The effects of direct thrombin inhibition with dabigatran on plaque formation and endothelial function in Apolipoprotein E-deficient mice

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Abbreviations: ApoE⁻/⁻: Apolipoprotein double knockout; Dabi: dabigatran; DHE: dihydroethidium; NO: nitric oxide; PAR: protease-activated receptor; ROS: reactive oxygen species; WT: wild type

Section: cardiovascular
Abstract

The recently developed oral anticoagulant dabigatran etexilate directly inhibits thrombin following activation by plasma esterases to dabigatran. Thrombin is involved in the pathogenesis of atherosclerosis. We investigated the effects of direct thrombin inhibition on atherosclerosis and endothelial function in a hypercholesterolemic mouse model with accelerated atherosclerosis (ApoE−/−-mice). ApoE−/− mice were treated with a cholesterol-rich diet for 12 weeks and either dabigatran etexilate (900mg/kg body weight) or vehicle. Wildtype (WT, C57/B6) mice served as control. Endothelial function was assessed with carbachol (endothelium dependent) using glyceroltrinitrate (endothelium independent) as control in aortic rings. Atherosclerotic lesion formation was evaluated with oil-red staining and vascular collagen content was determined by Sirius red staining. Reactive oxygen species (ROS) production was determined by semiquantitative immunohistochemical staining. Measurement of dabigatran plasma levels (622.3±169 ng/ml) and a performed coagulation test (diluted thrombin time, dTT) revealed a relevant anticoagulatory concentration. Dabigatran etexilate attenuated increased atherosclerotic plaque formation (ApoE−/−-Dabi: 16.1±3.8% of ApoE−/−-control, p<0.001), decreased collagen content (ApoE−/−-Dabi: 49.1±10% of ApoE−/−-control, p=0.01) and ROS production in DHE-staining (ApoE−/−-Dabi: 46.3±5.4% of ApoE−/−-control, p=0.005) in parallel to an improvement of endothelial function (ApoE−/−-control 42.6±2.7 vs. ApoE−/−-Dabi 62.9±3.3% of phenylephrine-induced contraction, p=0.001) at 100 µmol carbachol. These data suggest that direct thrombin inhibition in a relevant dosage improved endothelial function, and reduced atherosclerotic lesion size, collagen content and oxidative stress in hypercholesterolemic atherosclerosis. Interference with the coagulation system might provide a therapeutic target to modify atherosclerotic disease progression.
Introduction

Dabigatran etexilate is a novel oral anticoagulant drug inhibiting thrombin (factor-II) in its active form and thereby acting at the convergence of the intrinsic and extrinsic coagulation cascade (Schirmer et al., 2010). It has recently been shown to be at least non inferior or even superior in stroke prevention in atrial fibrillation compared to warfarin in the RE-LY-trial (Connolly et al., 2009). According to subgroup analyses of the RE-LY study it is currently a matter of debate whether dabigatran etexilate leads to increased coronary events compared to warfarin (Hohnloser et al., 2012; Uchino and Hernandez, 2012). However, linkage between coagulation and inflammation is well described (Esmon, 2005; van der Poll et al., 2011). The recent phase III trial might have been too short (median follow up in the RE-LY trial 2.0 years) to capture reliably atherosclerotic events. An experimental animal model can test the hypothesis whether novel anticoagulatory mechanisms affect atherosclerotic lesion formation. We therefore investigated the effects of direct thrombin inhibition on vascular function and atherosclerotic lesion formation in a well-established murine model of enhanced atherosclerosis.

Methods

Animals and Procedures

Animal procedures were performed in accordance with institutional guidelines, the German animal protection law and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). 10 weeks old male C57BL/6J mice (wild-type, WT) and apolipoprotein-E-deficient (ApoE−/−) mice (C57BL/6J, genetic background, Charles River, Sulzfeld, Germany) were used in this study. The animal studies were approved by local authorities (no.: 09/2009 and 26/2008).
Dose finding

According to recommendations by Boehringer Ingelheim, derived from the development program, dabigatran etexilate was used at a dose of 900 mg/kg bodyweight per day. This dosage was chosen due to a known bioavailability of 6%. This dose is also regarded to dabigatran etexilate being about 2-fold less potent in rat vs. human (Wienen et al., 2007) thus explaining why slightly supratherapeutic plasma concentrations were chosen for the experiments in mice. After scarification of the animals dabigatran plasma concentrations and the diluted thrombin time were determined.

Treatment

All mice were fed a high-fat, cholesterol-rich diet (24% fat, 24% protein, 41% carbohydrate, Research Diets, New Brunswick, NJ, D12451) for 12 weeks starting at the age of 10 weeks. ApoE\(^{-/-}\)-mice were treated with dabigatran etexilate (900 mg/kg body weight) via chow or served as control (cholesterol-rich diet only).

Heart rate and blood pressure

Systolic blood pressure and heart rate of mice were determined with the tail cuff-method on 5 consecutive days with 20 measurements per mouse per day at the end of the treatment period (BP-2000, Visitech-Systems, USA).

Endothelial function

The ascending aorta was dissected and immersed in ice-cold buffer of the following composition (mM): NaCl 118.0, CaCl\(_2\) 2.5, KCl 4.7, MgCl\(_2\) 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25.0, Na EDTA 0.026 and D(+)glucose 5.5, pH 7.4). Adventitial tissue was carefully removed. Experiments were conducted on aorta rings of 3 mm in length.
Endothelial function was measured using aortic rings (n=4/mouse) mounted in an organ-chamber filled with the above-described buffer (37°C; continuously aerated with 95% O2 and 5% CO2). Isometric contraction was determined with a force transducer. The aortic rings were gradually stretched over 20 min to a resting tension of 10 mN, which was maintained throughout the experiment, and was allowed to equilibrate. Pharmacologically-induced contraction of aortic rings was performed with phenylephrine (10 µmol). Drugs were added in increasing concentrations to obtain cumulative concentration-response curves for carbachol as an endothelium-dependent relaxing agent and nitroglycerin, as a NO donor. The drugs were washed out before adding the next substance. The relaxing effect of carbachol was abolished by adding L-NAME (1 µmol). Aortic rings with a phenylephrine-induced contraction below 5% of the baseline contraction (10 mN) and without any response to carbachol (relaxation <10%) were excluded from statistical analysis due to a presumable damage of the endothelium (Wassmann et al., 2004a).

Oxidative stress
Oxidative stress in the aorta was determined by assessing the vascular superoxide production in situ using dihydroethidium (DHE) fluorescence microscopy as described previously (Wassmann et al., 2004b).

Dihydroethidium fluorescence microscopy
Dihydroethidium (DHE) is the chemically reduced form of the commonly used DNA dye ethidium bromide. DHE itself is blue fluorescent (absorption/emission: 355/420 nm) in cell cytoplasm while the oxidized form ethidium is red fluorescent (absorption/emission: 518/605 nm) upon DNA intercalation. Aortas were carefully excised and placed in chilled, modified Krebs-HEPES buffer. Connective tissue was
removed and aortas were cut into 4-mm segments, which were embedded in Tissue Tek OCT embedding medium (Miles Laboratories), snap-frozen, and stored at -80°C. Samples were sectioned on a Leica cryostat (10 µm) and placed on glass slides. Then, Krebs-HEPES buffer containing 2 µmol/L DHE was topically applied to each tissue section and sections were incubated in a dark humidified chamber at 37°C for 30 min. In situ production of superoxide was visualized by fluorescence microscopy. Images were acquired with identical acquisition parameters and were stored digitally (Laufs et al., 2005).

**Atherosclerotic plaque formation**

Staining of the aorta (ascending aorta and aortic sinus) was performed by independent investigators to quantify plaque formation using oil-red O staining. After embedding of aortic tissue (see above), samples were sectioned on a Leica cryostat (10 µm), starting at the apex and through the aortic valve area into the ascending aorta. The samples at the ascending aorta were placed on slides. Five consecutive sections per animal were used for analysis. For the detection of atherosclerotic lesions, the sections were fixed with 3.7% formaldehyde for 1h, rinsed with de-ionized water, stained with oil red O working solution (0.5%) for 30 min or Sirius Red for 15 min, and were rinsed again. For morphometric analysis, hematoxylin staining was performed according to standard protocols. All sections were examined under a Nikon E600 microscope. Lucia Measurement Version 4.6 software was used to measure lipid-staining area and total area of the histological sections. The total lesion area of 4 cross-sections per mouse was calculated (Wassmann et al., 2004a).
Collagen content

To assess vascular collagen content, sirius red staining of aortic-sinus sections was performed. Aortic sections were incubated with the sirius red agent (picro-sirius red solution 0.1% containing sirius red 0.1%, picrin acid 1.2%) for 10 minutes after washing with descending ethanol concentrations (100%, 90% and 70%) and subsequent incubation with xylol for two minutes. Aortic collagen content was quantified using fluorescence microscopy through polarized light (G-2A-fluorescence filter, excitation-spectrum 510-560 nm, exposure time 83ms, 40-fold magnification). Aortic sections from each treatment group were processed in parallel, and images were acquired with identical acquisition parameters, especially a constant area of integration was recorded for each section. Collagen content was expressed as fraction (percentage) of the total cross-sectional area.

Statistical analysis

All data are expressed as mean±SEM. Following Kolmogorow-Smirnow testing for normal distribution, intergroup differences were assessed with an ANOVA and Dunnet’s post hoc analysis. SPSS 20.0 was used for statistical calculations. Statistical significance was assumed at a p-level <0.05.

Results

Heart rate, blood pressure and body weight

Dabigatran etexilate treated and control ApoE−/−-mice showed a decrease in heart rate and body weight compared to WT. Dabigatran etexilate treatment showed no difference in systolic blood pressure compared to WT and ApoE−/−-control. There were no obvious bleeding complications during the whole treatment period (table 1).
Dabigatran plasma levels and coagulation tests

Dabigatran plasma levels were determined at the end of the experiment (622.3±169 ng/ml, n=10 in ApoE mice). Under these concentrations a relevant increase in diluted thrombin time (dTT) could be observed (figure 1) (Love et al., 2007) by using HEMOCLOT (HYPHEN BioMed, France) as described previously (Stangier and Feuring, 2012).

Atherosclerotic plaque formation

Atherosclerotic lesion formation was quantified in the aortic sinus and the ascending aorta by histological analyses of oil-red-O-stainings. Following a cholesterol-rich diet, atherosclerotic lesion formation in ApoE-/--mice was significantly increased in the aortic sinus as well as the ascending aorta as compared to WT mice (WT: 32.4±8.3% of ApoE-/--control, p<0.001, n=10). Treating ApoE-/--mice with dabigatran etexilate showed a significant reduction of atherosclerotic lesion formation (ApoE-/--Dabi: 16.1±3.8% of ApoE-/--control, p<0.001, n=5) (figure 2).

Collagen Content

Collagen content in the vascular wall was quantified in the aortic sinus in all treatment groups by histological analysis of sirius red stainings. Vascular collagen was higher in ApoE-/- than in WT mice (WT: 66.2±9.6% of ApoE-/--control, p=0.014, n=8) Dabigatran etexilate decreased collagen content significantly (ApoE-/--Dabi: 49.1±10% of ApoE-/--control, p=0.002, n=5) (figure 3).

Oxidative stress

Semiquantitative detection of reactive oxygen species (ROS) release by DHE fluorescence microscopy revealed increased synthesis of reactive oxygen species in
ApoE−/− mice compared to wildtype mice (WT: 49.5±10.6% of ApoE−/− control, p=0.003, n=8). Treatment with dabigatran etexilate showed a significant reduction of ROS (ApoE−/− Dabi: 46.3±5.4% of ApoE−/− control, p=0.004, n=5) (figure 4).

Endothelial function:
Endothelium dependent relaxation of aortic rings was significantly impaired in ApoE−/− mice compared to WT mice at all carbachol concentrations (p<0.05). Treatment with dabigatran etexilate significantly improved endothelial dependent relaxation in ApoE−/− 42.6±2.7 vs. ApoE−/− Dabi 62.9±3.3%, p=0.001, n=10 of phenylephrine-induced contraction at 100 µmol carbachol (figure 5). Potency (pD2 (-log)) of endothelium dependent relaxation with carbachol significantly improved by treatment with dabigatran etexilate compared to ApoE−/− (table 2). Endothelium independent relaxation with glyceroltrinitrate of aortic rings was not different between treatment groups (table 2).

Discussion
In this study we demonstrate that the oral administration of the direct thrombin inhibitor dabigatran etexilate in relevant doses leads to a reduced atherosclerotic lesion size and improves endothelial function by reducing oxidative stress in Apolipoprotein E-deficient mice.

Thrombin plays a major role in the coagulation cascade cleaving fibrinogen to fibrin thereby promoting fibrin clot formation (Furie and Furie, 1992). In addition, thrombin can act as a modulator of endothelial function thereby promoting atherosclerosis at early stages (Borissoff et al., 2009). A link between coagulation and inflammation is well described (Esmon, 2005; van der Poll et al., 2011). Thrombin promotes
mechanisms of atherosclerosis such as the proliferation of vascular smooth muscle cells (Dabbagh et al., 1998; Ivey and Little, 2008), fibroblast stimulation (Chambers et al., 1998), nitric oxide synthesis (Eto et al., 2001; Watts and Motley, 2009), vascular tone (Derkach et al., 2000), recruitment of proinflammatory cells (Bizios et al., 1986), platelet aggregation, and thrombus generation (Eidt et al., 1989; Harker et al., 1995). Thrombin modulates vascular tone by generation of a biphasic vascular response with an early relaxation and a subsequent transient contraction mediated by the secretion of prostaglandin H₂ (PGH₂) or thromboxane A₂ (PGA₂) (Derkach et al., 2000). Prolonged incubation with thrombin has been reported to inhibit NO synthesis, which has a critical impact on endothelial function (Ming et al., 2004; Zhang et al., 2004). Once lesions in the endothelium are present, thrombin generation is concentrated at these sides of vascular injury (Hatton et al., 1989) and promotes a pro-inflammatory response mediated by protease activated receptor type 1 (PAR-1) (Hirano, 2007). In addition, elevated ROS levels correlate with endothelial dysfunction and progressive atherosclerosis (Harrison et al., 2003). Considering these pluripotent effects of thrombin on vascular biology, administration of a direct thrombin inhibitor might offer promising therapeutic options in treating vascular disease.

Dabigatran etexilate, an orally available direct thrombin inhibitor, has been shown to be superior to warfarin in the prevention of stroke in atrial fibrillation the RE-LY trial (Connolly et al., 2009). The initial results suggested a significant increase of non-fatal myocardial infarction in the dabigatran group compared to warfarin which was not robust after re-evaluation (Connolly et al., 2010). However, there was a concern about a potential promotion of atherosclerosis by inhibition of thrombin with dabigatran and a newly published meta-analysis of several randomized non-inferiority
trials regarding dabigatran seem to confirm these concerns (Uchino and Hernandez, 2012). Former experimental studies already showed beneficial effects on vascular pathology by direct thrombin inhibition with melagatran (Bea et al., 2006). They demonstrated a reduced atherosclerotic lesion size and a plaque stabilizing effect. Borissoff et al. revealed data regarding dabigatran etexilate having a strong and protective effect against atherosclerosis in mice with a pro-coagulant phenotype (TMPRO/PRO:ApoE−/−) (Borissoff et al., 2010). Preusch et al. found retardation in atherosclerotic lesion by treatment with dabigatran etexilate (Preusch et al., 2010). And Nagy et al. showed that treatment with dabigatran etexilate significantly inhibited atherosclerotic lesion formation in the aorta in low density lipoprotein-receptor deficient mice most likely due to remodeling of plaque extracellular-matrix (Nagy et al., 2011). These latter studies have hitherto only been published in abstract format.

Our findings are in line with these observations and additionally show a beneficial effect on endothelial function with reduction of oxidative stress and ROS production being the pathophysiological link. Furthermore we could demonstrate that dabigatran was present in relevant doses that yielded an anticoagulatory effect. Therefore our experimental studies do not provide support for mechanisms in favour of accelerated occurrence of atherosclerosis.

In conclusion, our study demonstrates protective effects of effective direct thrombin inhibition, on vascular remodelling at relevant doses. Thrombin inhibition by dabigatran leads to improved endothelial function and reduces oxidative stress, as well as inhibiting progression of atherosclerotic plaques and proliferation of collagen synthesis. Appropriately designed long term clinical trials will have to show whether
thrombin inhibition has beneficial effects on vascular outcomes to follow up on these experimental findings.

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Conducted experiments: Lee, Kratz, Baumhäkel

Performed data analysis: Lee, Kratz, Schirmer, Baumhäkel

Wrote or contributed to the writing of the manuscript: Lee, Kratz, Schirmer, Baumhäkel and Böhm
References


Footnotes:
Illkyu-Oliver Lee and Mario T. Kratz contributed equally to this work.

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Disclosures
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Legends for figures

**Figure 1:** Anticoagulatory effect was obtained by measuring diluted thrombin time (dTT) with HEMOCLOT. Plasma for performing this standard curve was obtained by pooling the control plasmas from WT mice, ApoE-mice respectively (n=10 for each group). dTT was determined at 0, 50, 125, 500, 1000 and 2000 ng/ml dabigatran.

**Figure 2:** Plaque formation after 12 weeks of treatment of WT and ApoE\(^{-/-}\)-mice with dabigatran etexilate (900mg/kg body weight) or vehicle (A). Histomorphometric changes in atherosclerotic lesion in WT (B) and ApoE\(^{-/-}\)-mice (C) (n=10). The effects of dabigatran etexilate (D) on atherosclerotic lesion formation depicted by representative sections with Oil Red O staining of the aortic sinus (n=5).

**Figure 3:** Vessel fibrosis after 12 weeks of treatment of WT and ApoE\(^{-/-}\)-mice with dabigatran etexilate (900mg/kg body weight), rivaroxaban (30 mg/kg bodyweight) or vehicle (A). Histomorphometric changes in vessel fibrosis in WT (B) and ApoE\(^{-/-}\)-mice (C) (n=10). The effects of dabigatran etexilate (D) on vessel fibrosis formation depicted by representative sections with sirius red staining of the aortic sinus (n=5).

**Figure 4:** Quantification of the effects on reactive oxygen species (ROS) in situ determined by dihydroethidium (DHE) fluorescence and representative fluorescence microscopy after 12 weeks of treatment of WT and ApoE\(^{-/-}\)-mice with dabigatran etexilate (900mg/kg body weight) (A). Increased synthesis of ROS in ApoE\(^{-/-}\)-mice (C) compared to WT (B). Reduction in ROS production by treatment with dabigatran etexilate (D).
Figure 5: Endothelial function. After 12 weeks of treatment of WT and ApoE^{-/-}-mice with dabigatran etexilate (900mg/kg body weight) or vehicle aortic rings were isolated, studied in organ chamber experiments, and compared with aortic rings of wild-type (WT) mice (A) *p<0.05 vs ApoE^{-/-}.
Table 1: Heart rate, blood pressure and body weight

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<th>systolic blood pressure [mmHg]</th>
<th>heart rate [bpm]</th>
<th>body weight [g]</th>
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<tr>
<td>WT</td>
<td>115.3±2.5</td>
<td>703.8±10.2**</td>
<td>35.7±1.1***</td>
</tr>
<tr>
<td>ApoE−/−</td>
<td>119.6±1.9</td>
<td>637.2±13.9</td>
<td>30.2±0.5</td>
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<tr>
<td>ApoE−/− + Dabi</td>
<td>123.7±2.6</td>
<td>602.0±14.0</td>
<td>29.3±1.0</td>
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</tbody>
</table>

Table 1: SBP and heart rate were determined in all animals (Mean±SEM). WT: n=20, ApoE: n=19, ApoE-Dabi: n=20. **p<0.01; ***p<0.001 vs. ApoE−/−
Table 2: Efficacy and potency of endothelium dependent relaxation

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ApoE⁻/⁻</th>
<th>ApoE⁻/⁻ + dabigatran</th>
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<td>pD₂ (-log) (Maximum</td>
<td>-5.8±0.2</td>
<td>-5.3±0.3</td>
<td>-6.3±0.3**</td>
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<tr>
<td>Relaxation (%) Aortic</td>
<td>(72.0±4.4)*</td>
<td>(42.6±2.7)</td>
<td>(62.9±3.3)**</td>
</tr>
<tr>
<td>tissue, response to</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carbachol)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>pD₂ (-log) (Maximum</td>
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<td>-5.6±0.1</td>
<td>-5.7±0.1</td>
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<tr>
<td>Relaxation (%) Aortic</td>
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<td>(214.9±12.4)</td>
<td>(234.7±10.4)</td>
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<tr>
<td>tissue, response to</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glyceroltrinitrate)</td>
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Table 2: Efficacy (maximum relaxation as percent of phenylephrine induced contraction at a concentration of 100 µmol carbachol and 10 µmol glyceroltrinitrate) and potency (pD₂) of endothelium dependent (carbachol) and independent (glyceroltrinitrate) relaxation of aortic tissue. Mean±SEM. *p<0.05 vs. ApoE⁻/⁻ **p=0.001 vs ApoE⁻/⁻.
Figure 2

(A) Plaque area (% of ApoE-Control) for WT, ApoE, and ApoE+Dabi groups. The graph shows a significant difference between the groups with ApoE having the highest plaque area and ApoE+Dabi having the lowest. The p-value is less than 0.001.

(B) Representative images for WT group.

(C) Representative image for ApoE group.

(D) Representative image for ApoE+Dabi group.
Figure 3

(A) Bar graph showing vessel fibrosis (% of ApoE-Control) for WT, ApoE, and ApoE+Dabi groups. The graph indicates a significant difference with p=0.002.

(B) Histological image of a WT vessel showing mild fibrosis.
(C) Histological image of an ApoE vessel showing moderate fibrosis.
(D) Histological image of an ApoE+Dabi vessel showing reduced fibrosis compared to ApoE.
Figure 4

A

Oxidative Stress (% ApoE control)

WT ApoE ApoE+Dabi

p = 0.004

B C D

Images B, C, and D show immunofluorescent staining patterns.