Quercetin attenuates warfarin-induced vascular calcification in vitro independently from Matrix Gla protein

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*Running title: Molecular mechanism of warfarin-induced calcification

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Keywords: vascular calcification; warfarin; quercetin; β-catenin; PKA; BMP; matrix gla protein

BACKGROUND: Molecular mechanism(s) of warfarin-induced vascular calcification are not well known.

RESULTS: Inhibition of β-catenin signaling with shRNA or quercetin prevents osteoblastic transformation and calcification in VSMCs and calcification in aortic rings treated with warfarin, independent from MGP and protein carboxylation.

CONCLUSION: Quercetin inhibits vascular calcification via β-catenin signaling.

SIGNIFICANCE: Quercetin may be instrumental in treatment of warfarin-induced vascular calcification.

SUMMARY

Warfarin can stimulate vascular calcification (VC) in vitro via activation of β-catenin signaling and/or inhibition of matrix Gla protein (MGP) carboxylation. Calcification was induced in vascular smooth muscle cells (VSMCs) with therapeutic levels of warfarin in normal calcium and clinically acceptable phosphate levels. Although TGF/BMP and PKA pathways are activated in calcifying VSMCs, pharmacologic analysis reveals that their activation is not contributory. However, β-catenin activity is important because inhibition of β-catenin with shRNA or bioflavonoid quercetin prevents calcification in primary human VSMCs, rodent aortic rings and rat A10 VSMC line. In the presence of quercetin, re-activation of β-catenin using the glycogen synthase kinase 3-beta (GSK-3β) inhibitor LiCl restores calcium accumulation, confirming that quercetin mechanism of action hinges on inhibition of the β-catenin pathway. Calcification in VSMCs induced by 10 μM warfarin does not associate with reduced levels of carboxylated MGP and inhibitory effects of quercetin do not involve induction of MGP carboxylation. Further, down-regulation of MGP by shRNA does not alter the effect of quercetin. These results suggest a new β-catenin-targeting strategy to prevent VC induced by warfarin and identify quercetin as a potential therapeutic in this pathology.

Vascular calcification (VC) contributes to increased cardiovascular mortality in patients with advanced renal failure and confers morbidity in atherosclerosis and diabetes (1,2). Cross-sectional
studies indicate an association between anticoagulant therapy with coumadin (warfarin) and calcium phosphate deposition in arterial media (3,4). In rodents, warfarin treatment induces rapid VC and decreased arterial compliance (5,6) akin to elastocalcinosis, a condition prevalent in the elderly population and characterized by calcium deposition along the elastic lamellae. Extensive research over the past decades has established that VC is at least in part a cell-mediated process that includes stimulation of osteogenic/chondrogenic differentiation in vascular smooth muscle (7,8), vesicle release, apoptosis, extracellular matrix degradation and loss of calcification inhibitors (9,10).

The best characterized mechanism of warfarin action involves inhibition of protein γ-carboxylation. Some endogenous arterial inhibitors of VC, such as matrix gla protein (MGP) secreted in the arterial tissue by VSMCs and macrophages, require carboxylation for full activity. Carboxylated MGP (GlaMGP) can prevent calcification by direct inhibition of hydroxyapatite formation in the extracellular matrix (11,12) and by inhibition of the pro-osteogenic activity of bone morphogenetic proteins (BMPs) 2 and 4 through their sequestration (13,14). Thus, a decrease of GlaMGP has been considered the major molecular mechanism underlying warfarin-induced calcification (5,15). However, it appears that the hallmarks of calcification induced by GlaMGP deficiency are not the same as in the warfarin-induced pathology. Genetic ablation of MGP leads to VC that is associated with chondrogenic transformation of the smooth muscle (16) and is mediated by activated BMP signaling (13,14,17). In contrast, warfarin-induced calcification of elastic lamellae in vivo does not involve ectopic chondrogenesis, and ex vivo studies indicate that it may not hinge on BMP activation (11). Therefore, additional research is warranted to elucidate the signaling mechanisms orchestrating warfarin-induced elastocalcinosis.

We have recently demonstrated a critical role for activated canonical β-catenin signaling in warfarin-induced calcification in vitro by showing that the antagonistic protein Dikkopf1 (Dkk1) attenuates calcium deposition (18). Given the importance of activated β-catenin signaling in this type of calcification, we evaluated the bioflavonoid quercetin, capable of inhibiting this pathway (19-21), as an attenuator of osteoblast-like transformation and mineralization in VSMCs. In addition, we examined whether β-catenin activation and quercetin effect depend on changes in GlaMGP levels, and assessed potential interactions of the β-catenin pathway with BMP, Notch, and PKA pathways, which have previously been implicated in phosphate-induced calcification of VSMCs in vitro (16,22,23) and are known to interact with β-catenin signaling in diverse biological systems (24-27).

**METHODS**

**Cell and aortic ring cultures** – The A10 clonal embryonic rat aortic smooth muscle cell line (A10 cells; ATCC) was maintained in complete growth medium [DMEM (Invitrogen) containing 10% FBS (Hyclone) and 100 ng/mL penicillin and streptomycin (Invitrogen)]. Primary human aortic smooth muscle cells (Lonza) were cultured in the medium recommended by the supplier. Calcification was induced by a pro-mineralizing medium containing 1% FBS, inorganic phosphate (Pi; final concentration 1.6 mM) and 10 μM warfarin (Sigma-Aldrich). 2-3 mm aortic rings were isolated from male Sprague-Dawley rats that weighed 150 to 300 g as previously described (28) and mineralization was induced using the pro-mineralizing medium supplemented with 7 U/mL alkaline phosphatase (Sigma-Aldrich). Aortic rings treated with 7U/mL alkaline phosphatase and 1.6 mM Pi in the absence of warfarin served as control. Mineralizing media were further supplemented with quercetin (10-100 μM, Quercegen Pharma, Newton, MA), N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl [H-89 (50 nM; EMD Biosciences)], Wnt3a (10 ng/mL; R&D Systems), Noggin (10 ng/ml; R&D Systems), forskolin (5 μM; EMD Biosciences), or lithium chloride [LiCl; 10-500 μM (Sigma-Aldrich)] as indicated. For cultured cells, vehicle [DMSO (Sigma-Aldrich)]-treated cells served as reference.

**Calcium Quantitation** – Calcium content was determined colorimetrically by the o-cresolphthalein complexone method using the Calcium (CPC) Liquicolor kit (Stanbio Labs) in 0.1M HCl extracts from cultured cells or from aortic rings dried overnight at 60°C, and was
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normalized either to total cellular protein or to dry weight of aortic rings.

Tissue analysis – For histologic analysis, aortic rings were fixed with 4% paraformaldehyde and processed for cryosectioning. 10 µm sections were stained for calcium phosphate deposition using the von Kossa method (silver nitrate plus nuclear fast red) according to standard protocols.

Real-time PCR – RNA was isolated using the Qiagen RNeasy kit. cDNA was synthesized with iScript reverse transcriptase (Bio-Rad) using a DYAD thermocycler (MJ Research) and real-time PCR amplification was performed with EVA green chemistry in a CFX96 thermocycler (Bio-Rad). Primers (Table 1) were designed using PrimerQuest software (Integrated DNA Technologies).

Luciferase analysis – Stable pathway sensor cell lines were established by transduction of A10 cells with lentiviral particles using the Cignal Finder Development 10-pathway Lentiviral Luciferase Reporter Kit (SA Biosciences) (Table 2) followed by a 2-week selection with puromycin (10 µg/mL; Sigma), according to manufacturer’s protocol. 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 lactate dehydrogenase (LDH) activity present in the same cell lysates.

shRNA – lentiviral particles encoding shRNA targeting rat MGP or rat β-catenin (Santa Cruz) were used according to manufacturer’s protocol.

Western blot – Protein lysates were prepared using ice-cold RIPA buffer containing EDTA-free Protease and Phosphatase Inhibitors (Thermo Fisher). Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and Western blot was performed using the standard protocol. Primary anti-γ-carboxyglutamyl (Gla) epitope antibody was from Santa Cruz, and HRP-conjugated mouse anti-GAPDH from Sigma.

Data analysis and statistics – Western blots were analyzed by densitometry using Scion Image (Scion Corp.). Real-time PCR and luciferase activity data were analyzed using Excel (Microsoft Corp.). Data are expressed as mean ± standard error (SEM) from at least 3 independent experiments (n≥3). 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’s honest significant difference test. A value of p<0.05 was considered statistically significant.

RESULTS

Attenuation of warfarin-induced VSMC calcification with quercetin

Previous research analyzed warfarin-induced calcification in vitro and ex vivo in either high calcium or high phosphate (11,15), both of which have strong pro-calcific influences on VSMCs (9,29) complicating the study of warfarin effects. Thus, we employed the previously established model of warfarin-induced calcification occurring at normal calcium and clinically acceptable phosphate levels (18) in cell and organ cultures. In A10 rat VSMCs (A10 cells) 10 µM warfarin significantly enhanced low levels of calcification observed in 1.6 mM Pi (124.61±18.84 vs 8.23 ±3.11 µg Ca²⁺/mg total protein) (Fig. 1A). The employed 10 µM (3.0 µg/mL) concentration of warfarin is within the range of commonly found therapeutic total plasma warfarin levels (30).

Given the critical role of β-catenin signaling in warfarin-induced calcification in VSMCs revealed earlier with Dkk1 treatment (18) and the demonstrated efficacy of quercetin as an inhibitor of β-catenin signaling in cancer cells (19,21), we hypothesized that quercetin would prevent calcium deposition stimulated by warfarin in VSMCs.

Quercetin, when administered concurrently with warfarin, attenuated the increase in calcification in a dose-dependent manner (Fig. 1A). A significant reduction was observed with 25 µM quercetin, while 50-100 µM quercetin [commonly used to study the effects of quercetin in vitro (31,32)] caused a dramatic almost 10-fold reduction in calcium accrual induced by warfarin (p<0.05) and curbed calcium at levels characteristic of the non-calcifying cells. A similar potent inhibitory effect of quercetin on warfarin-induced calcium deposition was also observed in primary human VSMCs (Fig. 1B). To confirm the anti-calcific effect of this flavonoid on primary VSMCs in their native niche, we employed aortic rings induced to calcify ex vivo in medium supplemented with 1.6 mM Pi and 7 U/mL alkaline phosphatase. In rat and mouse aortic
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rings, 10 μM warfarin stimulated an almost 250% increase in calcium accrual and deposition of calcium phosphate in the extracellular matrix along the elastin lamellae indicative of bona fide VC (Fig. 1C). Quercetin prevented calcium accumulation as detected by histological and quantitative analyses (Fig. 1C). Together, these studies demonstrate the high efficiency of quercetin in preventing warfarin-dependent VC in vitro and ex vivo.

Molecular analysis of osteogenic genes showed that quercetin fully abolished warfarin-induced expression of osteogenic markers osteocalcin, type I collagen, and Runx2 (Table 3), indicating prevention of the osteoblast-like transformation in VSMC. At the same time, quercetin increased expression of osteopontin (OPN) which can act as an endogenous inhibitor of VC (33,34). Thus quercetin appears to have a two-pronged effect, acting as both an antagonist of the positive regulators of calcification and an agonist of protective anti-calcific mechanism.

Regulation of intracellular signaling in VSMCs by warfarin and quercetin

Physiological osteogenic differentiation is regulated by a complex network of intracellular signaling (35). To identify the pathways that may be of essence in pathologic warfarin-induced VC, we systematically examined the effects of warfarin and its antagonist quercetin on intracellular signaling implicated in osteogenic transformation of VSMCs. A panel of nine stable A10 VSMC-based signaling pathway reporter lines was established, using lentiviral luciferase constructs driven by various transcription response elements (Table 2). Additional experiments showed that the TGFβ reporter coding for Smad-dependent luciferase expression also responds to purified BMP2 (1.95±0.08 fold induction over control, p<0.001), and it is referred to hereafter as TGF/BMP reporter. Further, the ability of the BMP antagonist Noggin to attenuate warfarin-induced activation of this Smad-dependent reporter (discussed below) indicates that warfarin activated BMP rather than TGF signaling.

Expression of the luciferase reporters was analyzed (1) in cells cultured in 1.6 mM Pi medium in which calcification is very low; (2) in calcifying cell cultures supplemented with 1.6 mM Pi and 10 μM warfarin; and (3) in cells cultured in calcification medium further supplemented with 100 μM quercetin. In addition, luciferase activity was analyzed in cell cultures supplemented with each compound alone to identify signaling pathways modulated by warfarin and quercetin.

In calcified 8 day old VSMC cultures, warfarin significantly activated three signaling conduits, β-catenin, PKA, and TGF/BMP (Fig. 2A, black bars, p<0.01 for β-catenin and PKA, and p<0.05 for TGF/BMP signaling). The anti-calcific activity of 100 μM quercetin was associated with complete attenuation of the β-catenin and PKA signaling but had no significant impact on the TGF/BMP signaling (Fig. 2A, grey bars). These results implied that activity of the TGF and/or BMP pathway may be not critical to support calcification induced by warfarin, while β-catenin and PKA pathways may regulate this process. Of note, quercetin alone had no effect on any examined signaling pathways in non-calcifying cells (Fig. 2B, grey bars), implying that quercetin specifically attenuates the effects of warfarin on intracellular signaling in calcifying VSMCs.

Next, we examined whether the activation of β-catenin, PKA, and TGF/BMP pathways in the calcifying cells was triggered by warfarin or by Pi, which is used in our cell cultures at 1.6 mM but is known to affect VSMCs at higher concentrations (16). While neither 1.6 mM Pi nor 10μM warfarin cause significant calcification on their own (18), warfarin activated all three pathways (Fig. 2B, black bars, p<0.01), and 1.6 mM Pi induced a noticeable albeit not statistically significant 30% activation of PKA but did not alter β-catenin and TGF/BMP signaling (Fig. 2B, light grey bars). These data indicate that warfarin activates these pathways independently of Pi.

Next, we examined regulation of β-catenin, PKA, and TGF/BMP pathways by warfarin and quercetin in cells exposed to the pro-calcifying medium for only 3 days, before the onset of detectable matrix calcification. This analysis detected induction of all three reporters by warfarin and attenuation of its effects on β-catenin and PKA by quercetin (Fig. 2C), implicating activation of these signaling conduits as early events of the osteoblast-like transformation in VSMCs causing enhanced calcification.

In contrast to the above three pathways, neither warfarin (Fig. 3A) nor quercetin (Fig. 3B)
had a significant effect on ERK, Notch, JNK, C/EBP, NFκB, and pRb signaling as compared to the activity of these pathways in cells cultured in 1.6 mM Pi alone. Additional experiments showed that these reporter cells respond to other stimuli (our unpublished data), confirming a minor, if any, role for these pathways in osteoblast-like transformation regulated by warfarin. Consistent with the lack of significant change in NFκB signaling, the lack of induction of the inflammatory cytokines TNFα, IL-1β, and IFNγ in the warfarin-treated VSMCs (Table 3) provides little evidence for the involvement of the inflammatory response in warfarin-induced VC, unlike the proposed role for inflammation in diabetic VC (36).

Cross-talk between PKA and β-catenin signaling has been reported in osteoblast differentiation (26). To investigate the possibility of cross-communication between the pathways activated in warfarin-dependent osteogenic transformation in VSMCs, the corresponding A10 VSMC reporter lines were cultured in 1.6 mM Pi for 8 days in the presence of either PKA activator, forskolin, or the canonical β-catenin activator, Wnt3a (Fig. 3C), or the BMP antagonist Noggin (data not shown). While these supplements activated their corresponding luciferase reporters as expected, no cross-activation of the other analyzed pathways was observed. These findings indicate a “parallel” and independent, rather than sequential, activation of the PKA, β-catenin and TGF/BMP conduits in our model.

Regulation of VSMC calcification by β-catenin, PKA, and TGFβ/BMP pathways

We further sought to identify the role for β-catenin, PKA and BMP in warfarin-induced calcification in VSMCs by using inhibitors of these pathways.

Suppression of PKA signaling with chemical inhibitor H-89 or BMP signaling with Noggin had no effect on warfarin-induced calcification even though these treatments efficiently inhibited expression of the corresponding luciferase reporters (Fig. 4A, B).

In contrast, down-regulation of β-catenin signaling with a lentiviral vector expressing shRNA targeting β-catenin completely attenuated warfarin-induced calcification in VSMCs (Fig. 4C). The efficiency of RNA interference was evident from the attenuated ability of infected cells to respond to warfarin treatment with activation of β-catenin-dependent gene expression (Fig. 4C, right panel). Cell viability was not affected by shRNA transfection in 8-day cultures as assayed by LDH activity (data not shown).

Inhibition of β-catenin signaling is essential for quercetin action

To further confirm that inhibition of β-catenin signaling is central in attenuation of warfarin-induced calcification by quercetin, we examined whether the effect of quercetin could be prevented by activation of this pathway. In this study we employed LiCl to activate β-catenin signaling via pharmacological inhibition of glycogen synthase kinase 3-beta (GSK-3β) (37).

We found that LiCl can reverse the β-catenin-quenching effect of quercetin in a dose-dependent manner (Fig. 5A) and 500 µM LiCl completely restored the warfarin-dependent activation of β-catenin even in the presence of quercetin.

Importantly, LiCl also dose-dependently attenuated the inhibitory effects of quercetin on warfarin-induced calcification with complete restoration of calcium deposition at 500 µM (Fig. 5B). These findings demonstrate that attenuation of β-catenin activity by quercetin is of essence in its inhibitory effect on warfarin-induced VSMC calcification.

Calcification induced by therapeutic levels of warfarin in VSMC is not linked to changes in GlaMGP

The warfarin-mediated reduction in MGP carboxylation has been considered a major mechanism of calcification in VSMCs (5,15). Therefore, we analyzed the levels of GlaMGP in our model of warfarin-induced calcification. The 18 kDa GlaMGP protein was detected by Western blot with a monoclonal antibody directed against the γ-carboxyglutamyl (Gla) epitope (11). Consistent with previous studies (10), the levels of GlaMGP were severely reduced in VSMCs treated with 100 µM warfarin (Fig. 6A, upper panel). However, in 10 µM warfarin, MGP carboxylation was surprisingly similar to that in the control untreated cells (Fig. 6A) even though both 10 µM and 100 µM warfarin induced extensive...
calcification in VSMCs (Fig. 6A, bar graph). Augmentation of GlaMGP by treatment of cells with vitamin K (38) (Fig. 6A, upper panel) did not significantly inhibit calcification induced by 10 μM warfarin (Fig. 6A, bar graph) indicative of a mechanism independent of GlaMGP. Further, addition of 50-100 μM quercetin to the 10 μM warfarin prevented warfarin-induced calcification but had no effect on GlaMGP (Fig. 6A) implying that the quercetin effect is also GlaMGP-independent.

To investigate this possibility further, we analyzed quercetin action in VSMCs in which MGP expression was 84-88% down-regulated by lentivirus-expressed shRNA (Fig. 6B, upper panel). Quercetin effectively attenuated warfarin-induced calcium accrual in the GlaMGP-ablated VSMCs (Fig. 6B, bar graph), confirming that the effect of quercetin on calcification in this model does not rely on GlaMGP levels.

DISCUSSION

PKA, BMP, ERK and Notch pathways and inflammatory cytokine signaling have been linked to VC induced by elevated phosphate or by diabetic milieu (22,36,39,40), while β-catenin activity is critical in warfarin-induced VSMC mineralization (18) and has been implicated in both diabetic calcification (41) and calcification of the heart valves (42). Based on the perceived mechanistic similarity between pathological and physiological calcification, C/EBP, JNK and pRb pathways associated with osteogenesis (43-45) may also contribute to vascular mineralization. However, studies on individual pathways usually employ diverse and often non-overlapping stimuli to induce calcium accrual in vascular cells, precluding a comprehensive understanding of the broad-range cell signaling network in VC.

In this study, we performed the first systematic analysis of the osteogenesis-related signaling pathways, coupled with analysis of the inflammatory cytokine gene expression and GlaMGP levels in a single model of VSMC calcification at physiological levels of calcium, phosphate, and warfarin. We have found that this type of calcification does not associate with noticeable changes in Notch, ERK, C/EBP, JNK, pRb and NFκB pathways or in inflammatory cytokine gene expression. Further, while PKA and BMP signaling is activated by warfarin in VSMCs, inhibition of these pathways does not prevent calcification. These data suggest that PKA and BMP signaling pathways are not required for calcification to occur. In addition, activated BMP signaling does not preclude inhibition of calcification by quercetin further supporting a minor role for this pathway in calcification (11). Consistent with the above findings, warfarin-induced calcification in VSMCs does not associate with either induction of transcription factor Msx2, which regulates diabetic calcification via the BMP-Msx2 axis (46), or induction of the phosphate transporter PiT-1 and osteopontin, both of which are markers of PKA-dependent calcification (47).

Our findings indicate that despite the many common features between physiologic differentiation of osteoblasts and pathologic osteoblastic transformation of VSMCs, these processes show differences that may potentially underlie the “bone-vascular paradox” in which bone loss in osteoporosis is accompanied by VC (50).

Two major mechanisms of warfarin-induced calcification in VSMCs emerged from the reported study. High (100 μM) concentrations of warfarin in physiological phosphate severely reduced the level of GlaMGP in VSMCs, consistent with the effect of 25 μM warfarin in high phosphate on aortic rings (10), and stimulated high levels of calcification. Taking into account that exogenously added GlaMGP inhibits calcification in VSMCs (14), these observations indicate that high doses of warfarin may induce calcification through the inhibition of vitamin K epoxide reductase and ablation of GlaMGP. However, in warfarin therapy plasma levels of this drug are much lower and seldom exceed 10 μM (3.0 μg/mL) (30). At this concentration, warfarin treatment has no discernible effects on expression of endogenous GlaMGP in VSMCs cultured in physiological phosphate and calcium. Moreover, augmentation of GlaMGP by vitamin K treatment (up to 100 μM, a ten-fold higher concentration than warfarin) was not efficient in reducing calcification in this system. The effects of vitamin K were not consistent or dose-dependent, reaching at most a 30% (not statistically significant) reduction in calcification with the remaining
calcium levels being still 7 times higher than in the control cells. This limited efficiency of vitamin K in vitro is in agreement with a 50% reduction of arterial calcification observed in the rat model of warfarin-induced elastocalcinosi supplemented with high dose of vitamin K2 (48). Altogether, these observations indicate that while ablation of GlaMGP probably contributes to the effects of high warfarin concentrations on vascular cells, the role for this mechanism in physiological warfarin-induced vascular calcification is relatively minor. Although the inhibition of vitamin K epoxide reductase resulting in reduced protein carboxylation is the best characterized effect of warfarin, this compound also directly affects other proteins including the transcription factor pregnane X receptor (49) and enzyme transglutaminase 2 (TG2) (18). Activation of TG2 mediates warfarin-induced stimulation of β-catenin signaling in VSMCs (18) likely due to the ability of this protein to interact with LRP5/6 receptors (50).

The major mechanism underlying the calcification of VSMCs in physiological warfarin appears to be the activation of β-catenin signaling. Its critical role is unambiguously shown by the effect of β-catenin ablation that completely prevents calcification, consistent with the inhibitory effect of the Dkk1 protein that antagonizes the LRP5/6 receptors of the canonical β-catenin signaling (18). In accordance with these findings, the flavonoid quercetin capable of β-catenin inhibition (19,21) caused a dramatic, approximately 10-fold decrease in warfarin-induced calcium accrual reducing calcium levels to those found in the control non-calcifying cells. While quercetin may have a multitude of effects on living cells, we found that inhibition of β-catenin is the key in its inhibitory effect on calcification because forced activation of β-catenin signaling by concurrent LiCl treatment completely negated the anti-calcific effect of this flavonoid. These results also indicate that quercetin intercepts the β-catenin pathway in warfarin-treated cells upstream of GSK-3β - most likely at the level of ligand-receptor interaction (37) although this hypothesis needs further evaluation. In contrast, the roles for anti-oxidant and anti-inflammatory activities of quercetin (51) appear minor because studies reported here and elsewhere (18,52) demonstrate little (if any) involvement of these mechanisms in vascular calcification induced by warfarin.

While the presented comprehensive study of cell signaling in warfarin-induced calcification of VSMCs converged on the critical role of β-catenin pathway, it is noteworthy that activation of β-catenin per se is not sufficient to induce calcification in the absence of other stimuli (53), and further studies are needed to identify additional mechanism(s) acting in concert with β-catenin signaling to orchestrate osteogenic transformation and calcification in VSMCs.

In conclusion, this study contributes to mechanistic analysis of warfarin effects on vascular cells by demonstrating for the first time that warfarin-induced calcification may be prevented independent from GlaMGP levels. A better knowledge of the cellular communication network (54) in warfarin-treated vascular cells will be instrumental in developing approaches to prevention and treatment of this disorder. Our results contribute to the mechanistic understanding of the beneficial effects of quercetin on cardiovascular disease (55,56) and expose β-catenin signaling as a novel major target for preventive therapy of warfarin-induced VC. In addition, our findings suggest that quercetin may be a promising therapeutic to test in pre-clinical models of elastocalcinosis taking into consideration the wide safety margin of quercetin (57), its broad testing in clinical trials, and its applicability as a daily dietary supplement. However, a caution should be exercised in interpretation of in vitro studies for clinical applications.

**Acknowledgements** – We thank Quercegen Pharma, Newton, MA for the generous gift of quercetin, and Dr. Dmitry Nurminsky for discussion of the results.
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**FOOTNOTES**

* This work was supported, in whole or in part, by the National Institutes of Health (Grant R01HL093305 to M.N. and Fellowship T32AR007592 to K.B.)

2 The abbreviations used are: VSMC, vascular smooth muscle cell; BMP, bone morphogenetic protein; PKA, protein kinase A; ERK, extracellular signal-regulated kinases; Pi, inorganic phosphate; LiCl, lithium chloride; GSK-3β, glycogen synthase kinase 3-beta; LDH, lactate-dehydrogenase
FIGURE LEGENDS

FIGURE 1: Attenuation of warfarin-induced VSMC calcification by quercetin. A-B, Calcium content in A10 cells (A) or human VSMCs (B) cultured in pro-mineralizing medium containing 1.6 mM Pi and 10 μM warfarin (black bar) supplemented with quercetin (grey bars), as indicated below graphs (n=3). C, von Kossa stain for mineralized matrix deposition (left) and calcium content in rat aortic rings (right) cultured in pro-mineralizing medium in the absence (black bar) or presence (gray bar) of 100 μM quercetin (random mix of 4-5 2 mm aortic rings from 3 rats used in each condition), *, p<0.05; **, p<0.01.

FIGURE 2: Identification of β-catenin, PKA and TGF/BMP signaling pathways as potential mediators of warfarin-induced calcification in VSMCs. Activity of specified luciferase reporters (above graphs) analyzed in A10 cells induced to calcify for 8 days in pro-mineralizing medium (1.6 mM inorganic phosphate and 10 μM warfarin) with or without 100 μM quercetin (A); in non-calcifying A10 cells cultured for 6 days in the presence of individual supplements (B); and in non-calcifying A10 cells cultured for only 3 days in pro-mineralizing medium with or without 100 μM quercetin. *, p<0.05; **, p<0.01.

FIGURE 3: Regulation of intracellular VSMC signaling by either warfarin or quercetin. A-B, Activity of indicated luciferase reporters (above graphs) analyzed in A10 cells cultured in 1.6 mM Pi supplemented with 10 μM warfarin (A) or 100 μM quercetin (B). C, Luciferase activity for indicated signaling pathways (above graphs) in A10 cells cultured in pro-mineralizing medium with the pharmacologic activators of PKA (forskolin) or β-catenin (Wnt3a) (E), (n=6). *, p<0.05; **, p<0.01.

FIGURE 4: Role of β-catenin, PKA and BMP signaling in warfarin-induced vascular calcification. Matrix calcium levels in A10 cells cultured in pro-mineralizing medium for 8 days to induce calcification. Cells were treated with the PKA inhibitor H-89 (A), with the BMP antagonist Noggin (B), or with lentivirus expressing shRNA for rat β-catenin (C) (n=4). Luciferase activity for indicated signaling pathways is shown in the right bar graphs (n=6). *, p<0.05; **, p<0.01.

FIGURE 5: LiCl rescues warfarin effects on β-catenin signaling and vascular calcification in the presence of quercetin. A10 cells were cultured for 6 days in of pro-mineralizing medium (1.6 mM Pi and 10 μM warfarin) and 100 μM quercetin (to block calcification), supplemented with increasing concentrations of LiCl from 10-500 μM (hatched bars). A, Luciferase assay for β-catenin activity (n=4). B, Matrix calcium levels (n=4). Bars show fold induction compared to cells cultured in 1.6 mM Pi alone. *, p<0.05; **, p<0.01.

FIGURE 6: Quercetin-mediated inhibition of calcification is independent from MGP. A, GlaMGP expression determined by Western blot (upper panel) and calcified matrix deposition (graph) in A10 cells cultured in pro-calcifying medium (+, 10 μM warfarin; ++, 100 μM warfarin) in the presence or absence of quercetin or vitamin K as indicated. B, Down-regulation of MGP expression in A10 cells with lentivirus expressing shRNA for rat MGP as determined by Western blot (upper panel) does not affect the ability of quercetin to attenuate warfarin-induced calcification (graph; n=3). *, p<0.05; **, p<0.01; NS, not significant;
# Table 1. Primer sequences for rat genes analyzed by real-time PCR.

| Target Genes                     | Accession | Forward primer                  | Reverse primer                  |
|----------------------------------|-----------|---------------------------------|---------------------------------|
| **Housekeeping**                 |           |                                 |                                 |
| Ribosomal protein L19 (Rpl19)    | NM_031103 | agcacatccacaaactgaagga          | cgtttcggtctctcttagtggc          |
| **Inflammatory Markers**         |           |                                 |                                 |
| TNFα                             | NM_012675 | gccaaagcatggtggatcttcaaa       | acgcttgctccttgaagagaaacct      |
| IL-1β                            | NM_031512 | acctgtgtgtgtgtgttccc           | gctttcagctcactgtggtcag          |
| IFNγ                             | NM_138880 | catgcgaagtctgaggtgaac          | tggtaacagctgtgtaactct           |
| **Osteogenic Genes**             |           |                                 |                                 |
| Osteocalcin (OCN)                | NM_013414 | gtctgaaagctctcatgtcag          | ggtcctcgatctcaatttgtgacga       |
| Type I collagen (col I)          | NM_053304 | agcaaaagcatagtcagtaacc         | tgccagatgttaagctctttcag         |
| Runx2                            | NM_053470 | caagccacacagtgaagctgcaact      | cctcaaccacgaagctgcaattt         |
| Osteopontin (OPN)                | NM_012881 | tatcaaggtcatcctgagctgcac       | atccagctgactgactcaggtgct        |
| Phosphate transporter (PiT-1)     | NM_031148 | atcacaccctccagttgccca         | ctgatgggaaaggcaatgttt          |
| Osterix                          | NM_181374 | gagctgcaaggaagccatacact        | agtcacattgggtctgtaagg           |
| Msx2                             | NM_012982 | gcgtgaggagggtagagatggaga       | gtggcctgtgagagatgtgtag          |
| Alkaline Phosphatase             | NM_013059 | aatccctgtcctctcactagcaaa      | aatccctgtcctctcactagcaaa        |
Table 2. Transcription Factor Response Elements for Luciferase Signaling Reporter Constructs.

| Signal Transduction Pathway | Transcription Factor Response Element |
|-----------------------------|--------------------------------------|
| Notch                       | RBP-Jκ                               |
| β-catenin                   | TCF/LEF                              |
| NFκB                        | NFκB                                |
| BMP/TGFβ                    | SMADs                               |
| pRb/E2F                     | E2F/DP1                              |
| C/EBP                       | C/EBP                               |
| cAMP/PKA                    | CREB                                |
| MAPK/ERK                    | Elk-1/SRF                            |
| MAPK/JNK                    | AP-1                                |

Table 3: Analysis of gene expression associated with regulation of calcification in VSMCs by warfarin and quercetin

| Target Gene         | control       | warfarin       | warfarin + quercetin |
|---------------------|---------------|----------------|----------------------|
| **Osteogenic markers** |               |                |                      |
| Osteocalcin         | 1.01 ± 0.10   | 2.32 ± 0.09*   | 1.21 ± 0.02†         |
| Type I Collagen     | 1.01 ± 0.10   | 1.60 ± 0.07*   | 1.03 ± 0.03†         |
| Runx2               | 1.00 ± 0.02   | 1.48 ± 0.02*   | 0.66 ± 0.08†         |
| Osteopontin         | 1.00 ± 0.02   | 1.02 ± 0.01*   | 2.23 ± 0.04*         |
| Pit-1               | 1.01 ± 0.07   | 1.13 ± 0.38    | 1.12 ± 0.02          |
| Msx2                | 1.01 ± 0.07   | 1.11 ± 0.02    | 0.70 ± 0.02          |
| Osx                 | 1.08 ± 0.23   | 3.05 ± 0.60*†  | 2.09 ± 0.23*         |
| Alk Phos            | ND            | ND             | ND                   |
| **Inflammatory cytokines** |       |                |                      |
| Interleukin 1β      | 1.00 ± 0.04   | 1.19 ± 0.16    | 0.98 ± 0.04          |
| Tumor Necrosis Factor α | 1.00 ± 0.04   | 0.57 ± 0.18    | 0.75 ± 0.15          |
| Interferon gamma    | 1.00 ± 0.02   | 0.52 ± 0.20    | 0.69 ± 0.28          |

* p-value < 0.05 compared to control; † p-value < 0.01 compared to control; ‡ p-value < 0.05 compared to WVK; †† p-value < 0.01 compared to WVK

Gene expression was analyzed by real-time PCR in A10 cells treated with warfarin or warfarin+quercetin. Presented as fold change in expression compared to VSMCs treated with 1.6 mM Pi (control) (N=4; gene expression normalized to Rpl19).
**Figure 1**

A

| Warfarin: | Quercetin: |
|----------|------------|
| -        | -          |
| +        | +          |
| + 10     | +          |
| + 25     | +          |
| + 50     | +          |
| + 100    | +          |

Calcium content (ug/mg protein)

B

| Warfarin: | Quercetin: |
|----------|------------|
| -        | -          |
| +        | +          |
| +        | +          |

Calcium content (ug/mg protein)

C

- **no warfarin**
- **warfarin**
- **warfarin + quercetin**

Calcium content (ug/mg dry weight)
Figure 3

A

![Bar chart showing luciferase activity for different transcription factors under Warfarin treatment.]

| Transcription Factor | luciferase activity (fold) |
|----------------------|----------------------------|
| C/EBP                | 1                          |
| ERK                  | 0.5                        |
| JNK                  | 0.5                        |
| NFkB                 | 1                          |
| notch                | 1.5                        |
| pRb                  | 2                          |

Warfarin: - - - + - - - + - - - + - - - + - - - + - - - +

B

![Bar chart showing luciferase activity for different transcription factors under Quercetin treatment.]

| Transcription Factor | luciferase activity (fold) |
|----------------------|----------------------------|
| C/EBP                | 1                          |
| ERK                  | 0.5                        |
| JNK                  | 0.5                        |
| NFkB                 | 1                          |
| notch                | 1.5                        |
| pRb                  | 2                          |

Quercetin: - - - + - - - + - - - + - - - + - - - + - - - +

C

![Bar chart showing luciferase activity for different transcription factors under forskolin and Wnt3a treatment.]

| Transcription Factor | luciferase activity (fold) |
|----------------------|----------------------------|
| PKA                  | 4                          |
| b-cat                | 3                          |
| TGF/BMP              | 2                          |

Forskolin: - - - + - - - + - - - + - - - + - - - +

Wnt3a: - - - + - - - + - - - + - - - + - - - +
Figure 4

A

**

B

**

C

**  **
Figure 5

A

**β-catenin-dependent luciferase activity (fold)**

| Warfarin: | - | + | + | + | + | + | + | + |
|-----------|---|---|---|---|---|---|---|---|
| Quercetin:| - | - | + | + | + | + | + | + |
| LiCl (µM):| 0 | 0 | 0 | 10 | 50 | 100 | 500 | 500 |

B

**Calcium content (fold)**

| Warfarin: | - | + | + | + | + | + | + | + |
|-----------|---|---|---|---|---|---|---|---|
| Quercetin:| - | - | + | + | + | + | + | + |
| LiCl (µM):| 0 | 0 | 0 | 10 | 50 | 100 | 500 | 500 |
Figure 6

A

| Calcium (µg/mg protein) | Warfarin | Quercetin | VitK | GlaMGP | GAPDH |
|-------------------------|----------|-----------|-----|--------|-------|
| 1                       | -        | -         | -   | -      | -     |
| 4                       | ++       | -         | -   |        |       |
| 16                      | +        | -         | -   |        |       |
| 25                      | +        | 50        | -   | 75     | 12.2% |
| 64                      | +        | 100       | -   | 50     | 16.2% |
| 1024                    | +        | 20        | -   |        |       |
| 256                     | +        | 50        | -   |        |       |
| 64                      | +        | 100       | -   |        |       |
| 1024                    | +        | 20        | -   |        |       |
| 256                     | +        | 50        | -   |        |       |
| 64                      | +        | 100       | -   |        |       |
| 1024                    | +        | 20        | -   |        |       |
| 256                     | +        | 50        | -   |        |       |
| 64                      | +        | 100       | -   |        |       |

B

| Calcium (µg/mg protein) | Warfarin | Quercetin | MGPsh | Quercetin | VitK |
|-------------------------|----------|-----------|-------|-----------|------|
| 0                       | +        | -         | -     | -         | -    |
| 100                     | +        | +         | +     | -         | -    |
| 200                     | +        | +         | +     | -         | -    |
| 300                     | +        | +         | +     | -         | -    |
| 400                     | +        | +         | +     | -         | -    |
| 500                     | +        | +         | +     | -         | -    |

* NS

**
Quercetin attenuates warfarin-induced vascular calcification in vitro independently from Matrix Gla protein
Kelly E. Beazley, Saman Eghtesad and Maria V. Nurminskaya

J. Biol. Chem. published online December 7, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.368639

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