Melagatran Reduces Advanced Atherosclerotic Lesion Size and May Promote Plaque Stability in Apolipoprotein E–Deficient Mice

Florian Bea, Joerg Kreuzer, Michael Preusch, Sandra Schaab, Berend Isermann, Michael E. Rosenfeld, Hugo Katus, Erwin Blessing

Objective—Inflammatory mechanisms are involved in atherosclerotic plaque rupture and subsequent thrombin formation. Thrombin not only plays a central role in thrombus formation and platelet activation, but also in the induction of inflammatory processes. We assessed the hypothesis that melagatran, a direct thrombin inhibitor, attenuates plaque progression and promotes stability of advanced atherosclerotic lesions.

Methods and Results—Melagatran (500 μmol/kg/d) or control diet was administered to apolipoprotein E–deficient mice (n=54) with advanced atherosclerotic lesions. Treatment reduced lesion progression in brachiocephalic arteries (P<0.005). Morphometric analysis confirmed that thrombin inhibition promoted plaque stability and resulted in thicker fibrous caps (28.4±14.2 μm versus 20.8±12.0 μm; P<0.05), increased media thickness (29.3±9.6 μm versus 24.4±6.7 μm; P<0.05), and smaller necrotic cores (73 537±41301 μm² versus 126 819±51730 μm²; P<0.0005). Electro mobility shift assays revealed reduced binding activity of nuclear factor κB (P<0.05) and activator protein-1 (P<0.05) in aortas of treated mice. Furthermore, immunohistochemistry demonstrated reduced staining for matrix metalloproteinase (MMP)-9 (P<0.05). Melagatran had no significant effect on early lesion formation in C57BL/6J mice.

Conclusions—The direct thrombin inhibitor melagatran reduces lesion size and may promote plaque stability in apolipoprotein E–deficient mice, possibly through reduced activation of proinflammatory transcription factors and reduced synthesis of MMP-9. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: direct thrombin inhibitor ■ atherosclerosis ■ plaque ■ inflammation ■ transcription factors ■ MMP-9

Angiogenesis, and vascular pathology such as atherosclerosis and inflammation.10–12

In the present study, we tested the hypothesis, whether administration of the direct thrombin inhibitor melagatran prevents initiation of atherosclerosis in C57BL/6J mice and/or alters size and composition of advanced atherosclerotic lesions in hyperlipidemic apolipoprotein E–deficient mice.

Methods

Animals and Drug Treatment

Thirty-week-old female apolipoprotein E–deficient mice13 (strain name B6.129P2) on a C57BL/6J background (n=54) were kept within the animal care facility of the University of Heidelberg. 28 mice were fed a chow diet supplemented with melagatran (500 μmol/kg/d) for 22 weeks, 26 mice received regular chow diet. Ximelagatran, which is rapidly bioconverted into its active form, melagatran is generally used in humans. Because of a better

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bioavailability, the use of melagatran is generally accepted in mouse studies. The housing and care of animals and all the procedures done in the study were in accordance with the guidelines and regulations of the local Animal Care Committee.

Animal Sacrifice and Preparation of Tissues
Animals were euthanized at 52 weeks of age, a time point where previous studies demonstrated characteristics of advanced lesions, such as thinning of fibrous cap and intra plaque hemorrhage in the brachiophecal arteries of apoE-deficient mice.14 Mice were sedated (Avertin, Aldrich), blood was collected from the inferior vena cava, and the animals were euthanized by exsanguination. Thoracic aortas were ligated distal of the brachiophecal artery and removed for subsequent preparation for gel shift analysis. Mice were then perfused with 10% buffered formalin for 4 minutes at physiological pressure via the left ventricle. Brachiophecal arteries from each animal were dissected out, embedded in paraffin, and serially sectioned (5 μm). Every fifth section was stained with a modified Movat pentachrome stain.15 To identify vascular calcification, adjacent slices were stained with van Kossa stain.16 For quantification of collagen content, additional sections were evaluated following standard Masson trichrome staining.

Determination of Plasma Lipid Levels and Melagatran Concentration
Total cholesterol, phosphotungstic acid/magnesium chloride–precipitated HDL cholesterol, and triglycerides were determined enzymatically in heparinized plasma, and LDL cholesterol was determined indirectly by Friedewald formula. Melagatran concentrations were determined by high performance liquid chromatography/mass spectrometry with protein precipitated plasma samples.

Evaluation of Plaque Composition and Lesion Size
Two independent investigators who were blinded to the study protocol evaluated each section for characteristic features of plaque instability. These included: thickness of the fibrous cap (thin fibrous cap was defined as 3 or fewer cell layers); size of the necrotic core (a large necrotic core was defined as occupying more than 1/3 of the volume of the plaque), intraplaque hemorrhage (defined as the presence of red blood cells independent of microvessels), calcification (defined on the basis of positive staining with the van Kossa stain), presence of cholesteral crystals, medial erosion (defined as enlargement of the media caused by infiltration of plaque tissue), and lateral xanthomas (defined as the presence of aggregates of macrophase-derived foam cells situated on the lateral margins of the plaques). These were recorded as binary outcomes and the frequency for each group was determined. Each cross sectional area of each plaque was analyzed using computer-assisted morphometry (Image Pro; Media Cybernetics). Maximum lesion area, maximum lesion thickness, maximum percent stenosis, maximum area of the necrotic core, and minimal thickness of the fibrous cap of and the media per animal were identified and stored.

Preparation of Nuclear Extracts
Each aorta was prepared from connective tissue and homogenized in 400 μL of hypotonic buffer (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 2 mmol/L dithiothreitol [DTT]) supplemented with proteinase and phosphatase inhibitors (5 μg/mL E. L 64, 1 mmol/L NaF, 0.2 mmol/L NaVO₃, 0.5 mg/mL Pefabloc), incubated for 15 minutes on ice, after which 25 μL of 10% NP-40 was added. The nuclei were recovered by centrifugation (14,000 rpm, 1 minute, 4°C). The nuclear pellets were resuspended in 50 μL ice cold buffer C (20 mmol/L HEPES, pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L DTT supplemented with 5 μg/mL E64, 1 mmol/L NaF, 0.2 mmol/L NaVO₃, 0.5 mg/mL Pefabloc). After centrifugation (14,000 rpm, 5 minutes, 4°C), the supernatants containing nuclear protein were collected and stored at −80°C until used.

Electrophoretic Mobility Shift Assay
Protein concentrations were measured by the Bradford method.17 Nuclear extracts (10 μg protein in each assay) were incubated with labeled oligonucleotide probes. The sequences of the oligonucleotides used in the present study were as follows: NFκB, 5′-AGTTAGGGGACTTCGACTGGC-3′, AP-1, 5′-CTGGGGTA-GTCATCCTT-3′. The oligonucleotides (1.75 pmol/μL) were la- beled with [γ32P]-ATP by using T4 polynucleotide kinase. Specific activities used in each assay were around 10 000 cpm. Lipopolysaccharide (LPS) treated RAW cells were used as positive, untreated cells as negative controls. 100-fold excess of unlabelled oligonucleotides were used for cold inhibition. Binding reactions were resolved on a 4% native polyacrylamide gel and exposed to x-ray film for 12 to 24 hours. Gels were analyzed using densitometric analysis (Bio-Rad Laboratories).

Immunohistochemistry
Tissue sections of the brachiophecal artery adjacent to the site of maximum lesion area were dewaxed and rehydrated. Endogenous peroxidase activity was inhibited by incubation with Peroxyblock (Invitrogen). After sections were blocked with 20% (vol/vol) goat serum in PBS, they were incubated overnight at 4°C either with an anti–Mac-2 mouse macrophase antibody (Chemicon), anti–smooth muscle actin antibody (Dianova), or anti-mouse MMP-9 antibody (Chemicon) according to the manufacturers’ protocols. Sections were then incubated with the biotinylated secondary antibodies, rinsed 3 × with PBS and incubated for 10 minutes with streptavidin at room temperature. AEC-chromogen substrate (Invitrogen, Karlsruhe, Germany) was used for visualization. Finally, sections were counterstained with hemalaun. The extent of positive staining within the lesions was determined by two independent investigators who were blinded to the study protocol, using computer-assisted morphometry (Image Pro; Media Cybernetics).

Evaluation of Early Atherosclerotic Lesions
For evaluation of early atherosclerotic lesions, 24 fourteen-week-old male C57BL/6 mice received either a high fat, high cholesterol diet supplemented with melagatran (500 μmol/kg/d) or the atherogenic diet alone for 8 weeks. Previous studies demonstrated that 8 weeks feeding of atherogenic diet causes significant lesion formation in aortic sinuses of C57BL/6 mice.18 After fixation perfusion with 10% buffered formalin for 4 minutes, hearts were dissected out. The base of each heart was frozen in OCT medium (Sakura Finetek) and cryosectioned as described by Paigen et al.19 For quantification of the maximum lesion area within the aortic sinus, the cross sectional area of oil red O staining was measured from the points of emergence to disappearance of the aortic valves using computer assisted morphometry (Image Pro; Media Cybernetics).

Statistical Analysis
All data were expressed as mean±SD. Significant differences between means in plasma lipid profiles were determined with the two-tailed unpaired Student t test. For analysis of plaque morphometry and areas of positive stainings, groups were compared using the two-tailed Mann–Whitney U test. For evaluation of plaque morphology, groups were compared using the χ² test. A probability value <0.05 was considered statistically significant.

Results
Plasma Lipid Levels and Melagatran Concentrations
Melagatran treatment did not alter concentrations of total cholesterol, total triglycerides, HDL, and LDL in apolipoprotein E–deficient and C57BL/6J mice (supplemental Table I, available online at http://atvb.ahajournals.org). Effects observed in the present study therefore did not appear to be related to any changes in plasma lipid profiles. Plasma levels of melagatran averaged 0.67±0.33 μmol/L (range 0.25 to...
1.23 μmol/L) in treated apolipoprotein E−/− mice and 1.21±0.56 μmol/L (range 0.55 to 2.13 μmol/L) in C57BL/6J mice. Plasma concentration was <0.1 μmol/L in all control animals. Pilot studies with mice exposed to the same dosing of melagatran showed significantly increased TCT (thrombin clotting time) in melagatran treated mice (data not shown).

**Lesion Progression**

Chronic administration of melagatran in the chow diet over 22 weeks significantly reduced progression of atherosclerotic plaque development in apolipoprotein E deficient mice. Maximum lesion area in the melagatran group averaged 234±876 μm² compared with 290±733 μm² in the control group (P<0.005) (Figure 1A). Melagatran-treated mice had maximum lesion stenosis of 71.5±1.9% compared with 78.0±2.0% in the group of control mice (P<0.02) (Figure 1B). Maximum lesion thickness was also significantly smaller in treated as compared with the control animals (274±8.8 μm versus 365±22.3; P<0.005) (Figure 1C).

**Plaque Morphology**

Evaluation of plaque morphology showed a significant reduction in the frequency of thin fibrous caps (P<0.005), frequency of large necrotic cores (P<0.01), and occurrence of medial erosions (P<0.05), signs of plaque instability with melagatran treatment in apoE−/− mice (Figure 3). There was no statistically significant difference in frequency of intraplaque hemorrhage, presence of cholesterol crystals, calcifications, and lateral xanthomas (Table 1). Morphometric analysis confirmed that melagatran treatment resulted in thicker fibrous caps (28.4±14.2 μm versus 20.8±12.0 μm; P<0.05), increased media thickness (29.3±9.6 μm versus 24.4±6.7 μm; P<0.05) and smaller necrotic cores (73,537±41,301 μm² versus 126,819±51,730 μm²; P<0.0005). Size of necrotic cores were significantly smaller, even if normalized to lesion area (31.3±16.5% versus 43.5±12.9%; P<0.01) (Table 2).

**Gel Shift Analysis**

Electrophoretic mobility shift assays of nuclear extracts and subsequent densitometric evaluation showed a significant reduction of DNA binding activity of the transcription factors AP-1 (P<0.05) and NFκB (P<0.05) in aortic tissue from melagatran-treated, as compared with control mice (Figure 2).

**Immunohistochemistry and Special Stainings**

Analysis of plaque composition by immunohistochemistry showed significant increase of staining against smooth muscle alpha actin (P<0.02) and significant decrease of staining against MMP-9 (P<0.05) in the melagatran group (Table 3; supplemental Figure IC and ID). Staining against smooth muscle alpha actin was predominantly located within the fibrous cap (supplemental Figure IA), MMP-9 positive staining was localized mainly in the shoulder regions of the plaque (supplemental Figure ID). There was no statistical significant difference in staining against Mac-2, collagen, and calcium between the two groups.
Early Lesion Formation
Administration of melagatran in an atherogenic diet over 8 weeks did not significantly reduce formation of early atherosclerotic lesions in the aortic sinus of male C57BL/6J mice. Maximum lesion area averaged $1929 \pm 4122 \mu m^2$ as compared with $3680 \pm 7234 \mu m^2$ in the group of control mice, who received the atherogenic diet alone ($P=\text{n.s.}$) (Figure 1D). Thrombin inhibition therefore does not seem to alter initiation of fatty streaks formation, but plays a crucial role in progression and destabilization of advanced atherosclerotic lesions.

Discussion
Thrombin has numerous nonthrombotic associations with atherosclerosis and heart disease. Along with being the key final enzyme in fibrin formation and the most powerful known platelet activator, thrombin also acts as a signaling molecule, through protease-activated receptors (PARs). At least three PARs are expressed by human cells, with attendant G protein–coupled signaling cascades and physiological effects. Taking the pluripotent effects of thrombin on vascular pathogenesis, administration of direct thrombin inhibitors might offer promising therapeutic options in treating vascular disease. Several studies using animal models could already demonstrate the beneficial effects of thrombin inhibition with hirudin in experimental restenosis. Data on the vascular effects of new generation direct thrombin inhibitors are very limited. Hemdahl et al reported protective effects of sc melagatran in a mouse model of hypoxic stress induced myocardial infarction.

In the present study, we show for the first time that oral administration of melagatran attenuated the progression of ath-

### TABLE 1. Morphologic Analysis Arteria Brachiocephalica

<table>
<thead>
<tr>
<th></th>
<th>Melagatran n=10</th>
<th>Placebo n=10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thin fibrous cap</td>
<td>8/28 (29%)</td>
<td>17/26 (65%)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Large necrotic core</td>
<td>11/28 (39%)</td>
<td>20/26 (77%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Medial erosion</td>
<td>4/28 (14%)</td>
<td>8/26 (31%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Calcification</td>
<td>12/28 (43%)</td>
<td>18/26 (69%)</td>
<td>ns</td>
</tr>
<tr>
<td>Cholesterol crystals</td>
<td>23/28 (82%)</td>
<td>25/26 (96%)</td>
<td>ns</td>
</tr>
<tr>
<td>Lateral xanthoma</td>
<td>21/28 (75%)</td>
<td>22/26 (85%)</td>
<td>ns</td>
</tr>
<tr>
<td>Plaque haemorrhage</td>
<td>12/28 (43%)</td>
<td>12/26 (46%)</td>
<td>ns</td>
</tr>
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</table>

### TABLE 2. Morphometric Analysis Arteria Brachiocephalica

<table>
<thead>
<tr>
<th></th>
<th>Melagatran n=28</th>
<th>Placebo n=26</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min. thickness fibrous cap, $\mu m$</td>
<td>28.4±14.2</td>
<td>20.8±12.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Min. thickness media, $\mu m$</td>
<td>29.3±9.6</td>
<td>24.4±6.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Max. area necrotic core, $\mu m^2$</td>
<td>73,357±4,1301</td>
<td>126,819±5,1730</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Area necrotic core/max. plaque, %</td>
<td>31.3±16.5</td>
<td>43.5±12.9</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data are mean±SD.
erosclerotic plaques in brachiocephalic arteries of apoE−/− mice. Melagatran had beneficial effects on both size and composition of the advanced atherosclerotic lesions. In contrast, long term treatment with a 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitor, a well established pharmacological tool in the treatment of patients with coronary artery disease, promoted plaque stability, but did not affect lesion size in the same mouse model. The present study suggests that the plaque-stabilizing effects of chronic thrombin inhibition melagatran might be the result of antiinflammatory mechanisms of melagatran treatment.

Initiation of fatty streak formation was not significantly altered by melagatran administration in the present study, indicating that thrombin-mediated mechanisms might not play a pivotal role in the early stages of atherogenesis. However, because data suggest a downward trend on lesion formation in the melagatran group, we cannot rule out that results did not reach significance level because of the small sample size and/or because of the rather short duration of treatment.

Increased presence of smooth muscle cells (SMCs), thicker fibrous caps, reduced staining against MMP-9, lower rates of medial erosions, and smaller necrotic cores, as observed in the present study after long term administration of melagatran, demonstrate protective plaque stabilizing effects of thrombin inhibition. Effects of melagatran on lesion progression were most likely, at least in part, unrelated to its role in the coagulation cascade. Expression of tissue factor, a potent activator of the extrinsic coagulation cascade, was not different in thoracic aortas between melagatran treated and control mice, as assessed by RT-PCR (data not shown). Significant thrombus formation was not observed in the present study, nor in our previous studies of advanced atherosclerotic lesions in the same model, possibly because of a high fibrinolytic activity in mice.

Interestingly, frequency of intraplaque hemorrhage, in contrast to our previous observations with simvastatin, was not reduced by administration of melagatran. It is possible, that in regard to plaque hemorrhage, plaque-stabilizing effects of melagatran were counteracted by its classic anticoagulatory properties, resulting in comparable patterns of erythrocyte deposition in otherwise stable lesions. However, other signs of hemorrhagic abnormalities were not observed among the melagatran-treated mice.

Although uncontrolled proliferation of SMCs can contribute to restenosis after vascular injury, presence of that cell type is generally considered to enhance stability in advanced lesions, in particular if predominantly located within the protective fibrous cap. The increased expression of alpha actin in lesions of melagatran treated mice, as observed in the present study, might be the result of diminished rates of programmed cell deaths in SMCs. Cell culture experiments indeed revealed proapoptotic effects of thrombin on vascular SMCs.

Our experiments demonstrate that long-term treatment with the direct thrombin inhibitor melagatran reduces DNA binding activity of the redox-sensitive transcription factors NFkB and AP-1, and reduces positive staining against MMP-9. It is well known that NFkB and AP-1 are both important transcriptional regulators of MMP-9. Our observations are consistent with in vitro studies of mesangial cell, where thrombin stimulates MMP-9 mRNA expression through an AP-1 pathway. Thrombin-induced expression of MMP-9 mRNA and AP-1

### Table 3. Immunohistochemistry and Special Stainings of Arteria Brachiocephalica

<table>
<thead>
<tr>
<th>Staining</th>
<th>Positive Staining/Total Lesion Area (%)</th>
<th>Mean ± SD</th>
<th>Placebo</th>
<th>Melagatran</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha actin</td>
<td>1.33 ± 1.64</td>
<td>0.21 ± 0.52</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
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<tr>
<td>MMP-9</td>
<td>11.06 ± 7.49</td>
<td>15.94 ± 5.77</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
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<tr>
<td>Mac-2</td>
<td>3.55 ± 4.83</td>
<td>2.80 ± 2.94</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium (van Kossa)</td>
<td>10.17 ± 15.15</td>
<td>11.98 ± 15.07</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen (Trichrome Masson)</td>
<td>32.24 ± 11.32</td>
<td>29.69 ± 8.95</td>
<td>ns</td>
<td></td>
<td></td>
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</tbody>
</table>

*Data are mean ± SD. MMP-9 indicates matrix metalloproteinase 9; Mac 2, mouse macrophage antibody.*
binding activity has previously been blocked by the direct thrombin inhibitor hirudin, as well as by the NFXb-inhibitor curcumin, and by c-fos antisense oligonucleotides. Another study showed that thrombin-induced endothelial-1 expression in human vascular endothelial cells could be inhibited by PPAR activators by blocking AP-1, supporting the hypothesis that thrombin induced vascular pathology, at least in part, is mediated via the AP-1 signaling pathway. Although monocyte recruitment is an important proinflammatory event in atherogenesis, and thrombin is reported to act as a chemoattractant for these cells, number of macrophages were not affected by melagatran treatment in the present study. Further studies are needed to evaluate whether thrombin inhibition reduces activity of macrophages rather than the actual number of inflammatory cells in advanced atherosclerotic lesions.

In conclusion, the present study demonstrates that the thrombin inhibitor melagatran reduces lesion size and promotes plaque stability in apolipoprotein E–deficient mice, possibly through inhibiting activation of the proinflammatory transcription factors AP-1 and NFXb and reduced synthesis of MMP-9. New generation direct thrombin inhibitors might therefore emerge as potential therapeutic tools in patients with advanced atherosclerotic disease.

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Disclosures

None.

References

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Table I  Effects of Melagatran Treatment on Plasma Lipids

<table>
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<tr>
<th></th>
<th>Total cholesterol (mmol/l)</th>
<th>LDL cholesterol (mmol/l)</th>
<th>HDL cholesterol (mmol/l)</th>
<th>Triglycerides (mmol/l)</th>
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<tbody>
<tr>
<td><strong>Apo E-/-</strong></td>
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<tr>
<td>Melagatran</td>
<td>13.67±3.62</td>
<td>6.16±1.37</td>
<td>6.92±2.35</td>
<td>2.95±1.34</td>
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<tr>
<td>Control</td>
<td>13.65±2.92</td>
<td>6.15±0.96</td>
<td>6.85±1.98</td>
<td>3.28±1.62</td>
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<tr>
<td><strong>C57BL/6J</strong></td>
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<tr>
<td>Melagatran</td>
<td>3.24±1.13</td>
<td>1.40±0.40</td>
<td>1.53±0.78</td>
<td>2.13±2.31</td>
</tr>
<tr>
<td>Control</td>
<td>2.95±0.64</td>
<td>1.50±0.35</td>
<td>1.29±0.34</td>
<td>0.85±0.27</td>
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</tbody>
</table>

Data are mean±SD. Differences were not significant between the treatment groups.