharmacokinetics data, a dose of 100 mg/kg is sufficient to block MRs in rats. It is likely that during full blockade of MRs, endogenous ALDO, as a non-selective agonist, could evoke non-MR-dependent action and bind to glucocorticoid receptor (GR). It is well known that the effects of GR activation are closely related to endothelial dysfunction, inhibition of NO production, NADPH oxidase stimulation and von Willebrand factor increase.[48] The precise mechanism in which acute EPL administration may reveal some deleterious effects of ALDO on fibrinolysis and oxidative stress in normotensive rats should be evaluated.

We demonstrated that EPL is not effective in reversing ALDO-induced changes in NO bioavailability and oxidative stress. However, this does not exclude the involvement of these pathways in ALDO's prothrombotic effect. Thus, the involvement of other mechanisms in ALDO action can be suggested. It was shown that some of the effects of ALDO are mediated by angiotensin II (Ang II). ALDO interacts with Ang II to increase PAI-1 expression in vascular smooth muscle cells.[49] Moreover, ALDO-induced increases in NADPH oxidase activity and ROS production in rats were attenuated by Ang II receptor (AT_1) antagonist.[41] Thus, it is likely that in our model the harmful effects of ALDO also depend on Ang II action. We previously showed that acute Ang II infusion promoted thrombosis in the same model.[50] Furthermore, in our preliminary study, the AT_1 receptor antagonist valsartan partially, although significantly, reduced ALDO-induced thrombus augmentation. Valsartan also diminished the ALDO-induced plasma haemostatic changes, expressed as a decrease in the t-PA level and an increase in the TAFI level, and markedly blunted the increase in PAI-1 level (data not shown). Thus, it would appear that in the prothrombotic effect of ALDO in venous thrombosis, Ang II is involved. Moreover, there is a growing recognition of non-genomic, rapid responses to ALDO, sensitive or insensitive to the classical MR inhibitors (spironolactone, EPL).[51] Non-genomic actions may represent a group of responses mediated by several different, non-classical ALDO receptors and/or responses to second messenger systems involved in this action.[52] Thus, we cannot exclude that both these pathways are involved in the ALDO-induced haemostasis disturbances. However, a distinction between genomic and non-genomic effects in our experiment has remained elusive.

For the first time, our data indicate that surgery and venous stasis may lead to a rise in serum ALDO levels in rats. We noticed, similarly to others, that the basal physiological ALDO serum level in the control non-operated rats was approximately 120 pg/ml.[53] The surgical procedure with concomitant stasis induction (local hypoxia and endothelium injury) increased the basal ALDO serum level fivefold. This may suggest an activation of RAAS, leading to a post-operative 'hyperaldosteronism' in rats undergoing surgery. This is in agreement with the observation that the levels of this hormone may substantially exceed the normal levels during surgical intervention.[5] We also demonstrated that

venous stasis caused an additional twofold increase in the ALDO serum level. These observations may have (1) a methodological implication for the model used; (2) indicate ALDO as a factor which may play a role in haemostatic disturbances occurring during stasis caused by, for example, immobilisation, congestive heart failure and obesity.

Conclusion

In conclusion, the present study provides strong evidence that short-term ALDO infusion enhances experimental venous thrombosis in the mechanism involving primary haemostasis, fibrinolysis, NO and oxidative stress-dependent pathways. The prothrombotic effect of ALDO is partially mediated by MR. Our data provide direct evidence pointing to ALDO as a risk factor for venous thrombosis development. Our study is a major step forward to better understanding the mechanisms involved in the thrombotic complications occurring during a short-lasting increase of ALDO.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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