Warfarin Induces Cardiovascular Damage in Mice

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Objective—Vascular calcification is an independent risk factor for cardiovascular disease. Once thought to be a passive process, vascular calcification is now known to be actively prevented by proteins acting systemically (fetuin-A) or locally (matrix Gla protein). Warfarin is a vitamin K antagonist, widely prescribed to reduce coagulation by inhibiting vitamin K–dependent coagulation factors. Recently, it became clear that vitamin K antagonists also affect vascular calcification by inactivation of matrix Gla protein. Here, we investigated functional cardiovascular characteristics in a mouse model with warfarin-induced media calcification.

Approach and Results—DBA/2 mice received diets with variable concentrations of warfarin (0.03, 0.3, and 3 mg/g) with vitamin K1 at variable time intervals (1, 4, and 7 weeks). Von Kossa staining revealed that warfarin treatment induced calcified areas in both medial layer of aorta and heart in a dose- and time-dependent fashion, which could be inhibited by simultaneous vitamin K2 treatment. With ongoing calcification, matrix Glα protein mRNA expression decreased, and inactive matrix Glα protein expression increased. TdT-mediated dUTP-biotin nick end labeling–positive apoptosis increased, and vascular smooth muscle cell number was concomitantly reduced by warfarin treatment. On a functional level, warfarin treatment augmented aortic peak velocity, aortic valve–peak gradient, and carotid pulse-wave velocity.

Conclusion—Warfarin induced significant calcification with resulting functional cardiovascular damage in DBA/2 wild-type mice. The model would enable future researchers to decipher mechanisms of vascular calcification and may guide them in the development of new therapeutic strategies. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: aortic valve stenosis • matrix Gla protein • pulse-wave analysis • vascular calcification • vitamin K • warfarin

Vascular calcification (VC) is an important independent risk factor for the development of myocardial infarction, stroke, and renal disease.1–2 If symptomatic cardiovascular disease is already apparent, the extent of VC is a potent indicator of unfavorable outcomes.3 Additionally, effective secondary preventive strategies for cardiovascular disease may translate into slower progression of VC.3

Physiologically, VC is prevented by a network of calcification-inhibitory proteins6: matrix Glα protein (MGP) is currently considered as the most potent local inhibitor of ectopic calcification in the artery wall. MGP is locally produced by vascular smooth muscle cells (VSMCs). MGP-deficient mice die ≈6 weeks after birth because of fractures of the heavily calcified aorta.7 These mice also exhibit a phenotypic change of VSMCs toward osteoblast-like cells, a common finding in various forms of advanced VC.

Vitamin K–dependent proteins need vitamin K as cofactor for post-translational γ-carboxylation to achieve full biological activity.10 Well-established vitamin K–dependent proteins are the blood coagulation factors II, VII, IX, and X and protein C, S, and Z.11 By interfering with the vitamin K–driven γ-carboxylation process, warfarin has become the mainstay of long-term anticoagulation therapy in humans. MGP also belongs to this group of vitamin K–dependent proteins. Recently, it became apparent that warfarin also inhibits γ-carboxylation of MGP, leading to inactive, uncaboxylated MGP (ucMGP) thereby potentially promoting VC. Observational studies have shown an association...
between long-term warfarin treatment and increased prevalence and extent of aortic valve and coronary calcifications, respectively.\textsuperscript{12,13} Potential mechanisms of MGP-mediated inhibition of VC represent inhibition of calcium-crystal growth,\textsuperscript{14,15} bone morphogenetic proteins,\textsuperscript{16,17} and transdifferentiation of VSMCs into an osteochondrogenic phenotype, respectively.\textsuperscript{18}

Here, we characterized the sequence of key events from initial VSMC apoptosis via vascular calcium loading toward alterations of functional cardiovascular parameters as a consequence of warfarin-induced VC in mice. This experimental animal model may serve as a valuable tool to test treatment strategies against VC applying transgenic approaches and to further decipher the functional network between the various calcification inhibitors and activators.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

#### Calcium Measurement

Warfarin treatment provoked a dose-dependent increase in calcium deposition within the aortic and myocardial tissue, respectively, as shown by von Kossa staining (Figure 1). Compared with the control group, the 0.3- and 3-mg/g warfarin with vitamin K1 groups exhibited significant increases of von Kossa–positive areas within the aortic wall (\(P<0.05\) and \(P<0.01\), respectively). The extent of calcified myocardial tissue in the 3-mg/g warfarin with vitamin K1 group was significantly increased compared with the control group (\(P<0.05\) as calculated by the Kruskal–Wallis test). This was associated with a significant increase of calcium levels in aortic tissues and a nonsignificant increase in myocardial tissues as detected by cresolphthalein measurement (Figure 2A and 2B). After 7 weeks of treatment, the calcium deposition was further increased as detected by colorimetric detection from tissue extraction (Figure 2D and 2E). Tissue calcium content concomitantly increased dose dependently in aorta and heart but nonsignificantly in lungs (Figure 2C). Using von Kossa staining, we failed to detect significantly positive-stained areas in lungs (Figure 2F and 2G). In kidneys, we did not detect substantial positive von Kossa staining at all (not shown). The additional treatment of 100 \(\mu\)g vitamin K2 with warfarin (3 mg/g) and vitamin K1 over 4 weeks resulted in significantly reduced calcium content in the aorta and myocardium compared with 3 mg/g warfarin with vitamin K1 alone.

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>MGP</td>
<td>Matrix Gla protein</td>
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<tr>
<td>PWV</td>
<td>Pulse-wave velocity</td>
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<tr>
<td>t-ucMGP</td>
<td>Total uncarboxylated MGP</td>
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<tr>
<td>ucMGP</td>
<td>Uncarboxylated MGP</td>
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<td>VC</td>
<td>Vascular calcification</td>
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<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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**Figure 1.** Cardiovascular calcification as detected by quantitative histomorphometry of von Kossa–stained aorta (A) and myocardium (B) in mice after 28 days of warfarin with vitamin K administration. Representative von Kossa–stained sections through the aortic root depict the stepwise increment of media calcification with increasing warfarin concentration from 0.03 via 0.3 to 3 mg/g food compared with control mice receiving standard chow (C). Additional vitamin K2 treatment reduced calcium content. The histograms below show higher magnifications of areas specified above. ***\(P<0.001\); **\(P<0.01\); *\(P<0.05\).
Serum Chemistry and Total-ucMGP

Warfarin with vitamin K1–treated animals did not show any significant changes in serum levels of calcium, phosphorus, CRP, and BUN (not shown). Serum levels of total (t)-ucMGP revealed a dose-dependent increase in groups receiving 0.03 and 0.3 mg warfarin with vitamin K1 during 4 weeks, which was significant compared with the control group (Figure 3A).

In the group with the highest warfarin concentration (3 mg/g warfarin with vitamin K1), t-ucMGP levels were lower compared with the group receiving 0.3 mg (Figure 3A). Addition of vitamin K2 to the 3-mg/g warfarin and vitamin K1 treatment did not result in a reduction of serum ucMGP levels compared with warfarin with vitamin K1 treatment alone; for osteopontin, we detect a nonsignificant increase (+136%, *P=0.22). Extending the treatment period to 7 weeks did not result in further significant alteration of the expression of the abovementioned genes (Figure 4).

Evaluation of Cell Viability

Starting at 4 weeks of 3 mg/g warfarin with vitamin K1 treatment, cleaved caspase-3 and TUNEL (TdT-mediated dUTP-biotin nick end labeling) staining detected positive cells within the aortic vessel wall (Figure 5A and 5B). In control animals, neither caspase-3 nor TUNEL staining revealed positive signals (not shown). The total cellularity was concomitantly significantly reduced as shown by decreased DAPI-positive nuclei within the aortic wall of 3 mg/g warfarin with vitamin K1–treated mice (−68%) and was similarly reduced in all other dosages of warfarin (Figure 4A). Osteopontin mRNA expression was dose dependently increased, reaching statistical significance in the 3-mg warfarin with vitamin K1 group after 4 weeks of treatment (+206%) compared with control (Figure 4B). The expression of SM22α mRNA, a VSMC marker, was significantly reduced in the 0.03-mg/g warfarin with vitamin K1–treated mice (−68%) and was also similarly reduced in all other dosages of warfarin (Figure 4C). Treatment with vitamin K2 in addition to warfarin with vitamin K1 did not change SM22α or MGP expressions compared with warfarin with vitamin K1 treatment alone; for osteopontin, we detect a nonsignificant increase (+136%, *P=0.22). Extending the treatment period to 7 weeks did not result in further significant alteration of the expression of the abovementioned genes (Figure 4).

Expression of Calcification-Related Genes

Vascular expression of the calcification-related genes MGP and osteopontin was altered in warfarin with vitamin K1–treated animals: MGP mRNA expression decreased significantly already with the lowest dose of warfarin (−76%) and was similarly reduced in all other dosages of warfarin (Figure 4A). Osteopontin mRNA expression was dose dependently increased, reaching statistical significance in the 3-mg warfarin with vitamin K1 group after 4 weeks of treatment (+206%) compared with control (Figure 4B). The expression of SM22α mRNA, a VSMC marker, was significantly reduced in the 0.03-mg/g warfarin with vitamin K1–treated mice (−68%) and was also similarly reduced in all other dosages of warfarin (Figure 4C). Treatment with vitamin K2 in addition to warfarin with vitamin K1 did not change SM22α or MGP expressions compared with warfarin with vitamin K1 treatment alone; for osteopontin, we detect a nonsignificant increase (+136%, *P=0.22). Extending the treatment period to 7 weeks did not result in further significant alteration of the expression of the abovementioned genes (Figure 4).
to 7 weeks compared with 4 weeks of treatment. Addition of vitamin K2 to warfarin and vitamin K1 during 4 weeks resulted in significantly higher cell numbers compared with 3 and 0.3 mg/g warfarin with vitamin K1 supplementation (Figure 5C).

Assessment of Echocardiographic Parameters
The peak velocity in the aorta, measured within the outflow tract, and the aortic valve–peak gradient increased significantly after 7 weeks of treatment with 3 mg warfarin with vitamin K1 (Figure 6A and 6B). Similarly, the pulse-wave velocity (PWV) within the common carotid artery was increased after 4 (trend), and significantly after 7 weeks of treatment (Figure 6C). Pulse-wave velocity in the abdominal aorta was not significantly altered after 7 weeks of treatment (data not shown). Ejection fraction and diastolic function of the heart were not altered by warfarin administration at any time point (not shown).

Blood Pressure Measurements
We detected no difference between mice fed 3 mg/g warfarin with 1.5 mg/g vitamin K1 compared with control mice on systolic blood pressure levels after 4 (140±5 mm Hg and 130±10 mm Hg) and after 7 weeks (134±1 and 130±10 mm Hg, respectively). These values also did not differ from baseline values (138±11 mm Hg).

Figure 3. Warfarin administration for 4 weeks leads to increased serum levels of total uncarboxylated matrix Gla protein (t-ucMGP). The increase of t-ucMGP serum levels is less pronounced in the high-dose warfarin group, which is characterized by advanced calcification. Vitamin K2 treatment and extended warfarin with vitamin K1 treatment did not significantly change t-ucMGP serum levels compared with 3 mg/g warfarin and vitamin K1 (A). As shown by immunohistochemical analysis for ucMGP, Gla residues, and total MGP, warfarin treatment induces accumulation of ucMGP in the vicinity of calcified vascular lesions, whereas the abundance of Gla residues and total MGP in the tunica media are reduced compared with control mice (B to G). Arrows in E indicate positive ucMGP staining. *P<0.05.

Figure 4. Phenotypic characterization of calcifying aortic tissue. Differential expression of (A) matrix Gla protein (MGP), (B) osteopontin (OPN), and (C) SM22α (vascular smooth muscle cell marker). mRNA was analyzed by quantitative reverse transcriptase polymerase chain reaction as detailed in the Methods section. Percentage change is shown compared with the control group, which was set at 100%. *P<0.05.

Discussion
We describe for the first time a model of warfarin-induced widespread cardiovascular damage in wild-type DBA/2 mice. This damage included VC with apoptosis in the vessel wall, reduced media cellularity, and impairment of functional cardiovascular parameters. Furthermore, this damage was blocked by treatment with vitamin K2. These effects seem to be mediated via blockade of MGP carboxylation. Price and coworkers created the first experimental model of warfarin-induced VC in rats using a regimen of warfarin+vitamin K1, the latter to prevent lethal bleeding problems.19

Carboxylated forms of MGP are found in intact vessel walls, and uncarboxylated forms colocalize with areas of VC.20 This offers the possibility to modify the activity of MGP, which may influence the development of VC. Here, we demonstrate tissue deposition of calcium in the medial layer of the aortic wall and in myocardial and pulmonary tissue after treatment with warfarin. The calcium content increased in a warfarin dose–dependent manner. In lungs, the absolute calcium content was by far lower compared with the other tissues explaining the lack of von Kossa–positive staining. We attribute the chemical calcium
detection to cartilaginous tissue because we did not detect overt von Kossa-positive staining in pulmonary vessel walls or alveoli. The procalcifying influence of warfarin on bronchi has been previously described in humans, and MGP is known to be synthesized in chondrocytes as well. Similarly, humans with mutations in the MGP gene, the so-called Keutel syndrome, display early diffuse pulmonary cartilage calcification, emphasizing the vitamin K–MGP–calcification axis within this organ system.

Fatal bleeding complications occurred with low dosages of vitamin K1 (0.015 and 0.15 mg/g food) and were absent in high vitamin K1 (1.5 mg/g food) dose. Nevertheless, high dosages (ie, 1.5 mg/g food) did not protect mice against vascular calcification, which is in agreement with results obtained in rats. Vitamin K2 coadministration reduced the development of calcification similar to a rat model of warfarin-induced calcification. Warfarin dosages applied to mice in this experiment are far higher than those used in humans for inhibiting the coagulation cascade (eg, in the 3-mg/g group, daily intake was approximately 10–15 mg per animal [25 g], the usual dose in humans is 5 mg per day/70 kg). This may account for the comparably rapid development of VC in these mice. Nevertheless, also in humans, there is growing evidence from observational studies, that long-term use of vitamin K inhibitors is associated with progression of coronary and VCs.

To identify an alteration of the carboxylation status of MGP, we first stained for MGP in tissue sections of the aorta of warfarin/vitamin K1–treated mice exhibited apoptosis as depicted by positive staining for cleaved caspase-3 (A) and TUNEL (TdT-mediated dUTP-biotin nick end labeling)-positive nuclei (B) of VSMCs (arrow). C, Medial VSMC cellularity detected by automated nuclei counting after DAPI staining was profoundly reduced by warfarin treatment over time, underscoring significant VSMC loss, already evident at day 7. Addition of vitamin K2 to warfarin/vitamin K1 treatment prevented cell loss detected in warfarin/vitamin K1–fed mice. ***P<0.001; **P<0.01; *P<0.05.
decreased t-ucMGP levels.\textsuperscript{10} Nevertheless, our results are in line with the hypothesis that warfarin inhibits γ-carboxylation of MGP. In fact, the proportion of t-ucMGP to total MGP cannot be calculated because an assay of total MGP in mice is not available. The reduction of t-ucMGP in the highest warfarin dose might be explained by a transdifferentiation of VSMCs toward a non–MGP-producing osteoblastic phenotype or by increased apoptosis or nonapoptotic cell death within the vascular medial layer. Another explanation for the reduction in t-ucMGP in the high-warfarin group may be that the established colocalization of ucMGP with areas of calcification may resemble circulating ucMGP. T-ucMGP serum levels were not reduced after treatment with vitamin K2. Similar findings have been made in other vitamin K supplementation studies in rodents and possibly relate to the assay available. This is in contrast to human trials demonstrating a significant decrease of desphospho (dp)-ucMGP serum levels after vitamin K2 treatment.\textsuperscript{50} For rodents, only an assay measuring t-ucMGP and not dp-ucMGP is available. The process of calcification is considered to be actively mediated\textsuperscript{31} as indicated by a transdifferentiation of resident vascular cells like VSMCs. In the present study, a transdifferentiation of resident VSMCs in the vascular wall can be suggested by the reduction of the expression of VSMC-specific SM22α and the concomitant upregulation of osteopontin, which is a marker for osteoblast activity. This is in line with previous findings in the generation of VC.\textsuperscript{52,53} Aortic expression of the upstream regulatory gene cbfa1, which influences the expression of several osteoblastic genes, was also increased in the group of mice treated with 3 mg/g of warfarin but lacked significance (not shown). This further supports the hypothesis of a transdifferentiation of VSMCs towards an osteoblastic phenotype. Whether osteogenic progenitor cells invade into the calcified areas as recently proposed remains speculative in our model.\textsuperscript{64} SM22α expression within the aortic wall decreased after 7 days of warfarin treatment, indicating that VSMC changes preceded detectable calcification. Vitamin K2 treatment together with warfarin and vitamin K1 supplementation did not significantly change the expression of abovementioned genes compared with warfarin and vitamin K1 alone.

The reduction of SM22α expression in warfarin- and vitamin K1–treated mice may be related to cell loss within the vascular wall as well. Because apoptosis is known to facilitate the generation and progression of VC,\textsuperscript{35} we tested for TUNEL-positive and caspase-3–positive staining in the vessel wall. Apoptosis may lead to calcification, and calcification stress may induce apoptosis.\textsuperscript{55,56} Here, apoptosis was only detectable after 4 weeks of warfarin and paralleled the occurrence of calcification in this experiment. Accordingly, we cannot answer the question whether calcification or apoptosis is the first finding in the development of VC. In control animals, we did not detect any positive TUNEL and caspase-3 staining. Vitamin K2 coadministration to warfarin with vitamin K1 resulted in preserved cell numbers in the aorta, which parallels the reduced calcification detected. Besides calcium deposition, apoptosis may also be induced by decreased carboxylation of the protein Gas6, which inhibits VSMC apoptosis.\textsuperscript{37} Because specific antibodies against carboxylated Gas6 are lacking, we were not able to verify the latter hypothesis. Because gene expression of osteoblastic parameters was moderately increased, we cannot fully exclude that development of VC might also be independent of osteoblastic transdifferentiation at the time points evaluated here as mentioned recently.\textsuperscript{15} Nevertheless, we speculate that we detected a start of transdifferentiation that might continue with longer duration of warfarin treatment and calcium accumulation in the aortic wall.

The assessment of functional cardiovascular parameters revealed indices of stenosis of the aortic outflow tract as mirrored by increased peak velocity in the aortic root (ascending aorta) and increased aortic valve gradient. This correlates with findings in humans in which the long-term use of warfarin leads to valvular calcification that in turn induces increased aortic valve gradients and velocities in the aorta.\textsuperscript{26} Because we detected an increased PWV induced by warfarin in the carotid artery in mice, this needs to be addressed in human trials as well. An increased peripheral arterial stiffness is an independent risk factor for cardiac events and mortality.\textsuperscript{36} This underlines the potential importance of local vitamin K availability in the vascular wall. We failed to detect significant differences in PWV in the abdominal aorta. This might be explained by lower degrees of calcification in the abdominal aorta than in the thoracic aorta. Indeed in the present study, the abdominal aorta was free from overt calcification (not shown). In humans, the assessment of PWV is performed between the carotid and femoral artery. Because of the lack of a comparable device for rodents, we could only measure PWV between shorter distances as described in the Methods section. These factors may account for the lack of difference in PWV in the abdominal aorta.

We have to point out that the described generation of VC in response to warfarin administration in mice is restricted to a genetic background of DBA/2 animals. The same protocol applied on C57BL/6 mice did not result in comparable generation of VC (data not shown). The DBA/2 mouse model is prone to VC possibly because of its diminished serum level of magnesium,\textsuperscript{59,60} which is considered as an antagonist of calcium effects. Only recently, warfarin has been proved to induce a vulnerable plaque phenotype in apolipoprotein E–deficient mice, expanding the influence of vitamin K antagonists from the vascular medial layer on intimal atherosclerotic lesions.\textsuperscript{31}

Acknowledgments
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Disclosures
None.

References
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Krüger et al


Significance

This work is the first to describe functional cardiovascular impairment in wild-type mice after the administration of the vitamin K antagonist warfarin. The pathological functional in vivo analyses were supported by histopathological alterations of the vasculature and myocardium, which could partially be inhibited by parallel vitamin K2 administration. This model of vascular calcification offers the opportunity of combining it with transgenic animals to further elucidate the role of vitamin K availability for vascular health.
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Materials and Methods

Animals and diets
DBA/2Nchr mice were purchased from Harlan Laboratories (Rossdorf, Germany) and kept under a 12h light-dark cycle. All procedures were approved by the governmental animal board. All animals received water and food ad libitum. Diets were composed on a vitamin K-free powder basis (Arie Blok, Woerden, the Netherlands). Warfarin and vitamin K1 (phyloquinone) were purchased from Sigma (Munich, Germany), vitamin K2 (menaquinone-4, MK-4) was from CHEMOS (CHEMOS GmbH, Regenstauf, Germany). Diets were mixed and pelleted. For the dose-finding experiment, diets were composed of 1) control food (no warfarin, 1.5 mg/g vitamin K1), 2) 0.03 mg/g warfarin + 1.5 mg/g vitamin K1, 3) 0.3 mg/g warfarin + 1.5 mg/g vitamin K1, and 4) 3 mg/g warfarin + 1.5 mg/g vitamin K1. Diets were administered for 4 weeks. To inhibit the development of calcification we added 100 µg/g vitamin K2 to that warfarin/vitamin K1 dose that offered the most pronounced calcification (i.e. 3 mg/g warfarin and 1.5 mg/g vitamin K1). This diet was also maintained for 4 weeks. For longitudinal measurement of cardiovascular parameters the high-dose warfarin/vitamin K1 diet was chosen (3 mg/g warfarin and 1.5 mg/g vitamin K1) and followed for 1, 4 and 7 weeks. No pair feeding was performed. Animals were sacrificed under anesthesia and perfused with ice-cold non-calcium containing PBS before organs were removed and stored for further analyses.

Calcium measurement
Tissue was freeze-dried using a vacuum freeze dryer (Christ, Osterode, Germany) for 12 hours, weighed and incubated in 500-fold excess of 10% formic acid for 24 hours. Supernatant was used for assessment of tissue calcium content by the colorimetric cresolphthalein method (Randox Laboratories Ltd., Crumlin, UK) according to the manufacturer’s manual. All measurements were performed in duplicate. For histochemical determination, methyl carnoy-fixed tissue specimens were embedded in paraffin using an automated tissue processor. Sections were cut in 5 µm thick slices and calcium deposition was determined by staining according to von Kossa’s method. Stained areas were measured by computer based morphometry (ImageJ, National Institutes of Health, USA).

Serum chemistry
Blood was obtained by retroorbital puncture and collected into tubes containing a clotting-aid. Serum was separated by centrifugation at 2000 × g and stored at −80°C until assayed for calcium, phosphate, C-reactive protein and BUN by standard laboratory methods. Levels of total ucMGP (t-ucMGP) were determined as previously described 1.

RNA Isolation and Quantitative Reverse Transcription
Real-Time PCR Messenger RNA was extracted using the commercial kits RNAlater and RNeasy (Qiagen, Hilden, Germany) with proteinase K digestion before RNA extraction to maximize mRNA yield. Integrity and amount of mRNA were analyzed by capillary electrophoresis (Agilent Bioanalyzer 2100; Agilent Technologies, Böblingen, Germany). Reverse transcription and real-time PCR were performed with the ABI 7700 sequence detection system (PE, Applied Biosystems, Foster City, CA) as described previously in detail 2. Intron-spanning primers were derived from EnsEmbl for OPN (ENSMUST00000031243; sense GACCATGAGATTGGCAGTGATTT, antisense GATCTGGGTGCAGGCTGAAAG, probe FAM-
ATTGCCCTCTCCCTCCCAGGTTG-TAMRA) to yield an amplicon length of 116 bp; for MGP (ENSMUST00000032342; sense GCAGAGTGGGCGAGCTAAAG, antisense AGCCGCTACACAGCTTGTAGTC, probe FAMAGAGTCCAGGAACGCAAAACGCTG-TAMRA) to yield an amplicon length of 104 bp and for SM22-α (ENSMUST00000034590; sense ACGATGGAAACTACCGTGGAGAT, antisense GCCTTCCCCTTCTAATCTGATGA, probe FAM‐GAATGCTCCTTATGCTCTGGCTCTTCT- TAMRA) to yield an amplicon length of 197 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were derived from Ensembl entry ENSMUST00000086934 (sense GGCAAATTCAACCGGACAGT, antisense AGATGGTAGGGGTTCCTCCC, probe FAM‐AAGGCGGAGATGGGAAGCTTGTCTAC- TAMRA) to yield an amplicon length of 74 bp. Absolute mRNA quantification of samples was achieved by co-amplification of known quantities of pGEM-T plasmids (Promega, Madison, WI) containing the cloned target genes (GAPDH, OPN, MGP, and SM22-alpha). Expression was normalized to 1 million copies of GAPDH mRNA determined from the identical mRNA sample in each case. The expression level in untreated mice was arbitrarily assigned the value 1.0, and all other expression values were expressed as fold changes thereof.

**Caspase-3, TUNEL, DAPI and MGP staining**

To assess the degree of apoptosis in the aortic wall, we stained for cleaved caspase-3 and with the TUNEL method. For cleaved caspase-3, paraffin-embedded tissue sections were heated in citric acid (pH 6.0), rinsed with PBS and blocked with 20% FBS. Specimens were then incubated overnight with the primary antibody against cleaved caspase-3 (1:500, Cell Signaling, Danvers, MA, USA). A biotinylated goat anti-rabbit antibody (1:300, Vectorlabs, Burlingame, CA, USA), served as secondary antibody and visualization was performed using an ABC kit according to the manufacturer’s instruction (Elite Vectastain ABC™, Vectorlabs, Burlingame, CA, USA).

The TUNEL staining was performed as described elsewhere². In brief, sections of the aortic arch were fixed with 4% PFA and permeabilized with 0.1% Triton X-100. Specimens were stained using a commercial kit (In Situ Cell Death Detection Kit, AP, Roche Diagnostics Corp., Indianapolis, USA) according to the manufacturer’s instructions. The cellularity of the tissue sections was determined by DAPI staining (1.5 µg/ml; Vectashield™, Vectorlabs, Burlingame, CA, USA) and subsequent fluorescence microscopy and automated cell nucleus counting (ImageJ, National Institutes of Health, USA).

Immunohistochemistry on MGP was performed as previously described⁴. Sections were embedded in paraffin and subsequently cut (4 mm thickness). Staining was performed using antibodies against total MGP, tMGP (5 µg), carboxylated MGP, cMGP (1 µg), and uncarboxylated MGP, ucMGP (1 µg, provided by Vascular Products BV, Maastricht, the Netherlands). Immunostaining was performed using either biotinylated sheep anti–mouse IgG (Amersham Biosciences, Little Chalfont, United Kingdom) or biotinylated swine anti–rabbit IgG (Dako, Golstrup, Denmark) as a second antibody, followed by incubation with avidin-linked alkaline phosphatase complex (Dako); staining was performed by the alkaline phosphatase kit I (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin and mounted with coverslips.

**Assessment of cardiovascular parameters**

Echocardiography was performed as previously described⁵. Briefly, mice were anesthetized using isoflurane and two-dimensional and M-mode measurements were performed with a 30-MHz linear phased-array probe connected to a Vevo 770 echocardiography unit (VisualSonics Europe, Amsterdam, the Netherlands). The animals were placed in the supine-
lateral position under mask anesthesia by an inhaled mixture of 1.5% (v/v) isoflurane and 100% oxygen. ECGs were obtained throughout the procedure. Body temperature was maintained at 37°C by a heating pad. Excessive pressure on the thorax was avoided. Parasternal long-axis and short-axis views of the left ventricle (LV) were obtained, ensuring that the mitral and aortic valves and apex were well visualized. Area fraction and wall area were determined by planimetry of end-diastolic and systolic volumes in parasternal short axis. Measurements of LV end-diastolic and end-systolic dimensions were obtained in M-mode at mid-papillary level from more than three beats and fractional shortening (FS) was calculated as FS (%) = ((LVIDd − LVIDs) / LVIDd) × 100, where LVID is LV internal diameter, s is systole and d is diastole. Diastole is defined as maximum measurable area; systole is defined as minimum measurable area. Doppler flow spectrum of the ascending aorta was recorded from the suprasternal view. Peak velocity was measured, and the waveform was also traced to obtain a velocity time-integral (VTI) calculation and peak gradient.

Pulse-wave velocity in the right common carotid artery and the abdominal aorta were measured using the transit-time method in a two-dimensional mode as described previously. In the carotid artery, the proximal pulse wave signal was obtained 1 mm behind the origin of the subclavian artery, the distal signal 1.5 mm before the carotid bifurcation. The transit time was found by subtracting the distal arrival time between the ECG R-wave peak and the foot of velocity upstroke from the similarly determined proximal arrival time (PWV = Δd / (Pt_{dist} − Pt_{prox})), where Pt is the time point of the proximal or distal pulse wave signal and Δd is the distance between the two measuring points. The aortic pulse wave velocity was determined between the renal arteries and 5 mm behind using the same calculation formula.

**Blood pressure measurement**

Blood pressure was measured at beginning and after 4 and 7 weeks of treatment with 3 mg/g warfarin with vitamin K1 and compared to control diet using tail plethysmography in adapted and conscious mice as described previously.

**Statistics**

If not otherwise noted, analysis of variance (ANOVA) with Tukey’s post-hoc analysis was used to test for overall differences in non-size-matched experimental groups. A p < 0.05 was regarded as significant.
Reference List


