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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 Gla protein mRNA expression decreased, and inactive matrix Gla 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;33:2618-2624.)**

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

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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 outcome.[3,4] Additionally, effective secondary preventive strategies for cardiovascular disease may translate into slower progression of VC.[5]

Physiologically, VC is prevented by a network of calcification-inhibitory proteins[6]: matrix Gla 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,[8] a common finding in various forms of advanced VC.[9]

Vitamin K–dependent proteins need vitamin K as cofactor for post-translational γ-glutamylcarboxylation 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, uncarboxylated MGP (ucMGP) thereby potentially promoting VC. Observational studies have shown an association

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Nonstandard Abbreviations and Acronyms	
MGP	matrix Gla protein
PWV	pulse-wave velocity
t-ucMGP	total uncarboxylated MGP
ucMGP	uncarboxylated MGP
VC	vascular calcification
VSMC	vascular smooth muscle cell

between long-term warfarin treatment and increased prevalence and extent of aortic valve and coronary calcifications, respectively.[12,13] Potential mechanisms of MGP-mediated inhibition of VC represent inhibition of calcium-crystal growth,[14,15] bone morphogenetic proteins,[16,17] and transdifferentiation of VSMCs into an osteochondrogenic phenotype, respectively.[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 µg 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

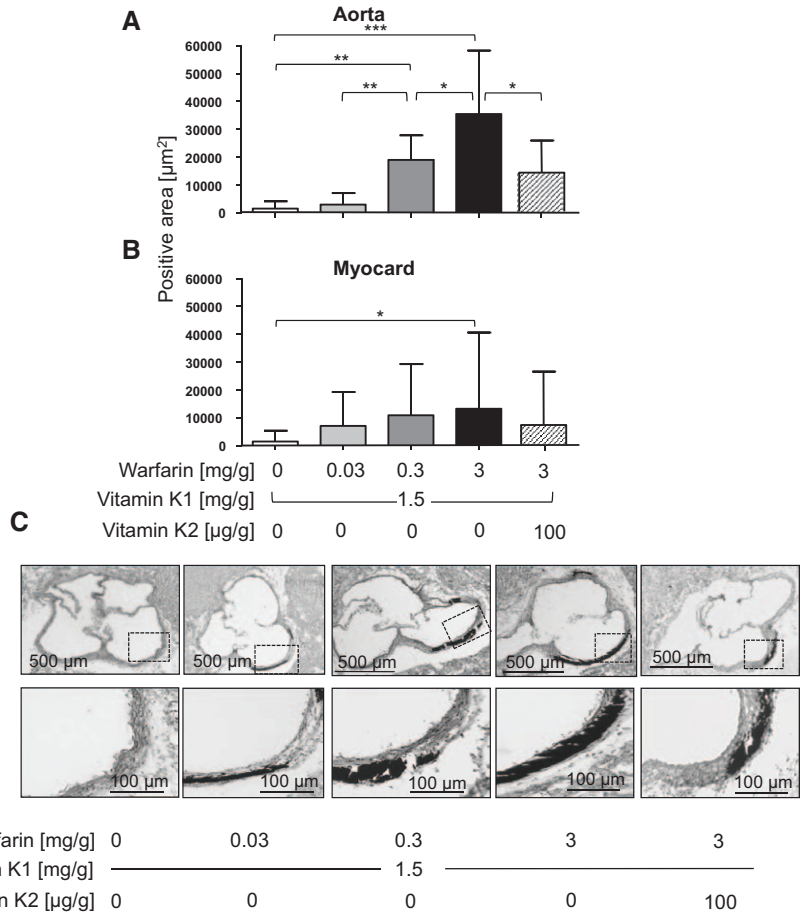

Figure 1. Cardiovascular calcification as detected by quantitative histomorphometry of von Kossa–stained aorta (**A**) and myocardium (**B**) in mice after 28 days of warfarin with vitamin K administration. Representative von Kossa–stained sections through the aortic root depict the stepwise increment 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 histographs below show higher magnifications of areas specified above. ***$P<0.001$; **$P<0.01$; *$P<0.05$.

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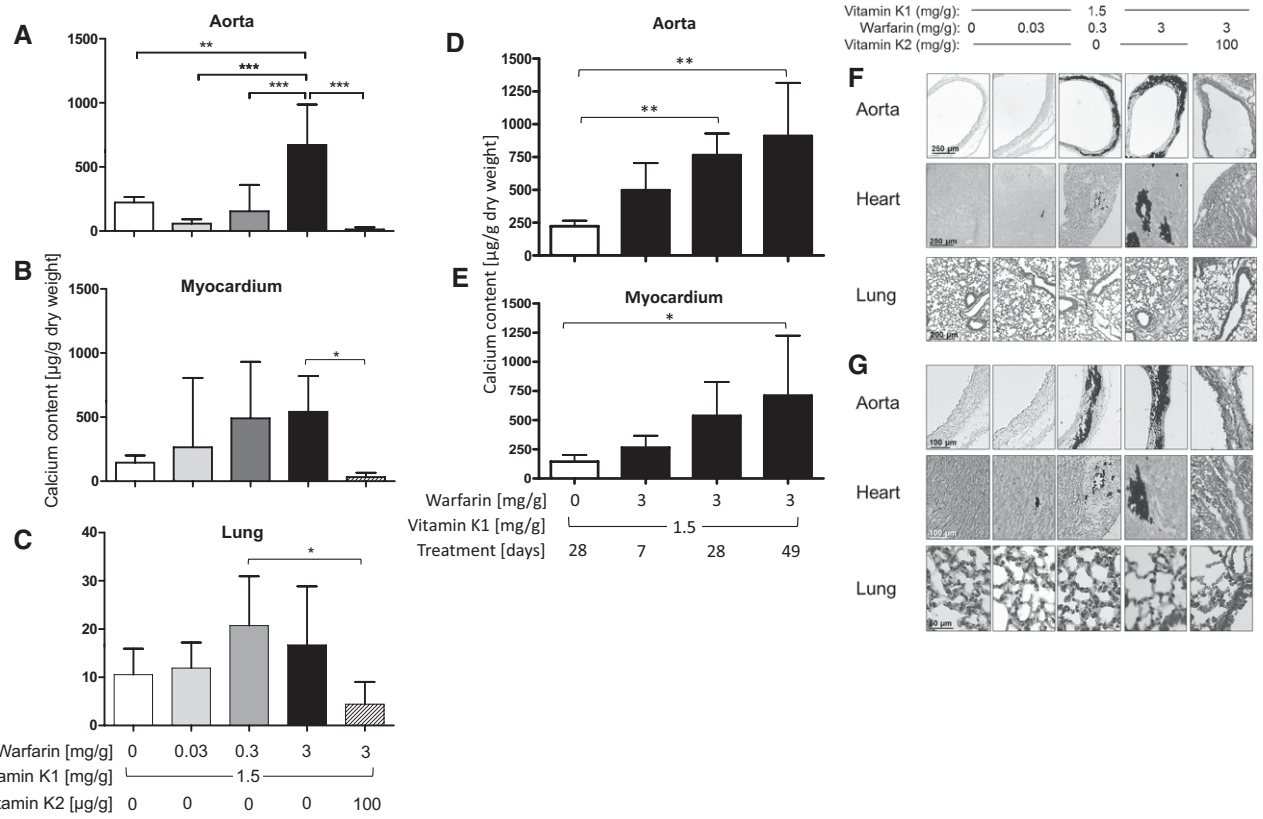

Figure 2. Extraosseous calcification after 4 weeks of warfarin and vitamin K treatment in various organs as detected by chemical calcium analysis (**A** to **C**). **D** and **E** depict evolution of aortic and myocardial calcium content over time by the same method. Quantitative assessment of calcified areas in various organs by von Kossa staining is shown in **F** and **G**, **G** showing higher magnifications. ***P<0.001; **P<0.01; *P<0.05.

(Figures 1A and 2A). In lungs, vitamin K2 addition led to significantly reduced calcium contents compared with 0.3 mg/g warfarin with vitamin K1 diet (Figures 1 and 2).

Serum Chemistry and Total-ucMGP

Warfarin with vitamin K1–treated animals did not show any significant changes in serum levels of calcium, phosphorus, C-reactive protein, and blood urea nitrogen (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 and K1 treatment alone (Figure 3A). Staining of the aortic wall for ucMGP and total MGP revealed an increase of ucMGP in warfarin-treated animals during 4 weeks compared with the control group, which is paralleled by a decrease of total MGP and total Gla residues (Figure 3B–G).

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).

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 4′,6-diamidino-2-phenylindole-positive nuclei within the aortic wall of 3 mg/g warfarin with vitamin K1–treated animals.

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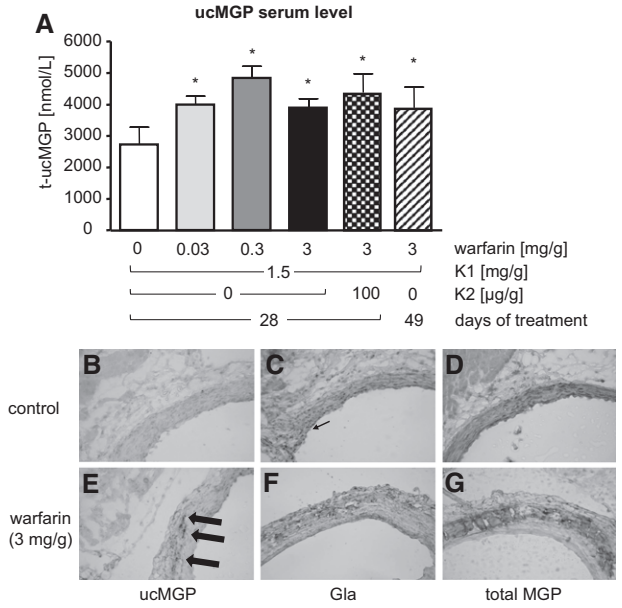

Figure 3. Warfarin administration for 4 weeks leads to increased serum levels of total uncarboxylated matrix Gla protein (t-ucMGP). The increase of t-ucMGP serum levels is less pronounced in the high-dose warfarin group, which is characterized by advanced calcification. Vitamin K2 treatment and extended 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.

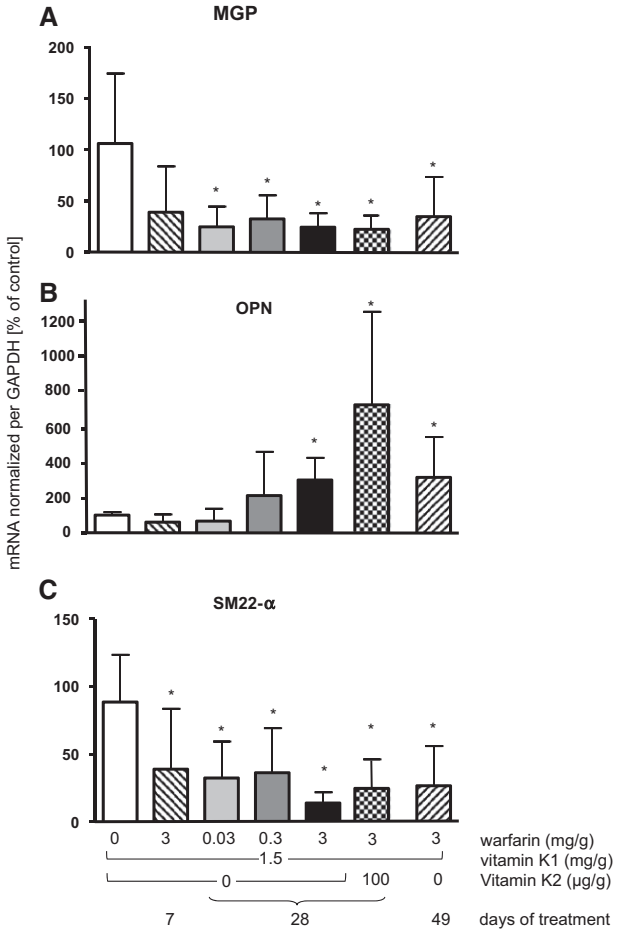

Figure 4. Phenotypic characterization of calcifying aortic tissue. Differential expression of (**A**) matrix Gla protein (MGP), (**B**) osteopontin (OPN), and (**C**) SM22α (vascular smooth muscle cell marker). mRNA was analyzed by quantitative reverse transcriptase polymerase chain reaction as detailed in the Methods section. Percentage change is shown compared with the control group, which was set at 100%. *P<0.05. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.

The vascular cellularity was further reduced significantly when extending the treatment phase 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).

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

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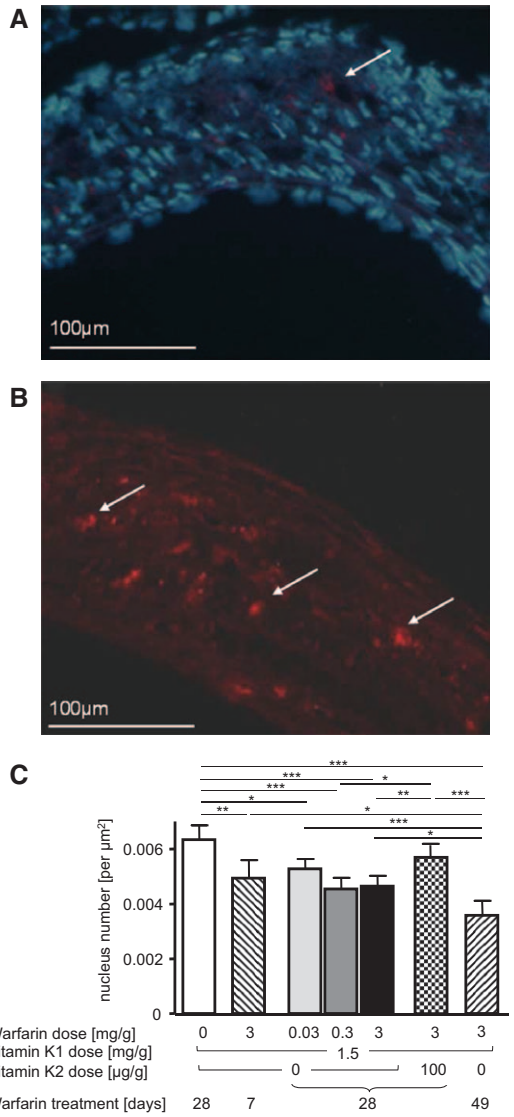

Figure 5. Warfarin administration induces vascular smooth muscle cell (VSMC)-specific apoptosis. Sections cut through the aorta of warfarin/vitamin K1–treated mice exhibited apoptosis as depicted by positive staining for cleaved capsase-3 (**A**) and TUNEL (TdT-mediated dUTP-biotin nick end labeling)-positive nuclei (**B**) of VSMCs (arrow). **C**, Medial VSMC cellularity detected by automated nuclei counting after 4′,6-diamidino-2-phenylindole 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.

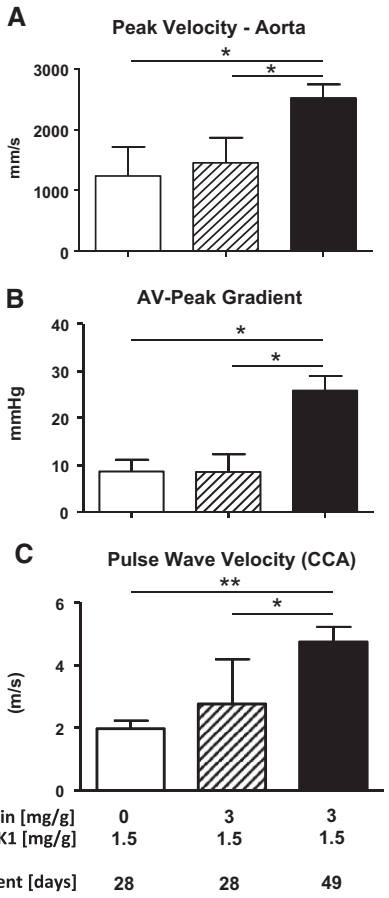

Figure 6. Warfarin administration during 4 and 7 weeks resulted in increased peak velocity (**A**), aortic valve (AV)–peak gradient (**B**) and an increased pulse-wave velocity in the common carotid artery (**C**). Concerning the peak velocity and AV-peak gradient, statistical significance was only reached after 7 weeks of treatment with 3 mg/g warfarin plus 1.5 mg/g vitamin K1. *P<0.05; **P<0.01. CCA indicates common carotid artery.

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 alveoles. The procalcifying influence of warfarin on bronchi has been previously described in humans,[21] and MGP is known to be synthesized in chondrocytes as well.[22] 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.[23,24] 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.[19] Vitamin K2 coadministration reduced the development of calcification similar to a rat model of warfarin-induced calcification.[25] 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 ≈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.[26–29]

To identify an alteration of the carboxylation status of MGP, we first stained for MGP in tissue sections of the aorta. We detected positive staining for the inactive form ucMGP in the medial vascular layer in warfarin-treated animals, whereas no staining was seen in control animals. This was paralleled by a reduction of total Gla residues and total MGP in warfarin-fed mice. Second, we assessed the levels of t-ucMGP in mouse sera. Compared with controls, we found a significant increase of t-ucMGP in low and medium dose levels of warfarin treatment

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(0.03 and 0.3 mg/g) with vitamin K1 but a reduction in the high-dose group compared with the medium group. This finding is in contrast to human data where warfarin treatment decreased t-ucMGP levels.[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.[30] For rodents, only an assay measuring t-ucMGP and not dp-ucMGP is available. The process of calcification is considered to be actively mediated[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.[32,33] 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 remain speculative in our model.[34] 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,[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.[35,36] 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.[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.[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.[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.[38] 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,[39,40] 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.[41]

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Disclosures

None.

References

1. Doherty TM, Asotra K, Fitzpatrick LA, Qiao JH, Wilkin DJ, Detrano RC, Dunstan CR, Shah PK, Rajavashisth TB. Calcification in atherosclerosis: bone biology and chronic inflammation at the arterial crossroads. *Proc Natl Acad Sci USA*. 2003;100:11201–11206.
2. Abedin M, Tintut Y, Demer LL. Vascular calcification: mechanisms and clinical ramifications. *Arterioscler Thromb Vasc Biol*. 2004;24:1161–1170.
3. Iribarren C, Sidney S, Sternfeld B, Browner WS. Calcification of the aortic arch: risk factors and association with coronary heart disease, stroke, and peripheral vascular disease. *JAMA*. 2000;283:2810–2815.
4. Greenland P, Lloyd-Jones D. Time to end the mixed–and often incorrect–messages about prevention and treatment of atherosclerotic cardiovascular disease. *J Am Coll Cardiol*. 2007;50:2133–2135.

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5. Achenbach S, Ropers D, Pohle K, Leber A, Thilo C, Knez A, Menendez T, Maeffert R, Kusus M, Regenfus M, Bickel A, Haberl R, Steinbeck G, Moshage W, Daniel WG. Influence of lipid-lowering therapy on the progression of coronary artery calcification: a prospective evaluation. *Circulation*. 2002;106:1077–1082.
6. Schinke T, McKee MD, Karsenty G. Extracellular matrix calcification: where is the action? *Nat Genet*. 1999;21:150–151.
7. Luo G, Ducy P, McKee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature*. 1997;386:78–81.
8. El-Maadawy S, Kaartinen MT, Schinke T, Murshed M, Karsenty G, McKee MD. Cartilage formation and calcification in arteries of mice lacking matrix Gla protein. *Connect Tissue Res*. 2003;44(suppl 1):272–278.
9. Shanahan CM. Inflammation ushers in calcification: a cycle of damage and protection? *Circulation*. 2007;116:2782–2785.
10. Schurgers LJ, Cranenburg EC, Vermeer C. Matrix Gla-protein: the calcification inhibitor in need of vitamin K. *Thromb Haemost*. 2008;100:593–603.
11. Chatrou ML, Winckers K, Hackeng TM, Reutelingsperger CP, Schurgers LJ. Vascular calcification: the price to pay for anticoagulation therapy with vitamin K-antagonists. *Blood Rev*. 2012;26:155–166.
12. Schurgers LJ, Shearer MJ, Hamulyák K, Stöcklin E, Vermeer C. Effect of vitamin K intake on the stability of oral anticoagulant treatment: dose-response relationships in healthy subjects. *Blood*. 2004;104:2682–2689.
13. Koos R, Krueger T, Westenfeld R, Kühl HP, Brandenburg V, Mahnken AH, Stanzel S, Vermeer C, Cranenburg EC, Floege J, Kelm M, Schurgers LJ. Relation of circulating Matrix Gla-Protein and anticoagulation status in patients with aortic valve calcification. *Thromb Haemost*. 2009;101:706–713.
14. Schurgers LJ, Spronk HM, Skepper JN, Hackeng TM, Shanahan CM, Vermeer C, Weissberg PL, Proudfoot D. Post-translational modifications regulate matrix Gla protein function: importance for inhibition of vascular smooth muscle cell calcification. *J Thromb Haemost*. 2007;5:2503–2511.
15. Khavandgar Z, Roman H, Li J, Lee S, Vali H, Brinckmann J, Davis EC, Murshed M. Elastin haploinsufficiency impedes the progression of arterial calcification in MGP-deficient mice. *J Bone Miner Res*. July 15, 2013. doi: 10.1002/jbmr.2039. http://onlinelibrary.wiley.com/doi/10.1002/jbmr.2039/abstract;jsessionid=717E16A93C0BD8956AC6C3EB2312563D.d03t04. Accessed September 5, 2013.
16. Zebboudj AF, Imura M, Boström K. Matrix GLA protein, a regulatory protein for bone morphogenetic protein-2. *J Biol Chem*. 2002;277:4388–4394.
17. Yao Y, Zebboudj AF, Shao E, Perez M, Boström K. Regulation of bone morphogenetic protein-4 by matrix GLA protein in vascular endothelial cells involves activin-like kinase receptor 1. *J Biol Chem*. 2006;281:33921–33930.
18. Speer MY, Yang HY, Brabb T, Leaf E, Look A, Lin WL, Frutkin A, Dichek D, Giachelli CM. Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. *Circ Res*. 2009;104:733–741.
19. Price PA, Faus SA, Williamson MK. Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler Thromb Vasc Biol*. 1998;18:1400–1407.
20. Schurgers LJ, Teunissen KJ, Knapen MH, Kwaijtaal M, van Diest R, Appels A, Reutelingsperger CP, Cleutjens JP, Vermeer C. Novel conformation-specific antibodies against matrix gamma-carboxyglutamic acid (Gla) protein: undercarboxylated matrix Gla protein as marker for vascular calcification. *Arterioscler Thromb Vasc Biol*. 2005;25:1629–1633.
21. Moncada RM, Venta LA, Venta ER, Fareed J, Walenga JM, Messmore HL. Tracheal and bronchial cartilaginous rings: warfarin sodium-induced calcification. *Radiology*. 1992;184:437–439.
22. Yagami K, Suh JY, Enomoto-Iwamoto M, Koyama E, Abrams WR, Shapiro IM, Pacifici M, Iwamoto M. Matrix GLA protein is a developmental regulator of chondrocyte mineralization and, when constitutively expressed, blocks endochondral and intramembranous ossification in the limb. *J Cell Biol*. 1999;147:1097–1108.
23. Keutel J, Jörgensen G, Gabriel P. [A new autosomal-recessive hereditary syndrome. Multiple peripheral pulmonary stenosis, brachytelephalangia, inner-ear deafness, ossification or calcification of cartilages]. *Dtsch Med Wochenschr*. 1971;96:1676–81 passim.
24. Schurgers LJ, Uitto J, Reutelingsperger CP. Vitamin K-dependent carboxylation of matrix Gla-protein: a crucial switch to control ectopic mineralization. *Trends Mol Med*. 2013;19:217–226.
25. Schurgers LJ, Spronk HM, Soute BA, Schiffers PM, DeMey JG, Vermeer C. Regression of warfarin-induced medial elastocalcinosis by high intake of vitamin K in rats. *Blood*. 2007;109:2823–2831.
26. Koos R, Mahnken AH, Mühlenbruch G, Brandenburg V, Pflueger B, Wildberger JE, Kühl HP. Relation of oral anticoagulation to cardiac valvular and coronary calcium assessed by multislice spiral computed tomography. *Am J Cardiol*. 2005;96:747–749.
27. Schurgers LJ, Aebert H, Vermeer C, Bültmann B, Janzen J. Oral anticoagulant treatment: friend or foe in cardiovascular disease? *Blood*. 2004;104:3231–3232.
28. Rennenberg RJ, de Leeuw PW, Kessels AG, Schurgers LJ, Vermeer C, van Engelshoven JM, Kemerink GJ, Kroon AA. Calcium scores and matrix Gla protein levels: association with vitamin K status. *Eur J Clin Invest*. 2010;40:344–349.
29. Weijs B, Blaauw Y, Rennenberg RJ, Schurgers LJ, Timmermans CC, Pison L, Nieuwlaat R, Hofstra L, Kroon AA, Wildberger J, Crijns HJ. Patients using vitamin K antagonists show increased levels of coronary calcification: an observational study in low-risk atrial fibrillation patients. *Eur Heart J*. 2011;32:2555–2562.
30. Westenfeld R, Krueger T, Schlieper G, Cranenburg EC, Magdeleyns EJ, Heidenreich S, Holzmann S, Vermeer C, Jahnen-Dechent W, Ketteler M, Floege J, Schurgers LJ. Effect of vitamin K2 supplementation on functional vitamin K deficiency in hemodialysis patients: a randomized trial. *Am J Kidney Dis*. 2012;59:186–195.
31. Schlieper G, Westenfeld R, Brandenburg V, Ketteler M. Inhibitors of calcification in blood and urine. *Semin Dial*. 2007;20:113–121.
32. Chen NX, Moe SM. Uremic vascular calcification. *J Investig Med*. 2006;54:380–384.
33. Steitz SA, Speer MY, Curinga G, Yang HY, Haynes P, Aebersold R, Schinke T, Karsenty G, Giachelli CM. Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfa1 and downregulation of smooth muscle lineage markers. *Circ Res*. 2001;89:1147–1154.
34. Naik V, Leaf EM, Hu JH, Yang HY, Nguyen NB, Giachelli CM, Speer MY. Sources of cells that contribute to atherosclerotic intimal calcification: an *in vivo* genetic fate mapping study. *Cardiovasc Res*. 2012;94:545–554.
35. Clarke MC, Littlewood TD, Figg N, Maguire JJ, Davenport AP, Goddard M, Bennett MR. Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. *Circ Res*. 2008;102:1529–1538.
36. Reynolds JL, Joannides AJ, Skepper JN, McNair R, Schurgers LJ, Proudfoot D, Jahnen-Dechent W, Weissberg PL, Shanahan CM. Human vascular smooth muscle cells undergo vesicle-mediated calcification in response to changes in extracellular calcium and phosphate concentrations: a potential mechanism for accelerated vascular calcification in ESRD. *J Am Soc Nephrol*. 2004;15:2857–2867.
37. Melaragno MG, Cavet ME, Yan C, Tai LK, Jin ZG, Haendeler J, Berk BC. Gas6 inhibits apoptosis in vascular smooth muscle: role of Axl kinase and Akt. *J Mol Cell Cardiol*. 2004;37:881–887.
38. Vlachopoulos C, Aznaouridis K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. *J Am Coll Cardiol*. 2010;55:1318–1327.
39. van den Broek FA, Beynen AC. The influence of dietary phosphorus and magnesium concentrations on the calcium content of heart and kidneys of DBA/2 and NMRI mice. *Lab Anim*. 1998;32:483–491.
40. van den Broek FA, Beems RB, van Tintelen G, Lemmens AG, Fielmich-Bouwman AX, Beynen AC. Co-variance of chemically and histologically analysed severity of dystrophic cardiac calcification in mice. *Lab Anim*. 1997;31:74–80.
41. Schurgers LJ, Joosen IA, Laufer EM, et al. Vitamin K-antagonists accelerate atherosclerotic calcification and induce a vulnerable plaque phenotype. *PLoS ONE*. 2012;7:e43229.

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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