Transglutaminase Inhibitors Attenuate Vascular Calcification in a Preclinical Model

Kelly E. Beazley,* Derek Banyard,* Florence Lima, Stephanie C. Deasey, Dmitry I. Nurmins, Mikhail Konoplyannikov, Maria V. Nurinskaya

Objective—In vitro, transglutaminase-2 (TG2)—mediated activation of the β-catenin signaling pathway is central in warfarin-induced calcification, warranting inquiry into the importance of this signaling axis as a target for preventive therapy of vascular calcification in vivo.

Methods and Results—The adverse effects of warfarin-induced elastocalcinosis in a rat model include calcification of the aortic media, loss of the cellular component in the vessel wall, and isolated systolic hypertension, associated with accumulation and activation of TG2 and activation of β-catenin signaling. These effects of warfarin can be completely reversed by intraperitoneal administration of the TG2-specific inhibitor KCC-009 or dietary supplementation with the bioflavonoid quercetin, known to inhibit β-catenin signaling. Our study also uncovers a previously uncharacterized ability of quercetin to inhibit TG2. Quercetin reversed the warfarin-induced increase in systolic pressure, underlying the functional consequence of this treatment. Molecular analysis shows that quercetin diet stabilizes the phenotype of smooth muscle and prevents its transformation into osteoblastic cells.

Conclusion—Inhibition of the TG2/β-catenin signaling axis seems to prevent warfarin-induced elastocalcinosis and to control isolated systolic hypertension. (Arterioscler Thromb Vasc Biol. 2013;33:43-51.)

Key Words: β-catenin ■ quercetin ■ transglutaminase 2 ■ vascular calcification ■ warfarin

Vascular smooth muscle is characterized by phenotypic plasticity that contributes to cardiovascular disease. Perhaps, the best-studied type is an osteoblast-like vascular smooth muscle transformation in vascular calcification, the condition known as a risk factor for cardiovascular mortality in the general population and in patients with diabetes mellitus and end-stage renal disease. Vascular calcification also associates with atherosclerotic plaque burden, cardiac valve calcification, and isolated systolic hypertension, which is prevalent in the elderly population. At present, vascular calcification is not curable, emphasizing the need for a better understanding of its molecular mechanism to advance prevention and therapy.

Cross-sectional studies indicate a link between anticoagulant therapy with Coumadin (warfarin) and calcium phosphate deposition in arterial media and in the rat model, warfarin treatment induces elastocalcinosis and leads to isolated systolic hypertension. A commonly considered mechanism of warfarin-induced calcification involves inhibition of the vitamin K epoxide reductase enzyme, thereby deactivating carboxylation-dependent vascular proteins, including matrix Gla protein (MGP). Carboxylated MGP prevents vascular calcification directly by inhibiting hydroxyapatite formation and indirectly by inhibiting bone morphogenetic proteins, potent enhancers of osteogenesis. However, despite the efficacy of elevated carboxylated MGP to reverse warfarin-induced calcification ex vivo in aortic rings and in vitro, high-dose vitamin K treatment aimed to restore the extrahepatic levels of protein carboxylation in warfarin-treated animals had limited efficacy.

Previously, we established in vitro a critical role for canonical β-catenin signaling in warfarin-induced osteoblast-like transformation and calcification of vascular smooth muscle cells (VSMCs). We have also shown that warfarin activates β-catenin in VSMCs via enzyme transglutaminase-2 (TG2), adding to the growing list of non-Wnt agonists of this signaling pathway. Genetic ablation of TG2 protected against aortic calcification in warfarin-treated mice, identifying this enzyme as a potential therapeutic target. Indeed, specific pharmacological inhibition of TG2 prevented warfarin-induced calcification in vitro. However, cultured VSMCs may differ from smooth muscle cells in their vascular niche, and thus the effects of pharmacological TG2 inhibition in vivo may differ from the in vitro observations. In this study, we test the hypothesis that warfarin-induced calcification in vivo associates with activation of the TG2/β-catenin signaling axis and that inhibition of this signaling conduit can prevent elastocalcinosis. We report potent prevention of vascular calcification by the TG2-specific inhibitor
KCC-009.20 In addition, we demonstrate that 3,3′,4′,5,7-pentahydroxyflavone (quercetin), which is a known β-catenin inhibitor in various cells,21–23 efficiently prevents warfarin-induced medial calcification and its corollaries, and this effect may be mediated by the newly described ability of quercetin to directly inhibit TG2.

Materials and Methods
A detailed description of materials and experimental methods is available in the online-only Data Supplement. Reagents are from Sigma-Aldrich, unless otherwise specified.

Animals
Maintenance and procedures were performed in accordance with the guidelines and regulations of the University of Maryland School Medicine Institutional Animal Care and Use Committee. In vivo studies were performed on 6- to 8-week-old male Wistar rats (Charles River). Animals were treated daily for 4 to 6 weeks with 20 mg/kg vitamin K, 20 mg/kg warfarin, 10 mg/kg quercetin (QU995), 50 mg/kg KCC-009,20 or 30% dimethyl sulfoxide vehicle. At the end points, animals were anesthetized with isoflurane, and blood pressure was measured using a nylon catheter inserted into the left femoral artery. All animals were euthanized using CO₂ inhalation, followed by thoracotomy.

Statistical Analysis
Data are expressed as mean±SEM. Student t test was used for comparison between 2 groups. For >2 groups, significance was determined using 1-way ANOVA, with comparison between groups by Tukey-Kramer honestly significant difference test. P<0.05 was considered statistically significant; * denotes P<0.05, ** denotes P<0.01, and NS denotes not significant.

Results
The TG2/β-Catenin Signaling Axis Is Activated in Warfarin-Induced Medial Calcification
Medial arterial elastocalcinosi (5.73±0.41 μg calcium/mg dry weight compared with 2.38±0.24 μg Ca/mg dry weight in untreated controls; Figure 1A) was induced in rats by a 4-week-long treatment with daily dietary supplement of warfarin, coadministered with vitamin K (WWK; n=16). B, Representative Sirius Red staining for collagenous extracellular matrix (ECM) and quantification of cell/ECM area (left graph) and total width of elastic lamellae (right graph) in aortae (measured at 4 equidistant points in cross section) from control (n=7) and WWK-treated animals (n=12). Scale bar, 10 μm. C, Top, Immunofluorescence for β-catenin (red; nuclei counterstained with diamidino-2-phenylindole [DAPI] [blue]), Bottom, Mineralized matrix detected by von Kossa stain (black) counterstained with nuclear fast red. Regions of low (right) and high (left) levels of calcification are shown. Scale bar, 15 μm.

Increased expression of osteoblastic genes (Table) indicates that WWK-induced aortic calcification was accompanied by a switch to an osteoblast-like phenotype in vascular cells. Although the essential regulator of the osteogenic program Runx2 is induced relatively moderately at 53%, a similar change in Runx2 levels has previously been defined as sufficient for the osteogenic transformation of vascular cells ex vivo25 and in vitro.26 In cultured VSMC, warfarin stimulates TG2 expression and activity.27 In agreement, in the calcified aortae of the warfarin-treated rats, increased TG2-mediated protein cross-linking has been detected in vivo27 and we detect accumulation of the TG2 protein (Figure IC in the online-only Data Supplement). An original finding in this study is accumulation of nuclear β-catenin protein in the calcified areas and in morphologically normal tissue adjacent to the calcified regions (Figure 1D, arrows), suggesting that β-catenin activation occurs before calcification rather than in response to extracellular mineral deposition. Activation of the β-catenin pathway was further supported by increased expression of β-catenin/T-cell transcription factor

Figure 1. Activation of transglutaminase-2 (TG2)/β-catenin signaling axis associates with arterial calcification. A, Aortic calcium content in control and animals treated with warfarin coadministered with vitamin K (WWK; n=16). B, Representative Sirius Red staining for collagenous extracellular matrix (ECM) and quantification of cell/ECM area (left graph) and total width of elastic lamellae (right graph) in aortae (measured at 4 equidistant points in cross section) from control (n=7) and WWK-treated animals (n=12). Scale bar, 10 μm. C, Top, Immunofluorescence for β-catenin (red; nuclei counterstained with diamidino-2-phenylindole [DAPI] [blue]). Bottom, Mineralized matrix detected by von Kossa stain (black) counterstained with nuclear fast red. Regions of low (right) and high (left) levels of calcification are shown. Scale bar, 15 μm.


Table. Fold Change in Gene Expression in WKV-Treated Rats and WKV-Treated Rats Receiving Quercetin Compared With Untreated Control Rats (n=8)

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Control</th>
<th>WKV</th>
<th>WKV+Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteogenic markers</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Osteocalcin</td>
<td>1.00±0.52</td>
<td>2.36±0.44*</td>
<td>0.80±0.20‡</td>
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<td>Type I collagen</td>
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<td>1.47±0.15*</td>
<td>1.14±0.03‡</td>
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<tr>
<td>Runx2</td>
<td>1.00±0.09</td>
<td>1.53±0.09*</td>
<td>0.96±0.14‡</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>1.00±0.09</td>
<td>7.93±1.79*</td>
<td>5.25±1.94*</td>
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<tr>
<td>β-catenin target genes</td>
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<td></td>
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<tr>
<td>axin2</td>
<td>1.00±0.11</td>
<td>2.10±0.38*</td>
<td>0.51±0.12§</td>
</tr>
<tr>
<td>cyclin D1</td>
<td>1.00±0.14</td>
<td>2.30±0.31*</td>
<td>0.88±0.21§</td>
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<tr>
<td>Tcf4</td>
<td>1.00±0.08</td>
<td>1.41±0.18*</td>
<td>0.74±0.13‡</td>
</tr>
<tr>
<td>numb</td>
<td>1.00±0.09</td>
<td>1.72±0.22*</td>
<td>0.66±0.10§</td>
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<tr>
<td>Inflammatory cytokines</td>
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<td></td>
</tr>
<tr>
<td>Interleukin 1β</td>
<td>1.00±0.33</td>
<td>5.13±0.61†</td>
<td>6.63±1.62†‡</td>
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<tr>
<td>Interleukin 6</td>
<td>1.00±0.12</td>
<td>4.78±0.78*</td>
<td>5.58±2.17*</td>
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<tr>
<td>Tumor necrosis factor-α</td>
<td>1.00±0.34</td>
<td>5.57±0.90*</td>
<td>4.13±1.25*</td>
</tr>
<tr>
<td>Vascular markers</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Smooth muscle actin</td>
<td>1.00±0.16</td>
<td>1.98±0.71</td>
<td>5.10±0.86†‡</td>
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<tr>
<td>Smooth muscle MHC</td>
<td>1.00±0.31</td>
<td>0.84±0.16</td>
<td>15.31±2.82†</td>
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<tr>
<td>sm22α</td>
<td>1.00±0.18</td>
<td>1.41±0.38</td>
<td>4.84±0.77†</td>
</tr>
<tr>
<td>Calponin</td>
<td>1.00±0.08</td>
<td>0.90±0.15</td>
<td>6.34±1.15†‡</td>
</tr>
</tbody>
</table>

WK indicates warfarin coadministered with vitamin K; MHC, myosin heavy chain.

*P <0.05 compared with control.
†P <0.01 compared with control.
‡P <0.05 compared with WK.
§P <0.01 compared with WK.

Inhibition of TG2 Attenuates Warfarin-Induced Calcification and Activation of β-Catenin

To test whether the catalytic activity of TG2 is essential for WKV-induced arterial calcification, animals were treated daily with 50 mg/kg body weight of the TG2-specific small-molecule inhibitor KCC-009. KCC-009 supplementation almost completely eliminated the WKV-induced mineral deposition in the aorta (Figure 2A and Figure II in the online-only Data Supplement), with a 96% reduction in the area of aortic sections positively stained for noticeable calcium phosphate deposits (from 18.8% in WKV-treated animals to 0.8% in animals that received both WKV and KCC-009 treatments; Figure 2B). Similarly, KCC-009 treatment prevented the WKV-induced increase in total calcium extracted from the aortic tissue compared with the untreated control rats (1.73±0.21-fold in WKV-treated rats to 0.76±0.10-fold in WKV- and KCC-009-treated group; Figure 2B). In addition, KCC-009 counteracted thinning of the tunica media in response to WKV treatment (Figure 2C). Whereas rats receiving WKV showed an ≈30% decrease in the thickness of the tunica media, in rats receiving both WKV and KCC-009 the overall thickness was similar to control animals.

Finally, KCC-009 blocked the accumulation of nuclear β-catenin protein in response to WKV treatment (Figure 2D), further confirming the critical role of TG2 in mediating warfarin-dependent activation of the β-catenin pathway. These data demonstrate the therapeutic efficacy of TG2 inhibition in warfarin-induced elastocalcinosis. However, KCC-009 treatment also caused an ≈25% widening of the aortae (Figures IA and II in the online-only Data Supplement), via a yet unknown mechanism that may have adverse effects. Combined with the limited data on the in vivo toxicity of KCC-009 in both animal models and human patients, these results justify the need for a better characterized compound to inhibit TG2 in the background of warfarin treatment.

Characterization of Quercetin as a TG2 Inhibitor

We have shown that warfarin-induced calcification in vitro involves direct activation of the TG2 enzyme by warfarin. Based on a known significant overlap between the sets of proteins able to interact with warfarin and the flavonoid quercetin, we hypothesized that quercetin may also interact with TG2 to alter its activity. Direct binding of quercetin to TG2 was demonstrated by a pull-down assay using quercetin-conjugated sepharose beads incubated with purified TG2 protein (Figure 3A). Quercetin binding inhibits the catalytic activity of purified TG2 (Figure 3B) and negates activation of TG2 by warfarin (Figure 3C) in a dose-dependent manner. In addition, in vitro quercetin prevented warfarin-induced activation of the TG2 enzyme in cultured VSMCs (Figure IIIA in the online-only Data Supplement), without affecting the elevated expression of TG2 gene caused by warfarin (Figure IIB in the online-only Data Supplement). These findings identified quercetin as a potent TG2 inhibitor.
control mock-treated cells). Quercetin counteracted the warfarin-induced accumulation of β-catenin protein (Figure 4A), increase in the percentage of cells with overt nuclear localization of β-catenin (Figure 4B), as well as enhanced transcription of the β-catenin–dependent luciferase reporter in a dose-dependent manner (Figure 4C) and tempered expression of the β-catenin target genes, axin2, cyclin D1, and Tcf4 (Figure 4IC in the online-only Data Supplement), indicating attenuation of the warfarin effects on β-catenin signaling.

Taking into consideration the essential role of the activated TG2/β-catenin signaling axis in warfarin-induced calcification in vitro17 and the ability of quercetin to inhibit both TG2 activity and β-catenin signaling (described above), we sought to determine whether quercetin prevents warfarin-induced calcification in VSMC, similar to its inhibitory effect on mineralization in osteoblasts.33 In wild-type mouse aortic rings, quercetin significantly reduced warfarin-induced calcification, and this effect was lacking in the TG2–/– aortic rings (Figure 4D), indicating potential therapeutic efficiency of quercetin in WVK-induced elastocalcinosis in vivo and implicating TG2 as a primary target. Ample accumulation of quercetin metabolites in the arterial wall14 suggests its potential bioactivity in the aortic tissue and further supports its evaluation in the therapy of medial calcification.

Quercetin Prevents Warfarin-Induced Arterial Calcification

Daily supplementation of the WVK treatment with dietary oral quercetin at 10 mg/kg body weight (dose approved by the Food and Drug Administration as safe for human dietary consumption) significantly reduced both the cross-sectional calcified area of the tunica media (from 45% in WVK treatment to 2.6% in WVK+quercetin) and total calcium accrual (from a 2.4±0.17-fold increase compared with untreated control animals down to 1.5±0.08-fold). The calcification values in WVK-treated, quercetin-supplemented animals were not significantly different from the untreated control group (Figure 5A and 5B). In addition to the newly described ability of quercetin to inhibit TG2, this flavonoid has been characterized as an inhibitor of β-catenin activity in several cell types.20–22 Here, we analyzed the impact of quercetin on β-catenin signaling in warfarin-treated VSMCs.

The canonical β-catenin signal transduction cascade has been described in several excellent reviews.19,28 Accumulation of β-catenin protein, its nuclear localization, and increased activity of the β-catenin/TCF/lymphoid enhancer factor transcriptional complex are all hallmarks of active β-catenin signaling and were analyzed in VSMCs exposed to warfarin alone or warfarin and quercetin (compared with the control mock-treated cells). Quercetin counteracted the warfarin-induced accumulation of β-catenin protein (Figure 4A), increase in the percentage of cells with overt nuclear localization of β-catenin (Figure 4B), as well as enhanced transcription of the β-catenin–dependent luciferase reporter in a dose-dependent manner (Figure 4C) and tempered expression of the β-catenin target genes, axin2, cyclin D1, and Tcf4 (Figure 4IC in the online-only Data Supplement), indicating attenuation of the warfarin effects on β-catenin signaling.

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actin, smooth muscle myosin heavy chain, sm22a, and calponin (cnn1), in animals receiving WVK, as well as in control rats receiving quercetin only (Table and Figure VA in the online-only Data Supplement). These findings suggest that quercetin acts to stabilize the VSMC phenotype, and this is further supported by the quercetin-induced increase in markers of activated Notch signaling\(^3\) in the VSMCs (Figure VB in the online-only Data Supplement). Of note, increase in myosin heavy chain gene expression did not translate into a noticeable increase in protein (Figure VC in the online-only Data Supplement). This finding, combined with the lack of significant quercetin effects on the VSMC proliferation\(^7\) and apoptosis (Figure IB in the online-only Data Supplement), and similar arterial wall thickness (Figure 5D) and lumen diameter (Figure IA in the online-only Data Supplement) in the quercetin-treated and untreated animals, indicated that quercetin did not affect the turnover of VSMCs. The ability of quercetin to stabilize collagen matrix\(^8\) may underlie attenuation of the warfarin-induced thinning of the vessel wall (Figure 5D), but further analysis is needed to address this possibility. Of note, the 4-week-long treatment with quercetin in the background of warfarin administration had no toxic effects on animal motor activity (Figure VD in the online-only Data Supplement) or overall well-being, consistent with little, if any, evidence for quercetin toxicity.\(^9\)

**Quercetin Attenuates Warfarin-Induced Activation of TG2 and β-Catenin in Arterial Tissue**

To determine whether quercetin affects the aortic TG2/β-catenin signaling axis in vivo similar to its effects in vitro, TG2 protein and activity and hallmarks of active β-catenin signaling were compared in aortae of rats treated with WVK alone or WVK+quercetin. TG2 protein levels (Figure 6A) and catalytic activity (Figure 6B) in the aortic tissue of animals treated with WVK and quercetin were not significantly different from the levels in untreated control animals and were significantly lower than those in the WVK-treated group. Similarly, quercetin inhibited the WVK-triggered phosphorylation of glycogen synthase kinase-3β\(^2\) (Figure 6C) and accumulation of nuclear β-catenin protein in the aortic media (Figure 6D and 6E) and significantly attenuated the warfarin-induced expression of β-catenin transcription (Figure 4C) and accumulation of nuclear β-catenin protein in the aortic media (Figure 6D and 6E) and significantly attenuated the warfarin-induced expression of
β-catenin target genes (Table). These results are consistent with inhibition of the TG2/β-catenin signaling axis contributing to the quercetin mechanism of action in vascular calcification in vivo.

Discussion

A critical role for TG2/β-catenin signaling axis in warfarin-induced osteoblast-like transformation of VSMCs has been demonstrated in vitro, and TG2 activity has been implicated in arterial calcification in warfarin-induced elastocalcinosis in rats. Whereas several transglutaminases are expressed in the aortic tissue, this study demonstrates for the first time the efficacy of the TG2-specific inhibitor KCC-009 in preventing warfarin-induced elastocalcinosis. The significance of these findings potentially extends beyond warfarin-induced arterial calcification, given the central role for TG2 in phosphate-induced vascular calcification ex vivo and the augmented transglutaminase activity in atherosclerotic plaque calcification in vivo. In addition, the efficacy of systemic use of KCC-009 has been demonstrated in vitro, and TG2 activity has been implicated in arterial calcification in warfarin-induced elastocalcinosis. The significance of these findings potentially extends beyond warfarin-induced arterial calcification, given the central role for TG2 in phosphate-induced vascular calcification ex vivo and the augmented transglutaminase activity in atherosclerotic plaque calcification in vivo.

Figure 5. Quercetin prevents warfarin-induced vascular calcification. A, Representative aorta sections from animals treated with warfarin coadministered with vitamin K (WVK), WVK and quercetin, or quercetin alone stained with mineral-detecting von Kossa. Scale bar, 100 μm. B, Quantification of calcified to total vessel area (left graph; analysis was performed on at least 4 sections per animal) and fold change in total calcium content (right graph) (n=7 control; n=12 WVK, quercetin, WVK+quercetin). C, Systolic blood pressure (SBP; top) and pulse pressure (PP; bottom) in WVK-treated animals (left) or animals receiving WVK and quercetin (right) compared with control (n=6). D, Quantification of the average width of tunica media (measured at 4 equidistant points in cross section).

We describe a promising new strategy for preventing warfarin-induced vascular calcification and its corollaries with quercetin. The advantage of this bioflavonoid over the synthetic inhibitor KCC-009 is that it has a wide safety margin in long-term treatments and has been thoroughly evaluated in clinical studies addressing its therapeutic efficacy in cancer, heart disease, and inflammation. Thus, quercetin is an attractive choice for long-term prevention of vascular calcification in chronic conditions or in the background of long-term warfarin treatment. In addition, quercetin-stimulated expression of VSMC markers probably manifests the phenotypic stabilization of VSMCs and thus potential efficacy of quercetin in other vascular pathologies, such as atherosclerosis, restenosis, and hypertension, which hinge on phenotypic switches in these cells. Indeed, a recent study in mice demonstrates the efficacy of quercetin in attenuation of atherosclerosis, and in humans, quercetin consumption correlates with lower blood pressure and low-density lipoprotein levels and overall reduced cardiovascular disease–related mortality. Although potential drug interactions between quercetin and warfarin have been suggested, there is little direct evidence to date, and concurrent administration of these compounds had no notable adverse side effects in our animal model.

Our aortic ring data are consistent with previous findings that warfarin-induced calcification hinges on TG2 and demonstrate that quercetin acts via TG2 as quercetin is not effective in the TG2-/- aortic rings. Taking into account that inhibition of TG2 by KCC-009 attenuates β-catenin activation and calcium accrual, suggesting the TG2/β-catenin axis is critical for warfarin-induced calcification in vivo similar to the in vitro model, the direct inhibition of TG2 by quercetin and quenching of β-catenin activation by this flavonoid are probably responsible for the high efficacy of quercetin in prevention of elastocalcinosis. Furthermore, quercetin therapy may also be beneficial to prevent calcification of the heart valves and in diabetes mellitus in which β-catenin signaling has been implicated.

In addition to the newly described TG2/β-catenin axis, warfarin has been assumed to induce aortic calcification through...
inhibiting MGP γ-carboxylation, thereby affecting the anticalcific effects of the carboxylated MGP. Indeed, genetic loss of arterial MGP causes extensive aortic calcification.52 However, warfarin treatment in rat animal model did not reduce arterial expression of MGP according to this study and earlier report,8 and no correlation has been found between circulating levels of carboxylated MGP and coronary arterial calcium in older adults.53 Furthermore, carboxylation of MGP may not be critical for attenuation of hydroxyapatite crystal growth by this protein,8 and the ability of carboxylated MGP to bind and deactivate bone morphogenetic proteins14 may have little relevance to warfarin-induced aortic calcification because of the limited role of bone morphogenetic protein signaling revealed by ex vivo studies.13 Thus, it is perhaps not very surprising that high intake of vitamin K aimed to rescue protein γ-carboxylation was only 50% efficient in preventing arterial calcification in rats on the WVK diet,16 indicating a significant role for other γ-carboxylation–independent mechanism(s), such as TG2/β-catenin activation. It is noteworthy, in this regard, that treatment with the TG2 inhibitor KCC-009 was 100% efficient in blocking warfarin-induced calcification.

In conclusion, we demonstrate that the TG2/β-catenin signaling axis is activated in vivo in warfarin-induced osteogenic transformation in vascular smooth muscle and is a novel therapeutic target in elastocalcinosis. This study advances general understanding of the molecular mechanisms of phenotypic instability in VSMCs and presents a novel preventive strategy for vascular calcification. However, young animals were used in this study to model the warfarin-induced elastocalcinosis that is more common in patients aged >50 years,9 suggesting that further research is needed to test the efficiency of quercetin in older animals and that caution should be exercised in the extrapolation of the results from the animal studies to clinical applications. In addition, quercetin-based therapy needs further optimization by addressing the potential difference in quercetin accumulation by anatomically distinct vascular beds, the possible impact of the known sex differences in vascular calcification,54 and the dose-dependent potency of quercetin. Also, the question still remains whether, in addition to inhibition of TG2/β-catenin signaling, quercetin treatment also rescues γ-carboxylation of proteins, including MGP.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Expanded Materials and Methods

Animal and Experimental Design. Animal maintenance and procedures were performed in accordance with the guidelines and regulations of the University of Maryland School Medicine Institutional Animal Care and Use Committee. Studies were performed on male Wistar rats (Charles River), 6 to 8 weeks old. The rats were housed in normal cages and allowed free access to water and rodent diet. Experimental groups of 10-12 animals received Vitamin K (phytonadione, 20 mg/kg, Spectrum Chemical Mfg. Corp, New Brunswick, NJ) diluted in sesame oil (Sigma-Aldrich, St. Louis, MO), administered daily via oral gavage for 7 days. For studies on quercetin, Vitamin K treatment was then continued for 6 weeks with the addition of either warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin sodium salt, 20 mg/kg, Sigma-Aldrich) or warfarin and 10 mg/kg quercetin (QU995 from Quercegen Pharma, Newton, MA, USA, > 99.5 % pure), administered daily via oral gavage. For studies on TG2, daily warfarin-Vitamin K treatment was continued for 4 weeks with the addition of either KCC-009 (benzyl ((S)-1(((S)-3-bromo-4,5-dihydroisoxazol-5-yl)methyl)amino)-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)carbamate, 50 mg/kg, a kind gift from Dr. Chaitan Khosla, Stanford University, CA) diluted in 6 mL 30% DMSO (dimethyl sulfoxide, Sigma-Aldrich) vehicle or an equal volume of 30% DMSO vehicle alone, administered via intraparitoneal (IP) injection. In quercetin studies, we used age-matched groups of 8-10 animals each, either untreated or treated with only quercetin. For controls in TG2 studies, we used age-matched groups of 8-10 animals each treated with KCC-009 or vehicle. All rats were euthanized using CO₂ inhalation followed by thoracotomy. Arterial blood was collected at the time of euthanasia via cardiac puncture.

Cell and Aortic Ring Cultures. For in vitro studies, the A10 clonal embryonic rat vascular smooth muscle cell line (ATCC, Manassas, VA) was used. Cells were seeded at a density of
1.2x10^4 cells/cm² and cultured for 8 days in calcification-inducing medium [Dulbecco’s modified Eagle’s medium (Invitrogen) containing 1% fetal bovine serum (Hyclone), 1.6 mmol/L inorganic phosphate, 1.51 mmol/L calcium, and 10 μM warfarin from DMSO stock solution (Sigma-Aldrich) or, as a control, medium containing an equal volume of DMSO]. 2-3 mm aortic rings isolated from wild-type C57b mice (WT) or transglutaminase 2 null mice (KO) and cleaned of adventitia were cultured ex vivo for 8 days in the aforementioned calcification-inducing medium further supplemented with 7 U/mL alkaline phosphatase (Roche).

**Calcium analysis.** For analysis of calcium in aortic tissue, a section of each aorta was dried in an oven at 55°C and then weighed. The dried sections were then incubated in 250 μL 0.1 mol/L HCl overnight at 4°C. For analysis of calcium in cell cultures, cells were fixed in 4% paraformaldehyde (Thermo-Fisher) for 1 hour at 4°C. Fixed cells were then incubated in 300 μL 0.1 mol/L HCl overnight at 4°C. Calcium in the resulting tissue extracts or extracts from cultured cells was quantified using Calcium Liquicolor kit (StanBio laboratories, Boerne, TX) and normalized to aortic dry weight or total cellular protein, respectively.

**Quantitative real-time PCR.** Freshly dissected aortic tissues, cleaned of endothelium and adventitia, were rinsed with DEPC-treated PBS and stored at −80°C. Total RNA was isolated by rapid agitation (4 times for 1 minute each) in Trizol reagent (Invitrogen) using a Micro-Mini Beadbeater with 1.0 mm zirconia/silica beads (BioSpec Products). mRNA was purified with RNeasy kit (Qiagen) according to the manufacturer’s protocol. Real-time PCR amplification was performed with EVA green chemistry (Bio-Rad) using a Bio-Rad CFX98 thermocycler. Reactions were performed in triplicate for each primer pair (Supplemental Table I). The results were analyzed by the relative quantification method using Microsoft Excel.
**Von Kossa, Sirius Red and immunostaining.** 5 mm long pieces of freshly-dissected descending aortas were fixed in 4% paraformaldehyde, embedded in OCT freezing medium (Tissue-Tek) and frozen. Frozen 10 µm sections of aortas were stained for mineral using the Von Kossa silver nitrate method. Briefly, samples were rehydrated in water and incubated in 5% silver nitrate solution (Sigma-Aldrich) for 2 hours under a 150 watt lamp. Samples were washed in water and destained in 5% sodium thiosulfate (Sigma-Aldrich) for 5 minutes at room temperature. Sections were counterstained with nuclear fast red (Sigma-Aldrich) for 5 minutes. Frozen 10 µm sections of aortas were stained for collagenous matrix using Sirius Red (Sigma-Aldrich). Samples were rehydrated and incubated in Sirius Red stain for one hour at room temperature, washed twice in acidified water, and dehydrated in 100% ethanol.

Immunofluorescence for β-catenin protein was performed with rabbit anti-β-catenin antibody (1:80; Santa Cruz Biotechnology) and immunofluorescence for TG2 utilized rabbit anti-TG2 antibody (1:250; Abcam). Secondary Dylight 549-conjugated goat anti-rabbit antibody (1:400; Jackson ImmunoResearch) was used to visualize immunofluorescence. Nuclei were counterstained with DAPI (Sigma-Aldrich). Adjacent sections acted as negative controls (omission of primary antibody) and showed no positive staining. Immunostaining and Von Kossa analysis was performed on every 25th section for the lengthwise analysis of aortic segments. Aortic sections were examined by conventional microscopy using a Leica DMIL microscope equipped with a SPOT-RT3 real-time CCD camera (Diagnostic Instruments). Representative sections are shown in the figures, while statistical analysis was performed on at least 4 sections for each animal.

**Quercetin binding assay.** Quercetin was conjugated to EAS (epoxy-activated Sepharose 6B) beads according to the published protocol. Quercetin-conjugated, or control mock-conjugated beads were incubated with purified recombinant human TG2 (Zedira) overnight at 4°C and the
bead-bound fractions were collected by centrifugation and analyzed by Western blot for the presence of TG2.

**Transglutaminase Activity.** TG2 activity in total cell lysates was assayed by incorporation of the biotinylated pentylamine EZ-link (Thermo Fisher) into N,N'-Dimethylcasein (Sigma-Aldrich) in the ELISA-like assay as previously described. Briefly, cells were lysed in 5 mmol/L Tris-HCl pH 7.5, 0.25M Sucrose, 0.2 mmol/L MgSO4, 2 mmol/L DTT, 0.4 mmol/L PMSF, 5 μg/mL Leupeptine and 0.4% Triton X-100, and purified guinea pig liver transglutaminase 2 (gplTG2) (Sigma-Aldrich) was used as a standard. Reactions were carried out in 100 mmol/L Tris-HCl pH 8.5, 6.7 mmol/L CaCl2, 13.3 mmol/L DTT and 2.5 mmol/L EZ-link Pentylamine-Biotin (Thermo Fisher) for one hour at 37°C. Incorporated EZ-link Pentylamine-Biotin was probed with 1:5000 ExtrAvidin-Peroxidase (Sigma) for one hour at room temperature and detected by peroxidase reaction with Super AquaBlue ELISA substrate (eBioscience). TG2 activity was determined by reading the absorbance of ELISA substrate at 405nm, using a Polarstar Optima plate reader.

**Western Blot.** Aortic tissue was cut into small pieces and lyzed by thaw-freeze cycles in RIPA buffer containing EDTA-free Protease and Phosphatase Inhibitors (Thermo Fisher). Denatured 40 μg protein samples were separated by SDS-PAGE. Proteins separated by SDS-PAGE were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and Western blot was performed using the standard protocol. Membranes were incubated for 1 hour at room temperature in solution consisting of 5% non-fat dry milk or 5% bovine serum albumin (for β-catenin antibody) in TBS containing 0.1% Tween-20 (TBST) to block non-specific signal. Primary antibodies were diluted in 50% blocking solution [goat anti-TG2 1:1000 (Millipore), rabbit anti-β-catenin 1:1000 (Cell Signaling), rabbit anti-phospho-GSK-3β 1:1000 (Cell Signaling), rabbit anti-Histone 3 1:5000 (Thermo Fisher), rabbit anti-smooth muscle myosin
heavy chain 1:2000 (Abcam), or HRP-conjugated mouse anti-GAPDH 1:35,000 (Sigma)].
Membranes were incubated in primary antibody overnight at 4°C, washed 3 times for 10
minutes in TBST, and proteins were detected using HRP-conjugated secondary antibodies [goat
anti-rabbit 1:2000 (Thermo Fisher) or rabbit anti-goat 1:80000 (Sigma)] diluted in 2.5% non-fat
dry milk in TBST. Signal was visualized with SuperSignal West Pico chemiluminescent
substrate (Thermo Fisher). Protein bands were quantified by densitometry using Scion Image
(Scion Corporation) and the data were analyzed using Microsoft Excel.

**Luciferase Analysis.** A stable β-catenin reporter cell line was established by transducing A10
VSMCs with the Clignal Lenti TCF/LEF Luciferase Reporter (SA Biosciences), according to
manufacturer’s protocol, followed by a 2-week selection with puromycin (10 μg/mL; Sigma). For
the analysis of β-catenin signaling, the A10-βcat reporter cells were seeded at 3x10^3 cells/cm^2
and cultured for 48 hours in reduced-serum medium (1% FBS) containing 1.6 mmol/L
phosphate and 10 μmol/L warfarin in the presence or absence of quercetin at the indicated
concentrations. Luciferase activity in total cell lysates was measured in a 96-well plate
luminometer (Harta Instruments) using the Promega Luciferase Assay Kit and was normalized
to the total protein present in cell lysates.

**Blood Pressure Analysis.** At the endpoints, animals were anesthetized with isoflurane (3-5%
in balance O_2 for induction and 2.5% in balance O_2 for maintenance). A 2 mm incision was
made in the left thigh, the femoral artery was isolated by blunt dissection, and a short SPR-671
nylon catheter (Millar Instruments, Houston, TX) was inserted into the left femoral artery and
pushed into the distal abdominal aorta. The catheter was connected to a pressure transducer
(THM 150, Indus System), animals were allowed to stabilize in 1.5% isoflurane for 5 minutes,
and diastolic blood pressure (DBP), systolic blood pressure (SBP), and heart rate (HR) were
measured for 20 minutes. The pressure data were analyzed using MP 100 with AcKnowledge v3.7 (BioPac Systems). Mean arterial pressure (MAP) and pulse pressure (PP) were calculated from the SBP and DBP values. Mean arterial pressure (MAP) was calculated as \(((2 \times DBP) + SBP) / 3\) and pulse pressure (PP) was calculated as SBP - DBP.

**Oxidative Stress Analysis.** Cellular oxidative stress in frozen sections of aortas from control animals or WVK-treated animals was detected using the cell-permeable fluorogenic probe CellROX (Molecular Probes) that emits red fluorescence upon oxidation by reactive oxygen species (ROS). Immediately prior to fixation in 4% paraformaldehyde, aortic segments were incubated with 5 μmol/L CellROX for 30 minutes at 37°C. As a positive control for oxidative stress, aortas were first incubated with 100 μmol/L menadione (Sigma-Aldrich) for 30 minutes at 37°C prior to the CellROX reaction. Nuclei were visualized with DAPI (Sigma-Aldrich). Aortic sections were examined by conventional fluorescence microscopy using a Leica DMIL microscope equipped with a SPOT-RT3 real-time CCD camera (Diagnostic Instruments).

**Motor Activity Test.** The motor strength of representative rats from the experimental groups was evaluated using the hanging wire test \(^4\). The rats were placed on a metal wire cage lid and the lid was turned upside down. The latency to fall off the wire cage lid was measured up to a maximum of 120 seconds.

**Statistical Analysis.** Data are expressed as mean ± standard error (SEM). Student’s \(t\)-test was used for comparison between two groups. For more than two groups, mean values were compared using one-way analysis of variance (ANOVA) with comparison between groups by Tukey-Kramer HSD test. A value of \(p<0.05\) was considered statistically significant.
Supplemental Table I: Primers sequences for rat genes analyzed by real-time PCR.

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Supplemental Figure Legends

Supplemental Figure I. Effects of warfarin on the aorta in vivo. (A) Diameter of lumen in aortae of normal rats (N=13) and rats treated with WVK (N=18), KCC-009 (N=6), WVK+KCC (N=6), quercetin (N=12), or WVK+quercetin (N=12). (B) Expression of markers of apoptosis in WVK-treated animals (black bars) and WVK+quercetin-treated animals (dark grey bars) compared to untreated controls (N=5 animals per condition). (C) Expression of oxidative stress marker genes Hif-1a, Keap-1, and Nrf2 in WVK-treated animals (black bars) compared to untreated controls (grey bars) (N=8 animals per condition). Transcripts were analyzed by real-time PCR NS, not significant. (D) Reactive oxygen species were examined in aortic tissue with fluorogenic probe (red) and nuclei counterstained with DAPI (blue). Scale bar = 10 μm. Vitamin K3 (vit K3; 100 μmol/L) served as positive control for the induction of oxidative stress. (E) Immunofluorescence for TG2 [nuclei counterstained with DAPI (blue)] in aortae from control and WVK-treated animals. Mineral deposition detected by von Kossa staining of adjacent sections. Scale bar = 20 μm.

Supplemental Figure II. TG2 inhibitor KCC-009 prevents warfarin-induced aortic calcification. Sections of aortae from age-matched animals treated with vehicle (control), WVK (warfarin), KCC-009, or WVK + KCC-009 stained with mineral-detecting von Kossa (black) and counterstained with nuclear fast red. Scale bar = 100 μm. N=6.

Supplemental Figure III. Quercetin inhibits TG2 and β-catenin activity in cultured VSMCs. A10 cells were cultured in the presence of warfarin (10 μmol/L) and quercetin (100 μmol/L) as indicated. (A) Total transglutaminase activity. (B-C) Real-time PCR analysis of TG2 expression (B) and the expression of β-catenin target genes axin2, cyclin D1 (cycD1), and Tcf4 (C) normalized to Rpl19. *, p<0.05; **, p<0.01. N=4.
Supplemental Figure IV. Quercetin prevents warfarin-induced aortic calcification. Aorta sections from age-matched untreated control animals and animals treated with WVK (warfarin), Quercetin, or WVK + Quercetin stained with mineral-detecting von Kossa (black) and counterstained with nuclear fast red. Scale bar = 100 μm. N=7, control; N=12 per treatment condition.

Supplemental Figure V. Effects of Quercetin in vivo. (A-B) Increase in aortic transcripts of vascular markers – smooth muscle actin (sma), smooth muscle myosin heavy chain (MHC), sm22a, and calponin (cnn1) (A), and increase in aortic transcripts of Notch markers – hey1, RBP-J, and Dkk2 (B) in Quercetin-treated animals (hatched bars) compared to untreated controls (grey bars), measured by real-time PCR. N=7, control; N=8, quercetin. *, p<0.05. (C) Immunoblot for smooth muscle myosin heavy chain (smMHC) showing no significant difference between different treatment groups. Graph shows smMHC normalized to histone 3 (N=10). (D) Motor activity was not significantly altered in animals treated with WVK, WVK and quercetin, or quercetin alone compared to untreated animals. N=10.

Reference List


**Supplemental Figure I**

A.

Lumen diameter (μm)

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B.

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Oxidative stress marker expression (fold)

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D.

Control

WVK

Vit K3

E.

Control

WVK

TG2

TG2/DAPI

von Kossa

Bar scale
Supplemental Figure III

A

TG2 activity (μU/mg protein)

Warfarin: - + +
Quercetin: - - +

B

TG2 gene expression (fold)

Warfarin: - + +
Quercetin: - - +

C

β-catenin dependent gene expression (fold)

axin2 cycD1 Tcf4

Warfarin: - + + - + + - + +
Quercetin: - - + - - + - - +
Supplemental Figure V

A

[Bar chart showing vascular gene expression (fold) with x-axis: Querc: - + - + - + and y-axis: 0.5 1 2 4 8 for smMHC, MHC, sm22a, cnn1]

B

[Bar chart showing Notch marker expression (fold) with x-axis: Querc: - + - + - + and y-axis: 0.5 1 1.5 2.5 3.5 4.5 4.5 for hey1, RBP-J, Dkk2]

C

[Image showing Western blot analysis for MHC and His 3 with x-axis: WVK: - + + + + and y-axis: smMHC (fold) for - + + + +]

D

[Box plot showing Motor Activity over 10 days with x-axis: WVK: - + + + + and y-axis: Time (seconds) 0 20 40 60]