Effects of enalapril on disseminated intravascular thrombin formation during systemic inflammation

Claudia Marsik a,b, Monika Graninger a, Nigel Mackman c, Bjarne Osterud d, Thomas Luther e, Bernd Jilma a,*

a, *Department of Clinical Pharmacology, Vienna University, Vienna, Austria
b Institute for Medical and Chemical Laboratory Diagnostics, Vienna University, Vienna, Austria
c Department of Immunology and Vascular Biology, The Scripps Research Institute, La Jolla, CA, USA
d Institute of Biochemistry, University of Tromso, Tromso, Norway
e Institute of Pathology, University of Dresden, Dresden, Germany

Received 4 November 2002; accepted 27 January 2003

Abstract

Background: Tissue factor (TF), the main trigger of coagulation is important in the propagation of cardiovascular diseases. Based on an in vitro study, we hypothesised that enalapril may blunt the endotoxin-induced, TF-triggered coagulation in humans. Methods: In a randomised, controlled trial, 30 healthy male volunteers received 2 ng/kg of lipopolysaccharide (LPS) after pre-treatment with placebo or enalapril for 5 days or with enalapril 2 h before LPS infusion. Results: Infusion of LPS increased interleukin-6 levels 400 fold, and induced a 10-fold increase in prothrombin fragment, a fourfold increase in D-dimer, and a fivefold increase in plasmin–antiplasmin complexes. However, pre-treatment with enalapril did not blunt LPS-induced coagulation. Conclusions: Our trial provides evidence against a modulatory role of angiotensin converting enzyme in LPS-induced, TF-triggered coagulation.

Keywords: Angiotensin converting enzyme; Endotoxins; Enalapril; Prothrombin fragment; D-dimer; Randomised controlled trial

1. Introduction

Adverse cardiovascular events may be triggered by antecedent infections via an increased incidence of thrombotic events following infectious diseases [1–3]. While endotoxin responsive elements are currently subject of intensive investigation in the cardiovascular field [4], it is well known that innate immunity and coagulation are intricately linked [5].

In particular, systemic inflammation, including endotoxemia, induces tissue factor. Disseminated intravascular coagulation (DIC) causes changes in plasma levels of all coagulation factors: clinical studies reported that tissue factor (TF) induced coagulation increased levels of soluble TF, prothrombin fragment (F1+2), and soluble fibrin, resulting in increases of fibrin split products such as D-dimer. These markers are also increased in patients with unstable angina [6]. Lipopolysaccharide (LPS) induced thrombin generation is considered due to cytokines [7,8]: while TNF does not contribute [9], IL-6 may play a pivotal role [10], although experimental proof in humans is still lacking. Tissue factor forms a highly procoagulant complex with activated factor VII (FVIIa), which initiates the coagulation cascade [11]. Inappropriate in vivo expression of TF on monocytes is not only found in DIC, but also in patients with acute coronary syndrome [12].

Angiotensin-converting enzyme inhibitors (ACE-Is) have become a mainstay in the treatment of hypertension, congestive heart failure, and diabetic nephropathy. Although many of the effects of ACE-Is are linked to their...
anti-hypertensive action, numerous reports suggest alternative mechanisms of action related to the atherothrombotic process. Only recently, an in vitro trial suggested that ACE-I could possibly reduce NF-κB translocation and TF-expression in LPS-stimulated monocytes [13]. This in vitro study provided a model to further investigate whether inhibition of ACE activity could also modulate TF-induced coagulation in vivo.

As both NF-κB and TF are attractive targets for the treatment of DIC [14] and cardiovascular disease in general [12], we investigated whether the ACE-I enalapril could inhibit TF-induced coagulation in a well standardised model [15] of human endotoxemia [16–18].

2. Methods

2.1. Study design

The study was approved by the Institutional Ethics Committee. Written informed consent was obtained from all participants. The study was randomised, double-blind, placebo-controlled in three parallel groups (n=10/group).

2.2. Study subjects

Thirty healthy male volunteers were invited to participate in this trial. All subjects were 19–35 years of age with a body mass index between the 15th and the 85th percentile. Determination of health status included medical history, physical examination, laboratory parameters, virological and standard drug screening. In addition, study subjects were tested for hereditary thrombophilia, i.e., Factor V Leiden, protein C and S deficiency, to minimise potential risks imposed by endotoxin-induced coagulation activation. Exclusion criteria were regular or recent intake of medication including non-prescription medication, and relevant abnormal findings in medical history or laboratory parameters.

2.3. Study protocol

Group A (active) received enalapril 20 mg (Renitec, MSD) for 6 consecutive days (last dose 2 h prior to LPS infusion). Group B (single dose group) received placebo for 5 consecutive days and a single dose of 20 mg enalapril on the study day 2 h prior to LPS infusion. Group C (control) received placebo only. The experimental procedures of the endotoxin infusion studies have been described in detail previously [19,20]. Briefly, volunteers were admitted to the study ward at 8:00 a.m. after an overnight fast, because the response to endotoxin varies with daytime. Throughout the entire study period, subjects were confined to bedrest and kept fasting for 8.5 h following LPS infusion. A 5% glucose infusion (Leopold Pharma, Vienna, Austria) was started at 8:30 a.m. and continued over 8.5 h at 3 ml/kg/h to maintain adequate hydration. Two hours after enalapril (or placebo) intake, subjects received a bolus of 2 ng/kg LPS i.v. (National Reference Endotoxin, Escherichia coli; USP Convention, Rockville, MD, USA).

2.4. Sampling and analysis

Sampling times were selected based on the kinetics of coagulation effects seen in subjects challenged with LPS in previous LPS trials and on the kinetics of TF up-regulation [17,20]. Blood samples were collected by repeated venipunctures at all time points into EDTA anticoagulated Vacutainer tubes (Becton Dickinson [BD], Vienna, Austria) before LPS infusion, and thereafter at times indicated in the figures (except leukocyte counts which were obtained from an indwelling venous line on the contralateral arm from where LPS had been administered). Plasma samples were processed immediately by centrifugation at 2000 g at 4 °C for 15 min and stored at −80 °C before analysis. TF expression and CD11b expression were quantified by flow cytometry on a BD FACS Calibur flow cytometer. The anti TF antibody (American Diagnostica, NJ, USA) was FITC labelled and the anti-CD11b antibody was phycoerythrin labelled (BD). Results are presented as mean fluorescence intensity (MFI) and percentage of positive cells [20]. Cell subpopulations were gated according to their location on the FSC/SSC dot plot, and subsets were then plotted against their fluorescence (FL1/FL2). Neutrophil counts were obtained with a cell counter (Sysmex, Milton Keynes, UK), and monocyte counts were estimated from FSS/SSC flowcytometry counts [20].

The following commercially available assays were used: prothrombin fragment F1+2 (Behring; Marburg, Germany; normal value <1.9 nmol/l), fibrinolysis was assessed with the following assays: plasmin–antiplasmin (PAP) complexes (Enzygnost PAP micro, Behring; normal range: 120–700 μg/l), measuring plasmin activity; the fibrin split product D-dimer (Boehringer Mannheim, Mannheim, Germany; normal values <400 ng/ml) which reflects fibrinolytic digestion of cross-linked fibrin, interleukin-6 (normal range: 0.4–2.2 pg/ml) [21] and tumour necrosis factor alpha (normal range: 1–6 pg/ml) high sensitivity EIA, R&D Systems, MN, USA). TF levels in human plasma were quantified as described previously [22]. ACE activity was measured with an ACE reagent (Sigma Diagnostics, St. Louis, MO, USA) on a Hitachi 911 analyzer. Expected mean values are 30 U/l and the coefficients of variation for intra- and inter-assay variability were<5%.

2.5. Data analysis

Data are expressed as mean and the 95% confidence intervals (C.I.s) for description in the text. Non-parametric statistics were applied. All statistical comparisons within
groups were done with the Friedman analysis of variance (ANOVA) and the Wilcoxon signed rank test for post hoc comparisons. The Kruskal–Wallis ANOVA and the Mann–Whitney U-test were used to test changes in endpoints between groups for statistical significance. A two tailed P-value of <0.05 was considered significant.

2.6. Power calculation

A power calculation was based on the equation by Stolley and Strom [23].

3. Results

3.1. Plasma ACE activity

As outlined above our trial included three treatment arms. A single dose of 20 mg enalapril decreased ACE activity by 95% (95% confidence interval: 91–99%; P<0.01) at 2 h after LPS infusion, i.e., when coagulation starts. Similarly, those subjects, who had ingested enalapril for 5 days prior to the study day, presented with an 80% lower ACE activity on the study day. A further decrease in plasma ACE activity to about 5% of the initial value was seen 4 h after intake of the last tablet.

3.2. Effect of enalapril on systemic coagulation

3.2.1. Coagulation

Because of difficulties in assessing TF activity on non circulating cells in-vivo, we concentrated on downstream coagulation factors to evaluate a potential dampening effect of ACE inhibition on LPS-induced coagulation. Enalapril and control groups exhibited the same level of thrombin formation as measured by prothrombin fragment (F$_{1+2}$) concentrations (Fig. 1). Plasma levels of F$_{1+2}$ increased almost 10-fold at 4 h (P<0.001 vs. baseline, Fig. 1). n-Dimer increased constantly and peaked fourfold over baseline in the placebo group at 24 h and PAP-levels increased 4–6-fold at 2 h (P>0.05 between treatment groups).

3.2.2. Cytokine levels

Pre-treatment with enalapril neither affected peak TNF-levels 296 pg/ml (95% C.I.: 234–359 at 90 min; n=30) nor peak IL-6 levels 494 pg/ml (95% C.I.: 376–612 at 3 h; n=30), data not shown.

3.3. Regulation of tissue factor

3.3.1. TF expression

As expected, neutrophils and monocytes became highly activated as measured by a threefold increase in CD11b expression; yet, mean TF fluorescence intensity of peripheral monocytes was only increased by 30% (C.I.: 2–60%) at 4 h and returned to baseline levels at 24 h (P=n.s. between groups; data not shown). Twenty-four hours after LPS infusion, monocyte counts returned to baseline and TF expression was no longer elevated.

3.3.2. Plasma TF

Baseline levels of plasma TF averaged 149 pg/ml (95% C.I.: 115–184) and did not significantly vary over time, although a trendwise increase was observed after 24 h (mean: 164; 95% C.I.: 126–203).

3.3.3. Monocyte counts

Baseline values for all parameters were similar in all groups. Monocyte counts fell to almost undetectable values 2 h after LPS infusion, and nearly recovered at 6 h in all study groups.
3.3.4. Mean arterial pressure (MAP)

The dose of enalapril given in this trial follows the European guidelines for treating hypertensive patients. In our trial, enalapril did not significantly lower blood pressure of healthy young volunteers under LPS. Overall, the effects of LPS with and without enalapril on blood pressure and heart rate were mild to moderate. Mean basal MAP levels were slightly lower in subjects pretreated with enalapril (81 mmHg, C.I. 76–86) compared to placebo (86 mmHg, C.I. 79–93), and single dose enalapril (87 mmHg C.I. 82–93). Minimal MAP levels were reached at 6–7 h in all groups (pretreated 71 mmHg C.I. 61–80, placebo 76 mmHg C.I. 69–83, single dose 68 mmHg C.I. 63–73). MAP levels below 60 mmHg were observed at 7 h in four subjects receiving enalapril (three in pretreatment group and one with single dose), but in none of the placebo treated subjects.

4. Discussion

It has been reported that ACE inhibitors decrease LPS induced NF-κB translocation and hence TF-activity in human monocytes [13]. Based on this in vitro study, we hypothesised that inhibition of ACE activity could translate into inhibition of LPS-induced TF-triggered coagulation. We therefore used the human endotoxin model, which unequivocally induces TF-mediated coagulation [16–18].

As expected, we observed only a small TF-up-regulation on circulating monocytes by flow cytometry despite marked activation of monocytes as measured by the increase in CD11b expression. The reason for this phenomenon could be the margination of TF positive monocytes in the microvasculature including the lung, which was observed in a mouse endotoxemia model (Luther et al., unpublished data). Therefore, in vivo activated TF-expressing monocytes are not accessible by flow cytometric analysis. Well aware of this limitation, we focused on the LPS effect on downstream coagulation as measured by F1+2 and D-dimer levels. As shown in Fig. 1, prothrombin fragment increased about 10 fold in all treatment groups. Similarly, enalapril did not alter the LPS induced PAP-release.

Hence contrary to the in vitro study [13] enalapril was not able to blunt TF-triggered coagulation in vivo. This could be due to the enalapril dose administered to our volunteers. The 20 mg enalapril dose is expected to yield plasma enalaprilat concentrations of <0.1 μg/ml, which however were ineffective in vitro [13]. Yet, the administered dose of enalapril is the highest recommended dose according to the Austrian summary of product characteristics, and we achieved 95% inhibition of ACE activity. This indicates that clinically relevant suppression of ACE activity does not alter LPS-induced, TF-mediated coagulation in humans. As this is a negative finding, we have performed a power calculation.

We had an 80% power to detect a 50% difference in F1+2 levels. This was deemed adequate in view of the 50% decrease in TF activity induced by captopril in vitro, and the demonstrated >500% increase in thrombin generation between anticoagulants and placebo usually found in this model [20].

Another explanation for the lack of effect of enalapril may be the differential behaviour of isolated monocytes and monocytes in whole blood [24]. This has been demonstrated for different physiological mediators and drugs. For example, salicylate inhibited TF-expression in LPS-stimulated isolated monocytes whereas aspirin or salicylate enhanced TF-expression in whole blood, and did not affect LPS induced coagulation in vivo [20,25–28]. The same may hold true for ACE inhibitors.

One may criticise the dose of enalapril administered. However, the selection of the dose was based on a number of large, randomised trials demonstrating a survival advantage like the ACESS [29] and OVERTURE trials [30] administering either 20 mg of enalapril once a day or 10 mg b.i.d.

While Nanas et al. [31] described a multicentre trial with high dose enalapril of 60 mg once compared to 20 mg in patients with congestive heart failure, it did not yield significant differences either in mortality or in HR and SBP between treatment groups. Thus the dose was based on large randomised controlled trials (RCTs). Additionally, we did not intend to further increase the dose of enalapril, because this may adversely affect blood pressure. We considered that the minimal MAP should not decreased below 60 mmHg because this MAP is regarded the minimum necessary for adequate renal perfusion and indeed MAP values fell below 60 mmHg in four out of 20 volunteers receiving enalapril but in none of the placebo group. Besides, we assumed that this problem may be accentuated in septic patients, who are hypotensive.

Although our findings are mainly negative, they may still have clinical implications. First ACE-Is may be safely used in patients with cardiovascular disease, even if they are critically ill. The unaltered coagulation and fibrinolysis profile in response to systemic inflammation contributes to a good safety profile. Secondly, it has been suggested that adverse cardiovascular events may be triggered by antecedent infections. From our data it can be anticipated that the use of ACE-Is will not lower the increased incidence of thrombotic events following infectious diseases [1–3]. The concept of ACE-Is in DIC was initially supported by an animal trial. The trial describing the effects of captopril was performed in an intravascular coagulation model in rats [32]. The amount of fibrin measured was significantly lower in thrombin challenged animals treated with captopril compared to controls. However, our own data do not support the concept that the selected ACE-I (enalapril) would be beneficial in endotoxin induced DIC or other coagulopathies.

In conclusion, our trial provides evidence against a
modulatory role of ACE in LPS-induced, TF-triggered coagulation.

Acknowledgements

Supported in part by grant No. 8917 from the Jubiläumsfonds der Österreichischen Nationalbank.

References