The Effects of Direct Thrombin Inhibition with Dabigatran on Plaque Formation and Endothelial Function in Apolipoprotein E-Deficient Mice

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ABSTRACT

The recently developed oral anticoagulant dabigatran (Dabi) etexilate directly inhibits thrombin after 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 ([apolipoprotein E-deficient (ApoE(−/−))] mice). ApoE(−/−) mice were treated with a cholesterol-rich diet for 12 weeks and either dabigatran etexilate (900 mg/kg body weight) or vehicle. Wild-type (C57/B6) mice served as control. Endothelial function was assessed with carbachol (endothelium dependent) by 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 semi-quantitative immunohistochemical staining. Measurement of dabigatran plasma levels (622.3 ± 169 ng/ml) and a performed coagulation test (diluted thrombin time) 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 dihydroethidium 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 versus 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 been shown to be at least noninferior or even superior in stroke prevention in atrial fibrillation compared with 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 with warfarin (Hohnloser et al., 2012; Uchino and Hernandez, 2012). However, linkage between coagulation and inflammation has been well described previously (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 years) to reliably capture atherosclerotic events. An experimental animal model can test whether novel anticoagulatory mechanisms affect atherosclerotic lesion formation. We therefore investigated the effects of 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.

Materials and Methods

Animals and Procedures. Animal procedures were performed in accordance with institutional guidelines, German animal protection law, and the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Re-

ABBREVIATIONS: ApoE, apolipoprotein E; ApoE(−/−), apolipoprotein E deficient; Dabi, dabigatran; DHE, dihydroethidium; dTT, diluted thrombin time; NO, nitric oxide; ROS, reactive oxygen species; WT, wild type.
sources, 1996). Ten-week-old male C57BL/6J mice [wild-type (WT)] and apolipoprotein E-deficient [ApoE(−/−)] mice (C57BL/6J, genetic background; Charles River Breeding Laboratories, Sulzfeld, Germany) were used in this study. The animal studies were approved by local authorities.

**Dose Finding.** According to recommendations by Boehringer Ingelheim GmbH (Ingelheim, Germany), derived from the development program, dabigatran etexilate was used at a dose of 900 mg/kg body weight per day. This dosage was chosen because of a known bioavailability of 6%, and dabigatran etexilate is approximately 2-fold less potent in rat versus human (Wienen et al., 2007), which is 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 (dTT) were determined.

**Treatment.** All mice were fed a high-fat, cholesterol-rich diet (24% fat, 24% protein, and 41% carbohydrate; Research Diets, New Brunswick, NJ) 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 rates 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, Apex, NC).

**Endothelial Function.** The ascending aorta was dissected and immersed in ice-cold buffer composed of 118 mM NaCl, 2.5 mM CaCl₂, 4.7 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 0.026 mM Na EDTA, and 5.5 mM HEPES, pH 7.4. Adventitial tissue was carefully removed. Experiments were conducted on aorta rings of 3 mm in length. Endothelial function was measured by 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 measured 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 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 nitro-glycerin, as a NO donor. The drugs were washed out before adding the next substance. The relaxing effect of carbachol was abolished by adding N⁵-nitro-L-arginine methyl ester (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 because of 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 by using dihydroethidium (DHE) fluorescence microscopy as described previously (Wassmann et al., 2004b).

**Dihydroethidium Fluorescence Microscopy.** 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, whereas 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 (Bayer Corp., Elkhart, IN), snap-frozen, and stored at −80°C. Samples were sectioned on a Leica (Wetzlar, Germany) cryostat (10 μm) and placed on glass slides. Then, Krebs-HEPES buffer containing 2 μM DHE was topically applied to each tissue section, and sections were incubated in a 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 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 by 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 1 h, rinsed with deionized water, stained with Oil Red O working solution (0.5%) for 30 min or Sirius Red for 15 min, and rinsed again. For morphometric analysis, hematoxylin staining was performed according to standard protocols. All sections were examined under a Nikon (Tokyo, Japan) E600 microscope. Lucia Measurement version 4.6 (Nikon UK Ltd., Surrey, UK) software was used to measure lipid-staining area and total area of the histological sections. The total lesion area of four 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 (0.1% Picro-Sirius Red solution containing 0.1% Sirius Red and 1.2% picrin acid) for 10 min after washing with descending ethanol concentrations (100, 90, and 70%) and subsequent incubation with xyloc for 2 min. Aortic collagen content was quantified by using fluorescence microscopy through polarized light (G-2A-fluorescence filter; excitation spectrum 510–560 nm; exposure time 83 ms; 40-fold magnification). Aortic sections from each treatment group were processed in parallel, and images were acquired with identical acquisition parameters. 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 ± S.E.M. After Kolmogorov-Smirnow testing for normal distribution, intergroup differences were assessed with an analysis of variance and Dunnet’s post hoc analysis. SPSS 20.0 (SPSS Inc., Chicago, IL) was used for statistical calculations. Statistical significance was assumed at p < 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 with WT mice. Dabigatran etexilate treatment showed no difference in systolic blood pressure compared with 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 dTT could be observed (Fig. 1) (Love et al., 2007) by using HEMOCLOT

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<th>TABLE 1</th>
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<tr>
<td>Heart rate, blood pressure, and body weight</td>
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<tr>
<td>Systolic Blood Pressure</td>
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<tr>
<td>mm Hg</td>
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<tr>
<td>WT, n = 20</td>
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<tr>
<td>ApoE(−/−), n = 19</td>
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<tr>
<td>ApoE(−/−) + Dabi, n = 20</td>
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**, p = 0.001; ***; p < 0.001 vs. ApoE(−/−).
**Atherosclerotic Plaque Formation.** Atherosclerotic lesion formation was quantified in the aortic sinus and the ascending aorta by histological analyses of Oil Red O stainings. After a cholesterol-rich diet, atherosclerotic lesion formation in ApoE(−/−) mice was significantly increased in the aortic sinus and the ascending aorta compared with 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] (Fig. 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(−/−) mice 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] (Fig. 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 with WT 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] (Fig. 4).

**Endothelial Function.** Endothelium-dependent relaxation of aortic rings was significantly impaired in ApoE(−/−) mice compared with WT mice at all carbachol concentrations (p < 0.05). Treatment with dabigatran etexilate significantly improved endothelial-dependent relaxation in ApoE(−/−) mice [42.6 ± 2.7 versus ApoE(−/−) Dabi 62.9 ± 3.3%; p = 0.001; n = 10] of phenylephrine-induced contraction at 100 μmol carbachol [Fig. 5]. Potency [pD2 (−log)] of endothelium-dependent relaxation with carbachol significantly improved by treatment with dabigatran etexilate compared with ApoE(−/−) mice (Table 2). Endothelium-independent relaxation with glyceroltrinitrate of aortic rings did not differ 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 has been well de-
The secretion of prostaglandin H2 or thromboxane early relaxation and a subsequent transient contraction mediated by the secretion of prostaglandin H2 or thromboxane A2 (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 sites of vascular injury (Hatton et al., 1989) and promotes a pro-inflammatory response mediated by protease-activated receptor type 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 in the RE-LY trial (Connolly et al., 2009). The initial results suggested a significant increase of nonfatal myocardial infarction in the dabigatran group compared with 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 noninferiority 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. (2010) revealed data regarding dabigatran etexilate having a strong and protective effect against atherosclerosis in mice with a pro-coagulant phenotype [MPRO/PRO:ApoE(−/−)]. Preusch et al. (2010) found retardation in atherosclerotic lesion by treatment with dabigatran etexilate, and Nagy et al. (2011) showed that treatment with dabigatran etexilate significantly inhibited atherosclerotic lesion formation in the aorta in low-density lipoprotein receptor-deficient mice, most likely caused by remodeling of plaque extracellular matrix. These latter studies have hitherto been published only 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 antiplatelet effect. Therefore our experimental studies do not provide support for mechanisms in favor of accelerated occurrence of atherosclerosis.

In conclusion, our study demonstrates the protective effects of effective direct thrombin inhibition on vascular remodeling at relevant doses. Thrombin inhibition by dabigatran leads to improved endothelial function and reduces

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**Table 2**

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<th>WT</th>
<th>ApoE(−/−)</th>
<th>ApoE(−/−) + Dabigatran</th>
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<tr>
<td>Maximum relaxation, %, aortic tissue, response to carbachol</td>
<td>72.0 ± 4.4*</td>
<td>42.6 ± 2.7</td>
<td>62.9 ± 3.3**</td>
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<tr>
<td>pD_{max} − log</td>
<td>−5.8 ± 0.2</td>
<td>−5.3 ± 0.3</td>
<td>−6.3 ± 0.3**</td>
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*p, p < 0.05 vs. ApoE(−/−); **, p = 0.001 vs. ApoE(−/−).
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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Authorship Contributions

Participated in research design: Lee, Schirmer, Baumphäkel, and Böhm.

Conducted experiments: Lee, Kratz, and Baumhäuser.

Performed data analysis: Lee, Kratz, Schirmer, and Baumphäkel.

Wrote or contributed to the writing of the manuscript: Lee, Kratz, Schirmer, Baumphäkel, and Böhm.

References


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