Regression of warfarin-induced medial elastocalcinosis by high intake of vitamin K in rats

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Word count: abstract: 193 // total: 4701
Tables: 2 // Figures: 7 (4 color figures)

L.J.S designed the study, compiled and analysed the data and wrote the first manuscript draft. H.M.H.S performed research and analyzed data. B.A.M.S performed research and analysed data. P.M.S performed research. J.G.R.DM performed analysis of the data and contributed to the manuscript writing. CV designed the study and contributed in the manuscript writing.
**Abstract**

Arterial calcification (AC) is generally regarded as an independent risk factor for cardiovascular morbidity and mortality. Matrix Gla-protein (MGP) is a potent inhibitor of AC and its activity depends on vitamin K (VK). In rats, inactivation of MGP by treatment with the vitamin K-antagonist warfarin leads to rapid calcification of the arteries. Here we investigated whether pre-formed AC can be regressed by a VK-rich diet. Rats received a calcification-inducing diet containing both VK and warfarin (W&K). During a second 6-week period, animals were randomized to receive either W&K (3.0 mg/g & 1.5 mg/g, subsequently), a diet containing normal (5 µg/g) or high (100 µg/g) amount of VK (either K₁ or K₂). Increased aortic calcium concentration was observed in the group that continued to receive W&K, and also in the group changed to the normal dose of VK, AC progressed. Both the VK-rich diets decreased the arterial calcium content by some 50%. Additionally, arterial distensibility was restored by the VK-rich diet. Using MGP antibodies, local VK-deficiency was demonstrated at sites of calcification. This is the first study in rats demonstrating that AC and the resulting decreased arterial distensibility are reversible by high VK intake.
Introduction:

Arterial calcification is an important independent risk factor for the development of atherosclerosis, myocardial infarction, stroke, and renal disease \(^1,2\). Patients with manifest arterial calcification have an unfavorable prognosis compared to patients with no or mild calcification \(^3,4\). Therefore, the prevention or reversal of arterial calcification may lead to improved patient outcomes.

For a long time it has been thought that calcification was a passive process and the end stage of cardiovascular disease. During the last ten years, however, it has become clear that several osteoregulatory proteins, both stimulatory and inhibitory, are involved in the calcification of vascular tissue \(^5-8\). One of the strongest \textit{in vivo} inhibitors of arterial calcification is matrix Gla protein (MGP). MGP was first discovered in bone \(^9\), but it is mainly produced by vascular smooth muscle cells and chondrocytes. Its function became clear in MGP deficient mice \(^10\), which died within 6-8 weeks after birth due to rupture of the large arteries. Histochemical evaluation demonstrated complete calcification of the elastic fibers in the arterial vessels and a phenotypic change of smooth muscle cells into chondrocytes. MGP acts by direct inhibition of calcium crystal formation, and regulates bone morphogenetic protein-2 (BMP2), a growth factor responsible for osteogenic differentiation \(^11-13\). Recently, Murshed \textit{et al.} \(^14\) demonstrated that restoration of MGP exclusively in the vascular smooth muscle cells of the MGP null-mice, completely rescued the vascular calcification phenotype. For this effect the MGP needed to be gamma-carboxylated because mutating the Gla residues into aspartic acid residues led to the synthesis of non-functional MGP and to the death of all animals.
Vitamin K is an essential cofactor in the $\gamma$-carboxylation of glutamate residues in a small group of proteins including MGP. The activity of these vitamin K-dependent or Gla-proteins is strictly dependent on the presence of $\gamma$-carboxyglutamate (Gla) residues at a number of well-defined positions. The oxidation of vitamin K-hydroquinone (KH$_2$) into vitamin K-epoxide (KO) provides the energy required for Gla-formation, and the KO formed can be re-used after subsequent reduction by the enzyme vitamin K-epoxide reductase (VKOR). Coumarin-derivatives such as warfarin specifically block VKOR, leading to exhaustion of the available vitamin K stores and to the synthesis of non-carboxylated, inactive Gla-proteins. Mainly in the liver, a second pathway for KH$_2$ formation is present. The key enzyme in this pathway is the NAD(P)H-dependent DT-diaphorase. In extra-hepatic tissues such as the arterial vessel wall, DT-diaphorase activity is low, which explains why high vitamin K intake effectively counteracts the effect of warfarin in the liver, but not in bone and the arterial vessel wall. Thus, by subjecting experimental animals to a regimen of warfarin + vitamin K, the synthesis of Gla-proteins can be blocked in the extrahepatic tissues, without affecting coagulation factor synthesis in the liver. Using this regimen of warfarin and vitamin K, Price et al. showed that this induced medial calcifications of the elastic lamellae in arteries and heart valves of rats within 3–5 weeks through inhibition of the $\gamma$-glutamyl carboxylation of matrix Gla protein.

Vitamin K consists of two forms, namely phylloquinone (vitamin K$_1$; K$_1$) and the menaquinones (vitamin K$_2$; K$_2$). It has been reported that K$_1$ can be converted into vitamin K$_2$ (specifically MK4, menaquinone-4), the accumulation appears to be specific for extra-hepatic tissues. A recent study by Thijssen et al. revealed that menadione
(2-methyl-1,4-naphthoquinone) is a product of vitamin K catabolism and the likely intermediate in the synthesis of MK4 that is found in the arterial vessel wall. Recently, we used this arterial calcification model \(^{20}\) (the calcification inducing regimen of warfarin and vitamin K) and found that high vitamin K\(_2\) supplementation was able to inhibit warfarin induced arterial calcification in rats \(^{25}\). Moreover, we and others have reported that the use of coumarin-type anticoagulants is associated with increased cardiac valve calcification \(^{26,27}\). Further studies using immunohistochemical analysis with conformation-specific antibodies demonstrated that in calcifying carotid arteries MGP predominantly occurs in the non-carboxylated form, suggesting that the local vitamin K status is sub-optimal \(^{28,13}\).

The rat arterial calcification model, as developed by Price et al \(^{20}\) and used by others \(^{21,25,29}\) has thus far only looked at the development of arterial calcification. The aim of the present study was to use the rat arterial calcification model to investigate whether maximal MGP activity, ascertained by high vitamin K intake may stop the progression or even induce a reversal of warfarin induced arterial calcification and the associated decrease in arterial distensibility.
Materials and Methods:

Animals & diet

Male Wistar Kyoto rats were purchased from the Maastricht University. Rats were 10 weeks old when entering the study and all animals were housed in normal cages with free access to water and the indicated foods. Irradiated (0.9Mrad) vitamin K-deficient food was from Hope Farms, Woerden, The Netherlands. Vitamins K\textsubscript{1} and K\textsubscript{2} were dissolved in corn oil prior to adding to the vitamin K-deficient food in the required amounts. Warfarin was added directly to the food. All experimental protocols were approved by the Experimental Animal Ethics Committee of the Maastricht University.

To induce vascular calcification, rats (n=30) received a diet containing warfarin (3 mg/g food) and vitamin K\textsubscript{1} (1.5 mg/g food: the minimal dose required for rats is 0.5 µg/g food), according to the method described earlier by our group\textsuperscript{25}. These animals are designated as the W&K group. Control rats (n=18) received no warfarin and a normal dose of vitamin K\textsubscript{1} (5 µg/g food; this is equivalent to the vitamin K amount in normal standard rat food). From the control group, six rats where sacrificed at the start of the experiment to measure the baseline calcium content of the abdominal aorta and left carotid artery. After six weeks of treatment, 6 control rats and 6 W&K rats were sacrificed to monitor the effect of treatment. The remaining rats in the W&K group (n=24) were subdivided into four groups of six rats for another six-week treatment. One group continued the W&K diet, whereas warfarin was discontinued in the remaining three groups: one group received normal vitamin K\textsubscript{1} (5 µg/g food), one group received high vitamin K\textsubscript{1} (100 µg/g food: the dietary vitamin K requirements for rats are 0.5 µg/g food to maintain normal blood clotting), and the last group received high vitamin K\textsubscript{2}.
(menaquinone-4, 100 µg/g food). In addition, the remaining 6 control rats continued their diet for another six weeks (see Figure 1 for schematic overview).

**Antibodies and chemicals**

Monoclonal and polyclonal antibodies against various epitopes of MGP were raised according to standard procedures. The following MGP-derived peptides were used for immunization: aminoacid residues 61-79 (tMGP), residues 35-53 (4 Gla-residues; cMGP) and residues 35-53 (4 Glu-residues; ucMGP). The corresponding antibodies are designated as poAb-anti-tMGP (recognizing all forms of MGP), moAb-anti-cMGP (recognizing carboxylated MGP; cMGP), and poAb-anti-ucMGP (recognizing non-carboxylated MGP; ucMGP), respectively. Vitamin K$_1$ and warfarin were purchased from Sigma (Saint Louis, USA); vitamin K$_2$ (menaquinone-4) was a kind gift from Eisai (Tokyo, Japan). All chemicals were of analytical grade or better.

**Experimental procedures**

Rats were anesthetized with sodium pentobarbital. Blood was collected in 105 mM trisodium citrate either by tail vein puncture or from the portal vein (at the end of the experiment) and plasma aliquots were frozen at -80 °C. Before collecting all required tissues, the vasculature was perfused with a sterile vasodilating saline solution (150 mM saline, 100 pM sodium nitroprusside) via the portal vein. The aortic arch, thoracic and abdominal aorta, and right and left carotid artery were dissected, transferred to a physiological salt solution in a silicon-coated Petri dish, and adipose and connective tissue were carefully removed. The abdominal aorta and left carotid artery were frozen in
liquid nitrogen for assessment of the calcium content. The aortic arch and thoracic aorta were fixed in 1% (v/v) Hepes-buffered formaldehyde overnight at 4 °C for immunohistochemistry. The right carotid artery was used for monitoring the distensibility and compliance.

**Biochemical and immunohistochemical measurements**

Tissue calcium was determined after lyophilization and expressed per g dry weight; the freeze-dried tissues were extracted with a ten fold excess (w/v) of 10% formic acid (overnight at 4 °C) and calcium concentrations were measured using atomic absorption-spectrometry (AAS, Department of Clinical Chemistry, University Hospital Maastricht, The Netherlands). Immunohistochemistry was performed after embedding the tissues in paraffin and subsequent sectioning (4 µm thick). Each seventh section was used for calcium detection by Von Kossa staining. Each subsequent section was stained for haematoxilin/eosin, macrophages (mouse anti-rat CD68, Serotec, Oxford, UK), apoptosis using ApopTag® apoptosis detection kit (Chemicon, Temecula, USA), poAb-tMGP (5 µg), moAb-cMGP (1 µg), and poAb-ucMGP (1 µg), respectively. Immunostaining was performed using either biotinylated sheep anti-mouse IgG (Amersham Biosciences, Little Chalfont, UK) or biotinylated swine anti-rabbit IgG (Dako, Golstrup, Denmark) was used as a second antibody (60 minutes at RT), followed by incubation with avidin-linked alkaline phosphatase complex (30 minutes at RT; Dako, Golstrup, Denmark); staining was performed by the alkaline phosphatase kit I (staining 5 minutes at RT; Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin and mounted with coverslips. Each antibody staining was performed in one batch.
The relative extent of MGP staining and apoptosis was measured using a microscope coupled to a computized morphometry system (quantimed 570, Leica, the Netherlands). Three sections (28 µm apart) were used for morphometric analysis, and quantification was expressed as percentage staining of the total arterial medial area. To reliably compare the different antibodies (anti-tMGP, anti-cMGP, and anti-ucMGP) both microscope and camera adjustments were kept constant. 26,30

MGP in plasma was measured using a commercially available ELISA based assay using a moAb MGP antibody recognizing the n-terminal 3-15 sequence of MGP (Biomedica, Vienna, Austria) 3128.

**Vitamin K and KO Determination**

Concentrations of vitamin K1, K1-O, MK-4, and MK-4-O were analyzed by HPLC as described previously 25. Briefly, part of the aorta was weighed and homogenized in ethanol / water (v/v) at a ratio 1:2 using a blender (Ultra Turrax, Janke and Kunkel, Staufen, Germany). Samples were supplemented with 100 ng vitamin vitamin K1(25) (GLSynthesis, Worcester, USA) as an internal standard, extracted with 4 volumes of n-hexane, and pre-purified on silica columns as described previously 32. Quantification of vitamin K was performed by HPLC with fluorescence detection (excitation at 244 nm, emission at 430 nm) after post-column reduction on a 10 x 0.2 cm column filled with zinc powder (Riedel-DeHaën, Seelze, Germany) at 40° C. The mobile phase consisted of ethanol/acetonitrile/reduction buffer/water at a ratio (v/v) of
360:90:4:3 and was degassed continuously with helium. Reduction buffer contained: 1 M ZnCl₂, 1 M NaOAc, and 1 M AcOH in MeOH.

**Arterial distensibility**

The right carotid artery from all animals (t = 12 weeks point) was used to determine arterial distensibility as described 33. Artery segments (3-4 mm) were mounted in an arteriograph (Living System Instrumentation, Burlington, USA) in which the arterial diameter could be continuously monitored. Both ends of the vessels were cannulated on 120 to 150 μm wide glass micropipettes and tied with two 17 μm thin nylon threads. Arterial segments were bathed in a 10 mL organ chamber filled with calcium-free physiological salt solution (composition in mmol/L: NaCl 144, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, HEPES 14.9, and glucose 5.5, pH 7.4) which was maintained at 37°C and gassed with 95% O₂ and 5% CO₂. After administration of 10 μmol/L Na-nitroprusside to assure maximal vasodilatation, intra-arterial pressure was gradually increased from 10 to 200 mm Hg. Arterial distensibility or the relative change in arterial lumen volume induced by a given increase in pressure, was estimated by: DC = ΔA/Aₙ₋₁* ∆P (DC = distensibility, A = area, P = pressure) 34.

**Statistical Analysis**

Values are expressed as mean ± SD. The difference between two groups was determined by Wilcoxon ranked non-paired test. Differences for multiple comparisons were determined by ANOVA with Bonferroni correction. Differences were considered to be significant at p < 0.05.
Results

Arterial calcification

The plasma calcium concentration was not affected by the different regimens and ranged between 2.25 and 2.33 mmol/L (mean ± SD: 2.28 ± 0.03) (see Table 1). During the first six weeks of W&K treatment, all six rats analyzed in this group displayed a significantly increased aortic calcium content (mean ± SD in µg/mg dry tissue: 0.24 ± 0.02 vs 1.62 ± 0.36; p<0.001). As is shown in Table 1, aortic calcification further increased when the W&K treatment was continued from week 7 to 12. Remarkably, calcification continued at a comparable rate in animals receiving a normal dose of K1 during this period. In contrast, high vitamin K intake (both K1 and K2) not only blocked the progress of further calcium accumulation, but lead to an over 37% reduction of previously accumulated arterial calcium precipitates within 6 weeks (53% as compared to the 12 weeks W&K time point). A similar observation was made in the left carotid artery (reduction of 44% compared to the 6 weeks time point W&K), and there was a good correlation between the calcium content of the abdominal aorta and that of the left carotid artery in the various groups (r² = 0.85, p < 0.001). In the thoracic aorta, calcification was visualized by Von Kossa staining (Figures 2, 3, 4). In the control rats (at 0, 6, and 12 weeks) no calcification was detected, however extensive calcifications were found in all animals receiving the W&K diet (both at 6 and at 12 weeks). Also all rats treated with normal K1 during weeks 7-12 displayed extensive calcifications. In rats treated with high K1 one rat did not have visible calcium precipitates, whereas 5 animals had decreased but still visible calcifications. In the K2 group, calcium precipitates were absent in two rats, whereas in four rats remaining calcifications were observed.
**MGP measurements**

To investigate matrix Gla-protein in relation to calcification of vascular tissue we used conformation specific antibodies against MGP. With poAb anti-tMGP, we identified MGP in the arteries from control animals, where it was primarily associated with the elastin fibers (data not shown). Subsequent staining with conformation-specific antibodies revealed that virtually all of this MGP consisted of carboxylated (i.e.: active) MGP (Figure 2). Much higher total MGP concentrations were found, however, in arteries from the W&K group, where it co-localized with the calcium deposits (Figure 2 and 3). Also in the arteries from animals treated with low and high vitamin K the total MGP was elevated compared to the controls (Figure 2). Staining with conformation-specific antibodies revealed that in animals from both the W&K group (Figure 2 and 3 and the normal-dose vitamin K group (Figure 2) most of the MGP occurred in the non-carboxylated (i.e.: inactive) form, whereas in rats subjected to high vitamin K treatment (both K1 and K2) the MGP deposits were mostly present in the carboxylated (i.e.: active) form (Figure 2 and Figure 4). Moreover, mainly the non-carboxylated form of MGP co-localized with vascular calcifications. The above described immunohistochemical staining of MGP was quantified (percentage staining of MGP / total arterial medial layer) and plotted in Figure 5.

Measurement of total MGP in plasma revealed a 4-fold decrease after 6 weeks of warfarin-treatment (from 6.9 ± 1.2 to 1.8 ± 0.3 nmol/L) as compared to the control rats. After another 6 weeks of the warfarin diet or normal vitamin K1 diet, MGP levels had not changed from the values at 6 weeks in the W&K and normal K1 groups. However, a
significant rise in plasma MGP was noticed after the subsequent high vitamin K treatment (Table 1). Staining for macrophages revealed that these were absent in the vascular media layer (Figure 6E, F). However, measuring the rate of apoptosis using TUNEL staining (Figure 6A - D) demonstrated that in the W&K treated animals significantly more (11%) apoptotic vascular smooth muscle cells were present than in those receiving the control diet (< 1%). During high vitamin K treatment the rate of apoptosis was significantly lower (4% lower in the K₁ group and 3% in the K₂ group) than during W&K (p < 0.02) and normal vitamin K₁ treatment (7%; p < 0.05).

Vitamin K measurements

To investigate whether both forms of vitamin K are transported equally well to the arteries, we have measured the arterial vitamin K content following the different food regimens. Control rats had accumulated both K₁ and K₂ in their arteries (Table 2); despite the fact that these animals had received only K₁, the tissue concentrations of K₂ were two times higher than those of K₁. During the W&K diet, the animals received high doses of K₁, and consequently high levels of both K₁ and K₁O had accumulated in the arteries (Table 2). In this case no K₂ species were found since warfarin blocks the conversion from K₁ to K₂.³⁵-³⁷ It is remarkable that, although K₁ was utilized (as measured by K₁O) substantial tissue calcification was observed in this group (see Figure 2). After stopping the warfarin treatment, animals receiving the normal dose of K₁ showed vascular K₁ and K₂ levels which were in the range of control rats. No vitamin K epoxides were found in this group. In animals receiving a high dose of K₁ the arterial concentrations of both K₁ and K₂ were approximately 8 fold higher (Table 2) than after normal dose K₁ treatment,
and trace amounts of the respective epoxides were found. Rats receiving the high K₂ diet had accumulated exclusively K₂ and trace amounts of K₂O (Table 2).

Mechanical properties of isolated carotid arteries

Figure 7 summarizes pressure-diameter relationships of isolated carotid arteries during maximal vasodilatation (four groups from the 7-12 weeks experiment). The minimal arterial diameter at low distending pressure (10 mmHg) was significantly larger in the W&K when compared to control rats and especially in comparison to the rats treated with either the high K₁ diet or the high K₂ diet. Furthermore, within a physiological pressure range (100 – 140 mmHg) the arterial distensibility was significantly smaller in the W&K group than in the control and high vitamin K groups. At 100 mmHg, the distensibility averaged 8.5 ± 0.5, 3.6 ± 0.6, 7.5 ± 0.6, and 10.0 ± 0.7 * 10⁻³ mmHg⁻¹ for control, W&K, high K₁ and high K₂, respectively (significance p < 0.05 compared to W&K). The maximal diameter at high distending pressure (200 mmHg) did not differ significantly between experimental groups (1391 ± 21, 1381 ± 19, 1377 ± 14, and 1351 ± 19 µm for control, W&K, high K₁ and high K₂, respectively). Collectively these findings indicate that the W&K treatment increased the arterial stiffness of the arteries and that this was reversed by both the high K₁ and K₂ intake.
Discussion

In this study we provide evidence that warfarin-induced medial vascular calcification in rats is preventable or even reversible by high vitamin K intake, with a putative role for the vitamin K-dependent protein MGP. Although it is well known that MGP is important in the prevention of calcification\textsuperscript{14,28}, its contribution to regression of arterial calcification is a novel finding.

Traditionally, vascular calcification has been thought to be a passive end-process and, once it was present, an irreversible feature. Now it is known that both intimal and medial calcification is an active process with inhibitors and stimulators of calcification. Thus far, research has mainly focused on the prevention or retardation of arterial calcification using lipid lowering drugs like statins or bisphosphonates\textsuperscript{38-42}, but all failed in regressing existing arterial calcification. Recently, however, it was shown that medial elastocalcinosis can be reversed\textsuperscript{43,44} suggesting that calcium resorption, like its deposition, is an actively regulated process.

It has been shown previously that arterial calcification can be induced with the warfarin-containing diet\textsuperscript{20,25}. Here we addressed the question of whether progression of further calcification could be stopped and whether existing mineral deposits could be diminished by a high vitamin K diet in rats. Our experiments indicate that in healthy, non-treated animals a relatively low vitamin K intake is sufficient for complete MGP carboxylation and for preventing arterial calcification (Figure 2), whereas warfarin initiated calcium accumulation in the arteries increased accumulation of MGP in these areas. This can be explained by assuming a feed-back mechanism by which increased local calcium stimulates MGP expression in an attempt to prevent calcification. It has
been shown in cell cultures and vascular tissue that warfarin up-regulates the mRNA expression of MGP. However, under the conditions employed in our experiment, this increase in MGP did not prevent calcium deposition in the vascular tissue. Using conformation specific antibodies, we demonstrated that during warfarin treatment most of the MGP had been synthesized as undercarboxylated, inactive species. The fact that the serum MGP concentration declined to approximately 20% of normal is in agreement with observations by Price et al. and suggests a different balance between tissue MGP and circulating MGP levels.

Continuation of the warfarin-containing diet between weeks 7-12 led to an almost linear increase of the arterial calcium content. An unexpected finding was that calcium accumulation also continued in the normal vitamin K group even after warfarin treatment had been stopped. We speculate that the calcium salt precipitates (as identified by Von Kossa staining) induce a high MGP expression level, causing a high local vitamin K requirement which is not met by the normal vitamin K diet. This can be seen in Figure 2 and 3 in which the majority of the newly synthesized MGP is produced in the under-carboxylated, inactive form. This is in agreement with work from Sweat et al, who showed that in aging rats ucMGP was associated with arterial calcification as demonstrated with polyclonal conformation specific MGP antibodies. The authors concluded that inactive MGP due to vitamin K deficiency could lead to arterial calcifications.

Arterial calcification could, however, be reversed by high vitamin K intake. After a 6-week period (weeks 7-12) some 40% of the pre-formed calcium salts had been removed. In an attempt to find out the mechanism underlying this observation, we
monitored the presence of macrophages. It is known that bone is resorbed by osteoclastic activity and that in the vessel wall also macrophages can clear hydroxyapatite by phagocytosis. Staining for macrophages revealed that the arterial media areas of rats in all groups were free from macrophage infiltration (Figure 6E, F), which can thus be excluded as possible mechanism of calcium removal in this experiment. Staining for apoptosis, however, demonstrated that during the warfarin treatment apoptosis was up-regulated (Figure 6A - D). It has been shown that apoptosis precedes calcification, and this seems to be the likely mechanism for calcium salt deposition in the W&K animals. Vascular smooth muscle cell (VSMC) derived apoptotic vesicles are loaded with calcification inhibitors, including MGP and these vesicles have pro-mineralizing properties when MGP function is impaired. Here we demonstrate that high vitamin K intake is associated with significantly less VSMC apoptosis and with significant regression of arterial calcification. It has been shown that another vitamin K-dependent protein synthesized by VSMCs, growth arrest specific gene-6 protein (gas6) is involved in the survival of VSMCs and in the clearance of apoptotic bodies from the vasculature. Also gas6 requires Gla residues and hence vitamin K for its activity. On the basis of our data we conclude that vitamin K and the vitamin K-dependent protein MGP are involved in the observed regression of arterial calcification. However, we cannot conclude whether and to which extent other vitamin K-dependent proteins such as gas6 are involved in the observed regression of arterial calcification. Because macrophages were absent in the vascular media during high vitamin K treatment, we postulate that the calcium deposits were removed by phagocytosis carried out by the surrounding VSMCs under conditions of maximal calcification inhibition provided by the high vitamin K diet.
This is consistent with a paper by Proudfoot et al, that reported that phagocytosis is a normal property of VSMCs\textsuperscript{53}.

Additionally, the regression of arterial calcification was accompanied by restoration of arterial distensibility to a similar level as in the control rats. The fact that $K_1$ and $K_2$ (MK4) had similar effects in this model seems to be in contradiction to previous data where it was demonstrated that $K_2$ (MK4) is more effective than $K_1$ in preventing calcification during warfarin treatment\textsuperscript{25}. An explanation for this apparent discrepancy may be found in the fact that certain tissues (including the vessel wall) specifically accumulate $K_2$, even when the diet contains exclusively $K_1$\textsuperscript{54}. The conversion of $K_1$ into MK4 is blocked, however, during warfarin treatment\textsuperscript{35-37}. In the experiments performed by Spronk et al\textsuperscript{25} as well as in our experiments, $K_2$ (MK4) or $K_2$-O (MK4-O) were nearly absent in arteries from rats treated with W&K. Also in rats fed the normal vitamin $K_1$ diet, only small amounts of $K_2$ (MK4) could be identified. In the high $K_1$ group, however, $K_1$ had been converted to $K_2$ to such an extent that in the high $K_1$ group, arterial $K_2$ had comparable tissue concentrations as in the $K_2$ (MK4) treated group. We conclude that at very high intakes of $K_1$, (200 fold the daily requirement of the liver) both vitamers may help decrease arterial calcification.

The decreased MGP levels in the plasma of animals with substantial arterial calcifications are consistent with the outcomes of previous studies in rats\textsuperscript{20}. Also in humans it was reported that calcification is associated with decreased circulating MGP levels\textsuperscript{55}. Both carboxylated and non-carboxylated MGP have a high affinity for hydroxyapatite, hence the most plausible explanation for this observation is that in case of arterial mineralization most of the MGP produced is directly bound to the calcium salts.
and not set free in the circulation. After feeding rats either a high K₁ or a high K₂ (MK4) diet for 6 weeks, their plasma MGP levels had increased significantly. This may be related to the decreased vascular calcium content providing fewer matrixes for MGP binding, but also to an increased transport of MGP that had bound to the dissolving matrix. Moreover, it has been reported that the transport of calcium from calcified tissue may occur via a fetuin-MGP-calcium-phosphate complex, which has a much longer plasma half-life than free MGP. If complexed MGP is detected in our assay, the slow elimination of such complexes might contribute to the relatively high MGP concentration in the serum of rats on a high vitamin K diet.

We measured the arterial distensibility as a clinical parameter of vascular elasticity. The experiment shown in Figure 7 demonstrates that warfarin induced stiffening of the arterial vessel wall. This is consistent with work from Essahili et al. which showed that warfarin treatment resulted in increases of aortic pulse pressure, pulse pressure, and systolic blood pressure. In our model, normal vitamin K₁ in the diet was not capable of affecting arterial distensibility, whereas during the high vitamin K diet (both K₁ and K₂) the vascular properties that were lost by warfarin induced calcification were restored.

The animal model we used mimics arterial media sclerosis (also known as Mönckeberg's sclerosis). Media sclerosis is particularly common in diabetes mellitus, end-stage renal disease, and aging. Notably patients with chronic kidney disease (CKD) are at high risk of cardiovascular disease. These patients often receive a high calcium diet (to complex phosphate), vitamin D, and warfarin (to prevent thrombotic events). Recently it was demonstrated, however, that each of these treatments is associated with
an increased risk for arterial calcification \(^{26,46,49,58}\). Given the fact that arterial calcifications are predictive of cardiovascular events, regression of arterial calcification may help to reduce the risk for death in patients with CKD and coronary artery disease. Whether increased vitamin K intake could have such an effect in humans has to be investigated. Obviously this is only possible in patients not receiving oral anticoagulant treatment.
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Legends to the figures:

Figure 1.

Flow diagram of the protocol used.

Figure 2.

Effect of different dietary treatments on aortic calcification at 12-week time point in male Wistar Kyoto rats. Rats (n = 6 per group) were treated with the following dietary regimens; row 1 represents 12 weeks normal vitamin K diet (5 µg/g K₁), row 2 represents 12 weeks W&K diet (3 mg/g warfarin and 1.5 mg/g vitamin K₁), row 3 represents 6 weeks normal vitamin K (5 µg/g K₁) after 6 weeks of W&K, row 4 represents 6 weeks of high vitamin K₁ (100 µg/g) diet after 6 weeks of W&K, and row 5 represents 6 weeks of high vitamin K₂ (100 µg/g) diet after 6 weeks of W&K. The thoracic aorta segment (between the aortic arch and the renal branch) was removed immediately after sacrificing the animals in each diet group and fixed in 1% buffered formalin. Subsequently, longitudinal sections of each aorta were stained for mineral by von Kossa’s stain (column 1), ucMGP (column 2) and cMGP (column 3). Red stain indicates MGP, black stain indicates calcium, and blue indicates cell nuclei. Magnification 100x.
Figure 3.

Effect of W&K treatment (3 mg/g warfarin and 1.5 mg/g vitamin K₁) on the presence of calcification and MGP at 12-week time point. Sections were prepared as described in the legend of figure 2. Longitudinal sections of each thoracic aorta were stained with von Kossa (3A) and immuno-histochemically with anti-t-MGP (3B), anti-ucMGP (3C) and anti-cMGP (3D)(see methods for details). It is clearly demonstrated that due to the W&K diet arterial calcification was significantly present. In panel B total MGP is upregulated in the calcified area. From panel 3D it can be seen that cMGP is almost absent, whereas significant amounts of the inactive ucMGP are present around the calcified area (panel 3C). Arrow indicates same area. Red stain indicates MGP, black stain indicates calcium, and blue indicates cell nuclei. Magnification 400x. Letters represent: A= adventitia, M= media, and L= lumen.

Figure 4.

Effect of the high K₂ treatment (100 µg/g vitamin K₂) on presence of calcification and MGP at 12-week time point. Sections were prepared as described in the legend of figure 2. Longitudinal sections of each thoracic aorta were stained with von Kossa (4A) and immuno-histochemically with anti-t-MGP (4B), anti-ucMGP (4C) and anti-cMGP (4D)(see methods for details). It is shown that due to the high K₂ diet cMGP (panel 4D) is up-regulated in the calcified area (4A; along the elastic fibers). From panel 4C it can be seen that ucMGP is almost absent. Arrow indicates same area. Red stain indicates MGP, black stain indicates calcium, and blue indicates cell nuclei. Magnification 400x. Letters represent: A= adventitia, M= media, and L= lumen.
Figure 5.
Quantification of MGP at the 12-week time point. Three sections, each 28 µm apart, were measured using a microscope coupled to a computized morphometry system (for details see method section). Quantification was expressed as percentage staining of the total arterial medial area.

Diets represent 12 weeks control, W&K (3 mg/g warfarin and 1.5 mg/g vitamin K₁), normal vitamin K diet (5 µg/g K₁), high vitamin K₁ (100 µg/g K₁) and high vitamin K₂ (100 µg/g K₂) after 6 weeks of W&K. Black bars represent total MGP, grey bars represent cMGP and white bars represent ucMGP. Quantification was performed by two independent persons. Bars represent mean values ± SEM (n = 6).

Figure 6.
Effect of different dietary treatments on apoptotic activity at 12-week time point. Sections were prepared as described in the legend of figure 2. Longitudinal sections of each thoracic aorta were stained by immuno-histochemical techniques with an anti-macrophage antibody and with a TUNEL staining for apoptosis (see Methods for details).
Panel A represents 12 weeks normal vitamin K diet (5 µg/g K₁), panels B and E represent 12 weeks W&K diet (3 mg/g warfarin and 1.5 mg/g vitamin K₁), panel C represents normal K (5 µg/g) after 6 weeks W&K, and panels D and F is 6 weeks high vitamin K (100 µg/g K₁ or K₂) after 6 weeks of W&K. Macrophages are absent in the medial layer of aortic tissue, both in W&K (E) and high vitamin K (F) treated rats. Also, in control animals macrophages were absent (data not shown). In panel E a positive control is shown as in-set (arrow indicates macrophage). The W&K treated animals (B), however,
have significantly increased apoptosis of VSMCs compared to control animals (A), in which apoptosis is hardly measurable. Also in the normal K treated animals, after 6 weeks of W&K, (C) apoptosis is clearly visible. The high vitamin K treated rats (D) showed significantly reduced apoptosis as compared to the normal K treated animals (C). See results section for details. Magnification 400x.

Figure 7.
Effects of vitamin K-status on the mechanical properties of isolated rat carotid arteries. The pressure-diameter relationship was monitored for animals receiving the control diet (n = 6; triangles), those after 12 weeks of the W&K diet (n = 6; circles) and animals after 6 weeks W&K and subsequently 6 weeks of high vitamin K (both K1 (open squares) and K2 (closed squares; n = 6 per group). The arterial diameter is shown as a function of increasing pressure. Data are shown as mean ± SD.
Table 1: Arterial calcium contents after various treatments. Calcium was measured in the abdominal aorta, in the left carotid artery, and in plasma. MGP was measured in plasma using the Biomedica assay. All values are expressed as mean ± SD per group of 6 animals. Significant differences compared to controls (compared to the same time point) are expressed as * (P< 0.01). Significant differences compared to the W&K diet are indicated as † (P < 0.01).
Table 2. Vitamin K concentrations in the rat aortas at time point 12 weeks (n = 6). All values are expressed as mean ± SD per group of 6 animals. nd means not detectable. Detection limit for K₁ and K₂ (MK4) is 0.05 ng/g.

<table>
<thead>
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<th>Diet</th>
<th>K₁ (ng/g)</th>
<th>K₁O (ng/g)</th>
<th>K₂ (MK4) (ng/g)</th>
<th>K₂O (MK4) (ng/g)</th>
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<td>Control</td>
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<tr>
<td>W&amp;K</td>
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<td>967 ± 419</td>
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<td>nd</td>
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<tr>
<td>K₁ (5 µg/g)</td>
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<td>24.4 ± 29.3</td>
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<tr>
<td>K₁ (100 µg/g)</td>
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<td>2.3 ± 1.9</td>
<td>183 ± 79</td>
<td>6.5 ± 2.6</td>
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<tr>
<td>K₂ (100 µg/g)</td>
<td>nd</td>
<td>nd</td>
<td>248 ± 112</td>
<td>5.2 ± 4.8</td>
</tr>
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</table>
Figure 5
Regression of warfarin-induced medial elastocalcinosis by high intake of vitamin K in rats

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