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Abstract—Arterial calcification, common in vascular diseases, involves vascular smooth muscle cell (VSMC) transformation to an osteoblast phenotype. Clinical studies suggest that magnesium may prevent this, but mechanisms are unclear. We assessed whether increasing magnesium levels reduce VSMC calcification and differentiation and questioned the role of the Mg^{2+} transporter, transient receptor potential melastatin (TRPM)7 cation channels in this process. Rat VSMCs were exposed to calcification medium in the absence and presence of magnesium (2.0 to 3.0 mmol/L) or 2-aminoethoxy-diphenylborate (2-APB) (TRPM7 inhibitor). VSMCs from mice with genetically low (MgL) or high-normal (MgH) $[Mg^{2+}]_i$ were also studied. Calcification was assessed by von Kossa staining. Expression of osteocalcin, osteopontin, bone morphogenetic protein (BMP)-2, BMP-4, BMP-7, and matrix Gla protein and activity of TRPM7 (cytosol:membrane translocation) were determined by immunoblotting. Calcification medium induced osteogenic differentiation, reduced matrix Gla protein content, and increased expression of the sodium-dependent cotransporter Pit-1. Magnesium prevented calcification and decreased osteocalcin expression and BMP-2 activity and increased expression of calcification inhibitors, osteopontin and matrix Gla protein. TRPM 7 activation was decreased by calcification medium, an effect reversed by magnesium. 2-APB recapitulated the VSMC osteoblastic phenotype in VSMCs. Osteocalcin was increased by calcification medium in VSMCs and intact vessels from MgL but not MgH, whereas osteopontin was increased in MgH, but not in MgL mice. Magnesium negatively regulates vascular calcification and osteogenic differentiation through increased/restored TRPM7 activity and increased expression of anticalcification proteins, including osteopontin, BMP-7, and matrix Gla protein. New molecular insights are provided whereby magnesium could protect against VSMC calcification. **(*Hypertension*. 2010;56:453-462.)**

Key Words: calcification ■ vessels ■ hypertension ■ chronic kidney disease ■ osteocalcin ■ osteopontin ■ BMP

Vascular calcification, prevalent in patients with atherosclerosis, aneurysms, diabetes, hypertension, and chronic kidney disease (CKD), is associated with increased risk of morbidity and mortality.[1–5] Calcification contributes to increased vascular stiffness, decreased elasticity and reduced distensibility, characteristic features of the vascular phenotype in hypertension.[6,7]

Vascular calcification is a tightly regulated process similar to bone formation.[8] Factors promoting calcification include abnormalities in mineral metabolism, particularly hyperphosphatemia and hypercalcemia.[8] In the setting of magnesium deficiency, this phenomenon may be exaggerated,[9,10] with studies demonstrating a positive correlation between hyperphosphatemia, hypercalcemia, and arterial calcification. In vitro studies support these observations because exposure of VSMCs to high phosphate and calcium concentrations show a dose-dependent increase in mineralization, which is associated with VSMC differentiation to an osteoblastic phenotype.[11] This is driven by upregulation of transcription factors such as cbfa1 (core-binding factor 1α)/Runx2, MSX-2, and bone morphogenetic protein (BMP)-2, involved in normal bone development, and which control the expression of osteogenic proteins, including osteocalcin, osteonectin, alkaline phosphatase, collagen-1, and bone sialoprotein.[12,13] In culture, VSMCs can produce these bone-forming transcription factors and proteins, an effect that is augmented with high concentrations of phosphorous, calcium, cytokines, glucose, oxidized lipids, and a low concentration of magnesium.[11] Another mechanism contributing to vascular mineralization is loss of calcification inhibitors, such as fetuin-A, matrix Gla protein, pyrophosphate, and osteopontin.[14,15]

High phosphate and calcium levels promote VSMC differentiation through various putative processes, including acti-

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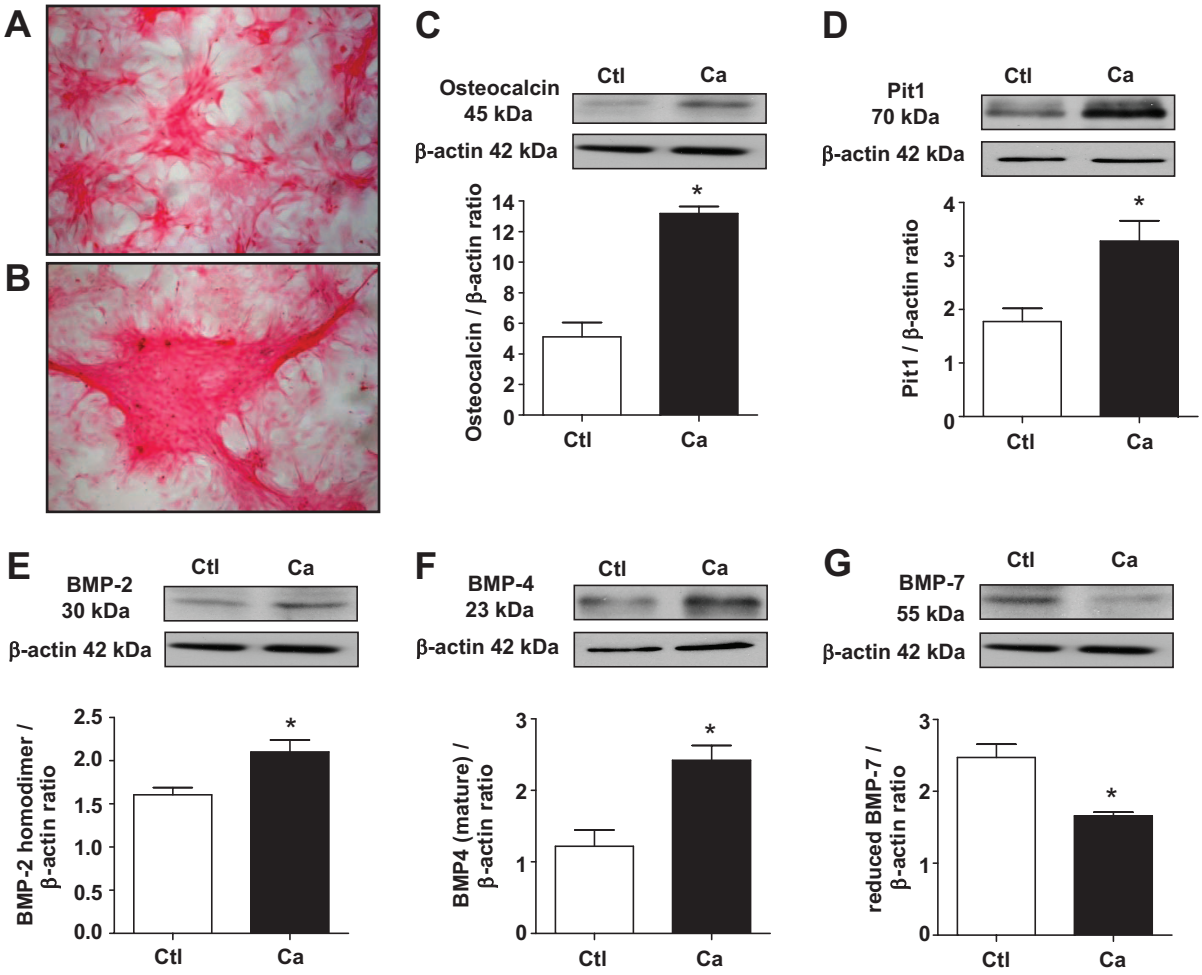

Figure 1. Exposure of VSMCs to high phosphate/calcium levels induces calcification and an osteogenic phenotype. VSMCs from WKY rats were exposed to calcification medium for 10 days. VSMCs were fixed and mineral deposition assessed by light microscopy using von Kossa staining (A and B). No deposits were found in control conditions (A). Black deposits indicate deposits of phosphate-containing mineral primarily in the extracellular regions (arrows) (B). C through G demonstrate expression of pro- and antiosteogenic proteins: osteocalcin, BMP-2, BMP-4, and BMP7 in VSMCs grown in control (Ctl) and calcification (Ca) media. BMP2 homodimer and reduced BMP-7 (active forms) were detected. D demonstrates expression of Pit-I. Immunoblots are representative of many blots. Open bars represent VSMCs exposed to control medium and closed bars represent VSMCs exposed to calcification medium. Data are presented as osteogenic protein:β-actin ratio. Results are means±SEM of 8 experiments. *$P<0.05$ vs control.

vation of a type III sodium-dependent cotransporter, Pit-I, which induces upregulation of cbfa1/Runx2,[16,17] VSMC vesicle formation, which contain dysfunctional mineralization inhibitors,[18] dysregulation of vitamin D receptor (VDR), and loss of functional calcium-sensing receptor.[19] In addition, decreased VSMC magnesium levels may be important, especially because magnesium antagonizes calcium effects.[20] We demonstrated that magnesium homeostasis in VSMCs is regulated by the transient receptor potential melastatin (TRPM)7 cation channel and that in hypertension VSMC TRPM7 activity and expression are downregulated.[21–23] TRPM7, which comprises an ion channel, containing a magnesium-permeable pore, fused to a kinase domain at the COOH terminus[24] has been implicated in osteoblast regulation.[25]

Clinically, management of vascular calcification is challenging. Antihypertensive treatment is associated with a reduced calcium index and decreased carotid intima:media ratio in high risk cardiovascular patients.[26] In experimental hypertension models, antihypertensive drugs prevent development of vascular calcification and decrease pulse pressure.[27] In CKD, phosphate control with oral phosphate binders, including aluminum- and calcium-based binders, is an important modality in the prevention/regression of vascular calcification.[28] However, these agents have unwanted side effects, and as such, there has been interest in alternative compounds, particularly magnesium-containing phosphate binders.[29]

Considering the fact that in cardiovascular disease VSMC $[Mg^{2+}]_i$ may be reduced, that low magnesium promotes calcification, especially in the context of high phosphate and calcium, that it is a very effective phosphate buffer and that it is safe (even in patients with renal disease, if monitored carefully) and inexpensive, magnesium may be an effective modality to prevent or regress vascular calcification in cardiovascular/renal disease. Here, we used an in vitro model of VSMC calcification to assess whether magnesium attenuates differentiation of VSMCs to an osteogenic phenotype and investigated putative molecular mechanisms underlying this process, focusing on TRPM7.

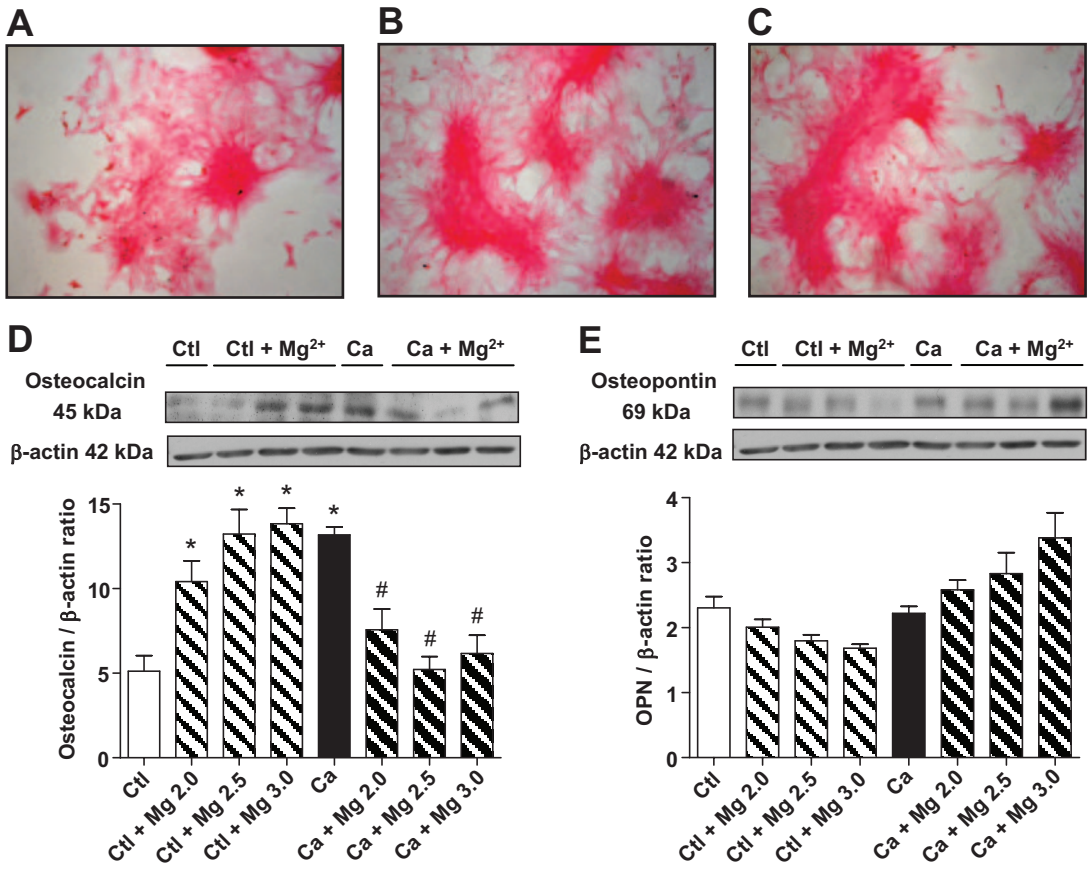

Figure 2. Magnesium attenuates VSMC mineralization effects of calcification medium. Treatment with magnesium concentration-dependently (2.0 to 3.0 mmol/L) reduced von Kossa–positive staining in VSMCs exposed to calcification medium (A through C). Black deposits indicate deposits of phosphate-containing mineral primarily in the extracellular regions (arrows). D demonstrates expression of osteocalcin in VSMCs grown in control (Ctl) and calcification (Ca) media in the absence and presence of magnesium (Mg) (2.0 to 3.0 mmol/L). E shows osteopontin expression in VSMCs grown in control (Ctl) and calcification (Ca) media in the absence and presence of magnesium (Mg) (2.0 to 3.0 mmol/L). Top, Representative immunoblots. Bottom, Corresponding bar graphs. Data are osteocalcin or osteopontin:β-actin ratio. Open bars represent VSMCs exposed to control medium and Mg^{2+}-enriched control medium (hashed lines). Closed bars represent VSMCs exposed to calcification medium and Mg^{2+}-enriched calcification medium (hashed lines). Results are means±SEM of 8 to 10 experiments. *$P<0.05$ vs control; #$P<0.01$ vs calcification group without magnesium.

Methods

An expanded Methods section is available in the online Data Supplement at http://hyper.ahajournals.org.

Cell Culture

VSMCs from WKY rats and from inbred mice (16 to 20 weeks old) selected for high-normal (MgH) or low (MgL) intracellular magnesium levels were investigated.[22,30,31]

In Vitro and Ex Vivo Calcification

Calcification of VSMCs was induced by high phosphate calcium–containing medium.[32] In some experiments, cells were exposed to calcification medium enriched with different concentrations of Mg^{2+}: 2.0, 2.5, and 3.0 mmol/L for 10 days in the absence and presence of the TRPM7 inhibitor 2-aminoethoxy-diphenylborate (2-APB). Isolated aortas from MgH and MgL mice were exposed to control or to calcification medium. Calcification was assessed by von Kossa staining.

Statistical Analysis

Experiments were repeated 8 to 10 times in duplicate. Data are expressed as means±SEM and were analyzed by ANOVA or by unpaired Student's t test as appropriate. $P<0.05$ was significant.

Results

Exposure of VSMCs to High Phosphate and Calcium Levels Induces Calcification

In VSMCs from WKY rats, Ca^{2+}-phosphate product accumulation was induced by calcification medium. von Kossa–positive staining for calcium was observed in only VSMCs exposed to calcification medium (Figure 1A and 1B). Calcification medium increased osteocalcin and Pit-I expression, osteogenic markers, compared with control conditions (Figure 1C and 1D, $P<0.05$). Expression of BPM-2 (Figure 1E) and BMP-4 (Figure 1F) was also increased by calcification medium. BMP-7 expression (Figure 1G) was decreased ($P<0.05$).

Magnesium Prevents VSMC-Induced Calcification

Magnesium dose-dependently reduced von Kossa–positive staining in VSMCs exposed to calcification medium (Figure 2A through 2C). Osteocalcin expression increase was also attenuated by magnesium (Figure 2D, $P<0.01$). The calcification process is an imbalance between production of proosteogenic factors and degradation of inhibitors of osteogenesis

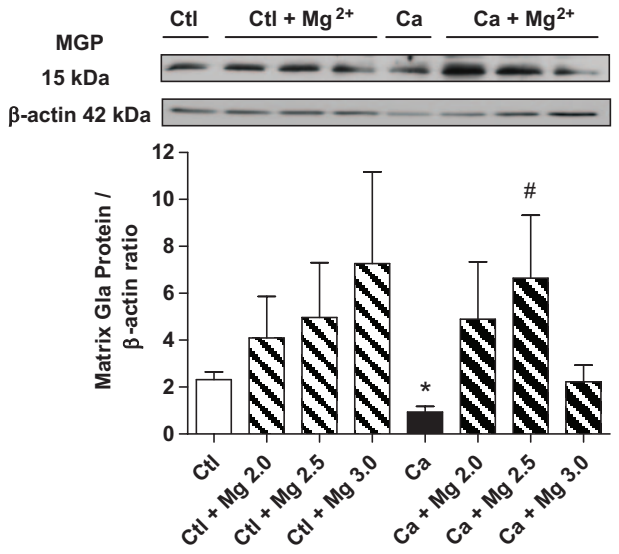

Figure 3. Effects of magnesium on the calcification inhibitor matrix Gla protein. VSMCs were exposed to calcification medium in the absence and presence of magnesium. Open bars represent VSMCs exposed to control medium and Mg^{2+}-enriched control medium (hashed lines). Closed bars represent VSMCs exposed to calcification medium and Mg^{2+}-enriched calcification medium (hashed lines). Data are presented as MGP:β-actin ratios. Results are means±SEM of 8 to 10 experiments. *$P<0.05$ vs control; #$P<0.05$ vs calcification group.

and calcium-phosphate product deposition. We assessed expression of osteopontin and matrix Gla-protein (MGP), anticalcification proteins, in VSMCs exposed to control and calcification medium in the presence or absence of magnesium. Osteopontin content was unchanged by the calcification medium compared with control (Figure 2E) but was increased by magnesium (3.0 mmol/L) ($P<0.05$). Matrix Gla protein expression was decreased by the calcification medium (Figure 3), an effect that was reversed by magnesium.

Figure 4 demonstrates effects of magnesium on expression of BMP-2 and BMP-7 in VSMCs exposed to calcification medium. High phosphate and calcium medium increased expression of the active form of BMP-2 (homodimer) (Figure 4A) and decreased expression of the proform and active form (reduced) of BMP-7 (Figure 4B, $P<0.05$), an effect reduced by magnesium. Exposure of VSMCs to the calcification medium also increased the precursor and mature form of BMP-4 (Figure S1 in the online Data Supplement), an effect reduced by magnesium treatment.

Calcification medium tended to increase Bax/Bcl-2 ratio, and cleaved caspase 3/caspase 3 ratios, but significance was not achieved. Mg^{2+} did not alter Bax/Bcl-2 or caspase 3 regulation (Figure S2).

Dysregulation of the Mg^{2+} Transporter TRPM7 Is Associated With Calcification

TRPM7 translocation from cytosol to membrane, an indirect measurement of transporter activity, was decreased by calcification medium compared with control conditions ($P<0.05$). Magnesium prevented the decrease in TRPM7 activity induced by calcification medium. The activity of TRPM7 was unchanged by magnesium in control conditions (Figure 5A). Despite changes in activity of the transporter, expression of TRPM7 was not affected by any of the treatments (Figure S3).

To better understand whether TRPM7 plays a protective role in vascular calcification, expression of osteocalcin, osteopontin, and matrix Gla protein was evaluated in VSMCs exposed to control and calcification medium containing 2-APB. As demonstrated in Figure 5B through 5D, treatment with the TRPM7 inhibitor recapitulated the calcification phenotype, where expression of osteocalcin was increased, whereas that of osteopontin and matrix Gla protein was decreased ($P<0.05$). TRPM7 inhibition blocked the protective effect of magnesium as seen in 2-APB–treated cells exposed to calcification medium plus magnesium (3.0 mmol/L) (Figure 6A and 6B, $P<0.05$).

VSMCs From MgL mice, but not MgH mice, Exhibit an Osteogenic Phenotype

TRPM7 content was reduced by calcification medium in VSMCs from MgL mice and MgH mice (Figure S4). In VSMCs from MgL mice, but not from MgH mice, calcification medium induced an increase in osteocalcin expression (Figure 7A), whereas osteopontin expression was increased in MgH VSMCs but not in MgL VSMCs (Figure 7B). Aortas from MgH and MgL mice did not exhibit calcium deposits when exposed to control medium. von Kossa–positive staining (Ca^{2+} deposits) was observed in all aorta sections (6 of 6) from MgL mice exposed to calcification medium (100%), whereas only 1 aortic section (1 of 5) was positive for Ca^{2+} deposits from MgH mice (20%) (Figure 8).

Discussion

Major findings from our study demonstrate that (1) a high phosphate/high calcium milieu induces osteogenic transformation of VSMCs as evidenced by increased calcification, upregulation of osteocalcin, BMP-2, BMP-4 and Pit-I, and downregulation of BMP-7; (2) magnesium dose-dependently attenuates VSMC calcification, an effect associated with increased expression of the anticalcification protein osteopontin and upregulation of the calcification inhibitor matrix Gla protein; (3) VSMC differentiation is associated with decreased activation of TRPM7, which is reversed by magnesium treatment; (4) TRPM7 inhibition in VSMCs recapitulates the phenotype of calcified VSMCs; and (5) VSMCs and intact vessels from MgH mice, but not from MgL mice, are protected against osteogenic transformation. These observations highlight the novel findings that magnesium has the potential to counteract molecular processes associated with vascular calcification and that the magnesium transporter TRPM7 may play a role in this process (Figure S5).

Arterial calcification is not a homogenous entity, but a complex manifestation influenced by derangements of calcium and phosphate homeostasis and by an imbalance between calcification inhibitors and promoters.[34] The in vitro model studied here has many characteristics of human vascular calcification in vivo. Of significance, the phosphate and

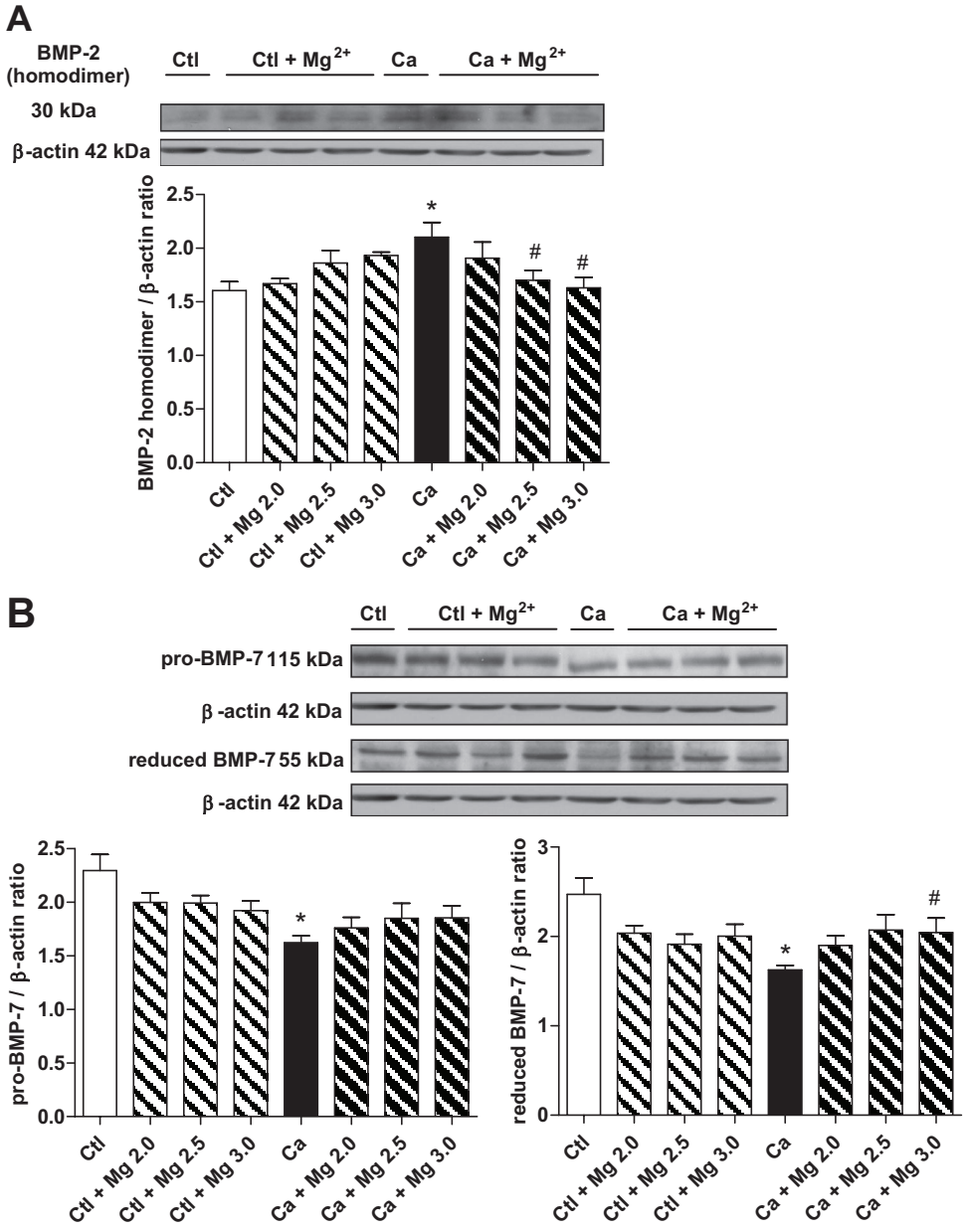

Figure 4. Effects of magnesium on expression of BMP-2 and BMP-7. VSMCs were exposed to calcification medium in the absence and presence of increasing concentrations of magnesium. High phosphate and high calcium medium increased expression of the active form of BMP-2 (homodimer) (A) and decreased expression of the proform and active form (reduced) of BMP-7 (B). Data are presented as BMP:β-actin ratios. Open bars represent VSMCs exposed to control medium and Mg^{2+}-enriched control medium (hashed lines). Closed bars represent VSMCs exposed to calcification medium and Mg^{2+}-enriched calcification medium (hashed lines). Results are means±SEM of 8 to 10 experiments. *$P<0.05$ vs control; #$P<0.05$ vs calcification group.

calcium concentrations in the calcification-inducing medium have clinical relevance because they are comparable to levels observed in patients with hyperphosphatemia or in patients on dialysis.[35] VSMCs exposed to calcification medium demonstrated granular deposits identified as phosphate-containing mineral by von Kossa staining, as well as increased expression of osteocalcin, BMP-2, and BMP-4, osteogenic proteins typically associated with osteoblastic differentiation and decreased expression of BMP-7 (also called osteogenic protein-1), which is important in bone metabolism and antifibrotic reactions. Moreover, expression of the sodium-dependent cotransporter Pit-I was increased in calcified VSMCs, as reported.[16,17] Osteoblastic markers are also elevated in the vasculature in experimental and clinical hypertension, diabetes and uremia as well as in patients with CKD.[36–40] Exposure of VSMCs to increasing concentrations of magnesium was associated with decreased calcification and reduced expression of osteogenic proteins, suggesting that magnesium inhibits calcification and osteoblast transformation of VSMCs. Interestingly, magnesium alone increased expression of osteocalcin without an effect on calcification but when combined with calcification medium, resulted in osteocalcin downregulation, suggesting that interactions between magnesium, calcium and phosphate may be important in the final

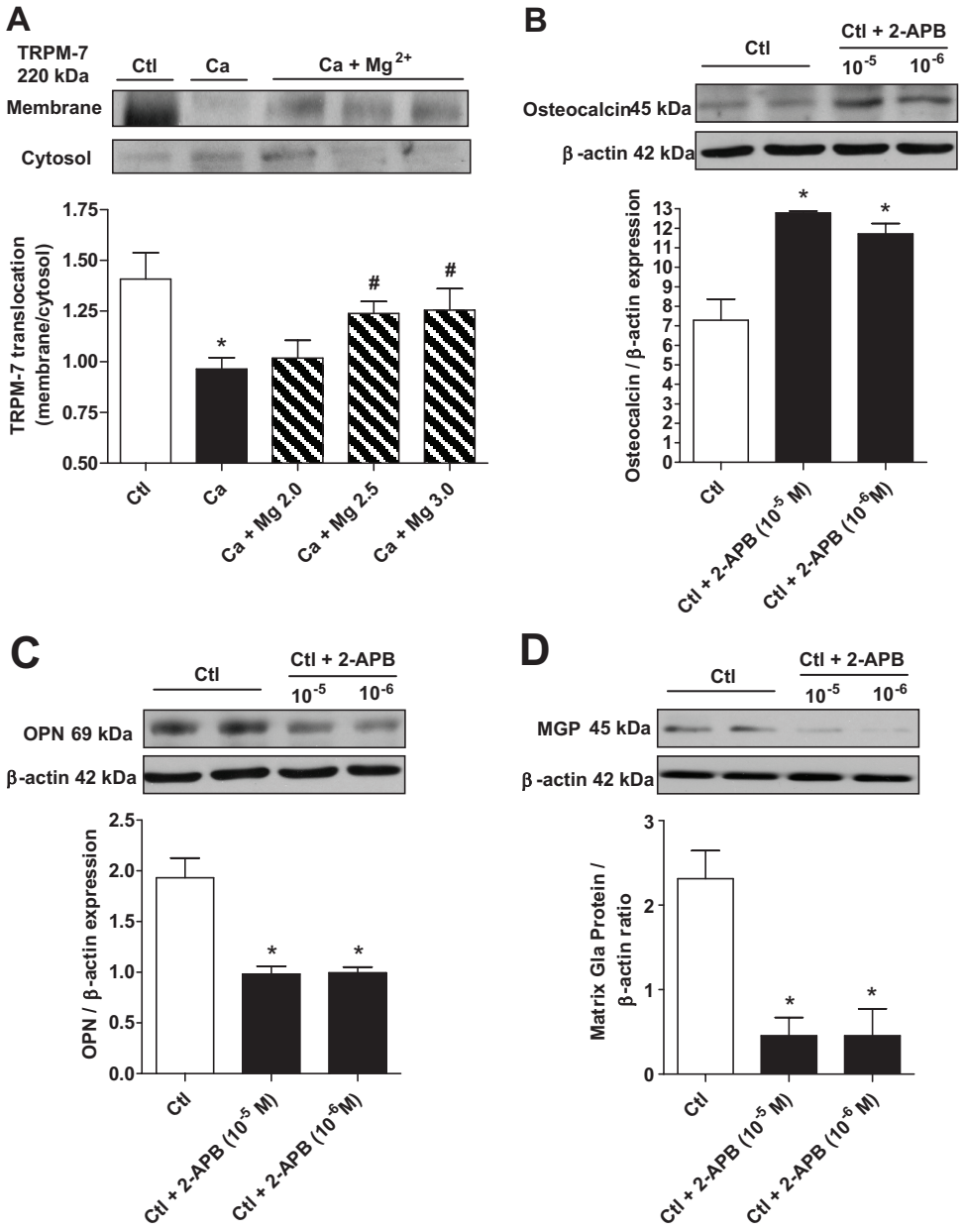

Figure 5. TRPM7 and calcification. A, TRPM7 activity in VSMCs as assessed by cytosol:membrane translocation. Top, Representative immunoblot of TRPM7 expression in cytosol and membrane fractions. Bottom, Corresponding bar graph. Data are presented as membrane TRPM7:cytosol TRPM7 content. Results are means±SEM of 8 to 10 experiments. **P*<0.05 vs control; #*P*<0.05 vs calcification group. B through D, Treatment with 2-APB recapitulated the calcification phenotype, where expression of osteocalcin was increased, whereas that of osteopontin and MGP was decreased in VSMCs grown in control (Ctl) medium. Top, Representative immunoblots. Bottom, Corresponding bar graphs. Open bars represent VSMCs exposed to control medium, whereas closed bars represent VSMCs exposed to calcification medium and Mg^{2+}-enriched calcification medium (hashed lines). Data are presented as osteogenic protein:β-actin ratios. Results are means±SEM of 8 experiments. **P*<0.05 vs control.

VSMC phenotype. The concentrations of magnesium used in our study, 1.0 to 3.0 mmol/L, have (patho)physiological relevance, because normal plasma levels range from 0.7 to 1.5 mmol/L and can be as high as 2.5 to 3.0 mmol/L in CKD.

Calcium phosphate crystals induce apoptosis, a process that has been implicated in the initiation of VSMC transformation to an osteoblastic phenotype.[41] However, unlike other studies, we did not find a significant effect of calcification medium on VSMC apoptosis, as assessed by Bax/Bcl and cleaved caspase3/caspase 3 ratios. Reasons for this may relate to differences in experimental protocols. Previous studies assessed direct effects of calcium phosphate crystals on VSMC growth/apoptosis and responses were investigated within 72 hours of crystal exposure.[41] In our studies, we did not treat VSMCs with crystals, and our studies were conducted over a longer time period.

Magnesium treatment was associated with an increase in osteopontin expression. Osteopontin, initially identified in osteoblasts as a mineralization-modulatory matrix protein, has now been identified to be multifunctional.[42] Although osteopontin is considered as a proinflammatory and proatherogenic molecule in inflammatory conditions, in vas-

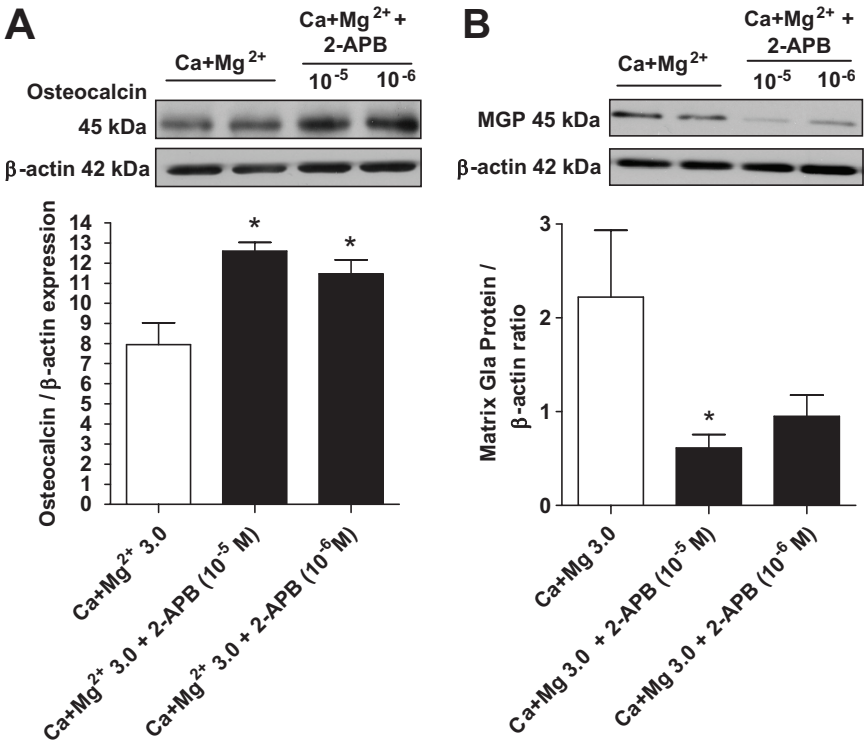

Figure 6. TRPM7 inhibition blocks the protective effect of magnesium. Expression of osteocalcin (A) was increased and that of MGP (B) was decreased in 2-APB–treated cells exposed to calcification medium (Ca) plus magnesium (3.0 mmol/L). Open bars represent VSMCs exposed to control medium, whereas closed bars represent VSMCs exposed to calcification medium. Data are normalized to β-actin content. Results are means±SEM of 8 experiments. *$P<0.05$ vs Ca+Mg group in the absence of 2-APB.

cular calcification, it acts as a negative regulator because it is an inhibitor of calcification and an active inducer of decalcification.[42] Hence, our findings suggest that magnesium may induce its protective effects, in part, by upregulating inhibitors of calcification, such as osteopontin and BMP-7. This was further confirmed by increased expression by magnesium treatment of matrix Gla protein, an endogenous calcification inhibitor.

The favorable anticalcification effects of magnesium are also observed in vivo. In a rat model of aortic transplantation, graft vessels exhibit massive media calcification and mineral accumulation.[43] This response was prevented by supplementation with magnesium, alkali citrate, and bases. In a model of nephrocalcinosis, oral administration of magnesium blunted progression of calcification.[44] Clinical studies have also shown protective anticalcification actions of magnesium.[45] In chronic dialysis patients, magnesium carbonate/calcium carbonate, used as a phosphate binder for 18 months, demonstrated a small change in calcium index.[46] Such observations have prompted an interest in using magnesium salts as phosphate binders, not only to treat hyperphosphatemia but also to inhibit development or progression of vascular calcification.[30,47–49] In further support of our thesis that magnesium protects against vascular calcification are the findings by Ishimura et al[50] that in nondiabetic hemodialysis patients, hypomagnesemia is associated with vascular calcification of hand arteries, independent of serum calcium and phosphate levels.

Exact mechanisms whereby magnesium interacts with calcium and other ions in the setting of high phosphate is complex, and we cannot rule out the possibility that there may be direct competition between magnesium and calcium for inorganic phosphate, which would reduce the calcium:phosphate interaction and hence alter the process of crystallization of hydroxyapatite. Peters and Epple demonstrated that the presence of additives, such as magnesium, distinctively alters the morphology of calcium:phosphate crystals in proatherosclerotic conditions.[51] von Kossa staining is not specific for calcium but is specific for anions of salt. Hence, although we cannot discern the exact composition of the crystals induced by calcification medium, it is clear that magnesium reduces the mineralization process in VSMCs. Future studies using spectroscopy or diffractometry will enable better characterization of the crystal composition.

To explore in greater detail putative molecular mechanisms underlying VSMC calcification in relation to magnesium-sensitive processes, the role of TRPM7 was probed. We focused on this chanzyme for three main reasons: (1) TRPM7 is functionally involved in magnesium homeostasis in VSMCs[33,52]; (2) TRPM7 is regulated by intracellular magnesium[24]; and (3) TRPM7 has recently been implicated to play an important role in osteoclast and osteoblast function.[53] Calcification of VSMCs was associated with decreased activity of TRPM7, as evidenced by decreased cytosol:membrane translocation. This may translate into decreased transmembrane magnesium transport. TRPM7 expression was unaltered by calcification medium, and magnesium treatment had no effect on TRPM7 content in calcified VSMCs. However, exposure of cells to increasing magnesium concentrations restored TRPM7 activity. Processes inducing blunting of TRPM7 activity are unclear but may relate to inhibitory actions of high calcium in the calcification medium, because TRPM7 is negatively regulated by high intracellular cation levels.[24] TRPM7 seems to be involved in the calcification/differentiation process, because 2-APB, which inhibits TRPM7

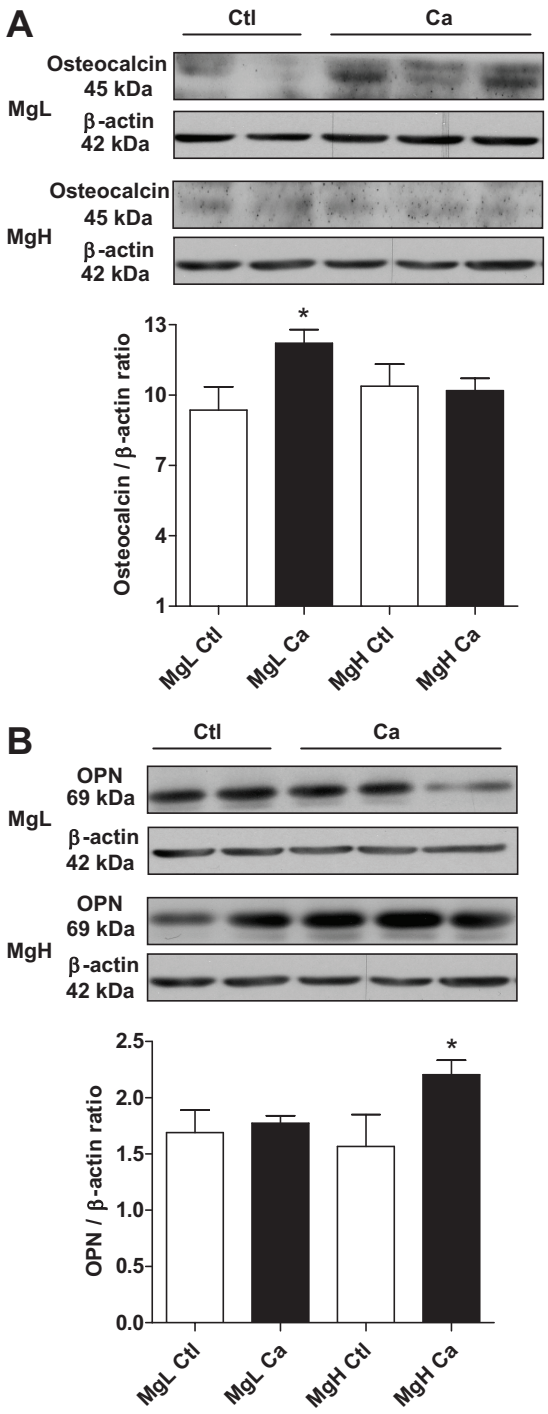

Figure 7. VSMCs from MgL mice, but not from MgH mice, exhibit an osteogenic phenotype by calcification medium. Expression of osteocalcin (A) and osteopontin (B) in VSMCs from MgL and MgH mice in the absence and presence of calcification medium (Ca) (10 days). Data are representative immunoblots, with corresponding bar graphs from 4 to 6 experiments. Open bars represent VSMCs exposed to control medium, whereas closed bars represent VSMCs exposed to calcification medium. Data are presented as protein:β-actin ratios. Results are means±SEM of 8 to 10 experiments. $^{**}P<0.05$ vs other groups.

activity and magnesium influx as we and others previously demonstrated,[21,54] recapitulated the osteoblast phenotype, without protective actions of magnesium treatment. This is evidenced by upregulation of osteocalcin and downregulation of osteopontin and matrix Gla protein in control conditions (without calcification medium), which mimicked effects in calcified VSMCs treated with magnesium. Our novel findings highlight the potentially important role of TRPM7 in calcification processes. Such phenomena extend beyond the vasculature, as recently shown in patients with osteoarthritis, where TRPM7 expression was found to be altered in articular chondrocytes.[55]

To examine the significance of our findings in an animal model of genetically low $[Mg^{2+}]_i$, we investigated VSMCs from MgL mice, which we previously characterized in detail.[30] These mice are hypomagnesemic, have increased blood pressure, and display endothelial dysfunction and vascular remodeling.[30] VSMCs from MgL mice, but not from MgH mice, when exposed to calcification medium, exhibit features of osteogenesis, as evidenced by increased osteocalcin, whereas VSMCs from MgH mice seem to be protected by showing increased expression of the antiosteogenic protein osteopontin. To further confirm the (patho)physiological significance of this, we examined effects of calcification medium on intact vessels from MgH and MgL mice. Similar to our findings in VSMCs, vessels from MgL were more susceptible to mineralization. Taken together, these findings suggest that VSMCs from mice that are hypomagnesemic (MgL) may be predisposed to osteogenic transformation, whereas VSMCs from mice with high-normal Mg^{2+} (MgH) may be protected from this process.

In summary, we demonstrate that high phosphate/calcium induces VSMC calcification and differentiation to an osteochondrogenic phenotype with associated decrease in TRPM7 activity. These processes were reversed by magnesium treatment. Blocking activity of TRPM7 with 2-APB recapitulated the osteogenic phenotype in VSMCs. Our findings suggest that calcification is associated with TRPM7 downregulation, and possibly associated decreased transcellular Mg^{2+} transport, an effect reversed by magnesium treatment. Our findings identify TRPM7 as a potentially new molecular player in VSMC calcification/osteogenic differentiation and suggest that magnesium, possibly through restoration of TRPM7 activity, may be a useful modality in the treatment of vascular calcification.

Perspectives

Identification of magnesium as a negative modulator of vascular calcification and the potentially important role of TRPM7 in processes associated with transformation of VSMCs to an osteogenic phenotype provide novel insights into molecular processes underlying vascular calcification. We elucidate some possibilities whereby magnesium may induce its protective actions: by increasing expression of anticalcification modulators, by counteracting calcium actions, and by restoring TRPM7 activity. These findings support the use of magnesium as a therapeutic strategy to prevent/ameliorate vascular calcification in patients with vascular disease.

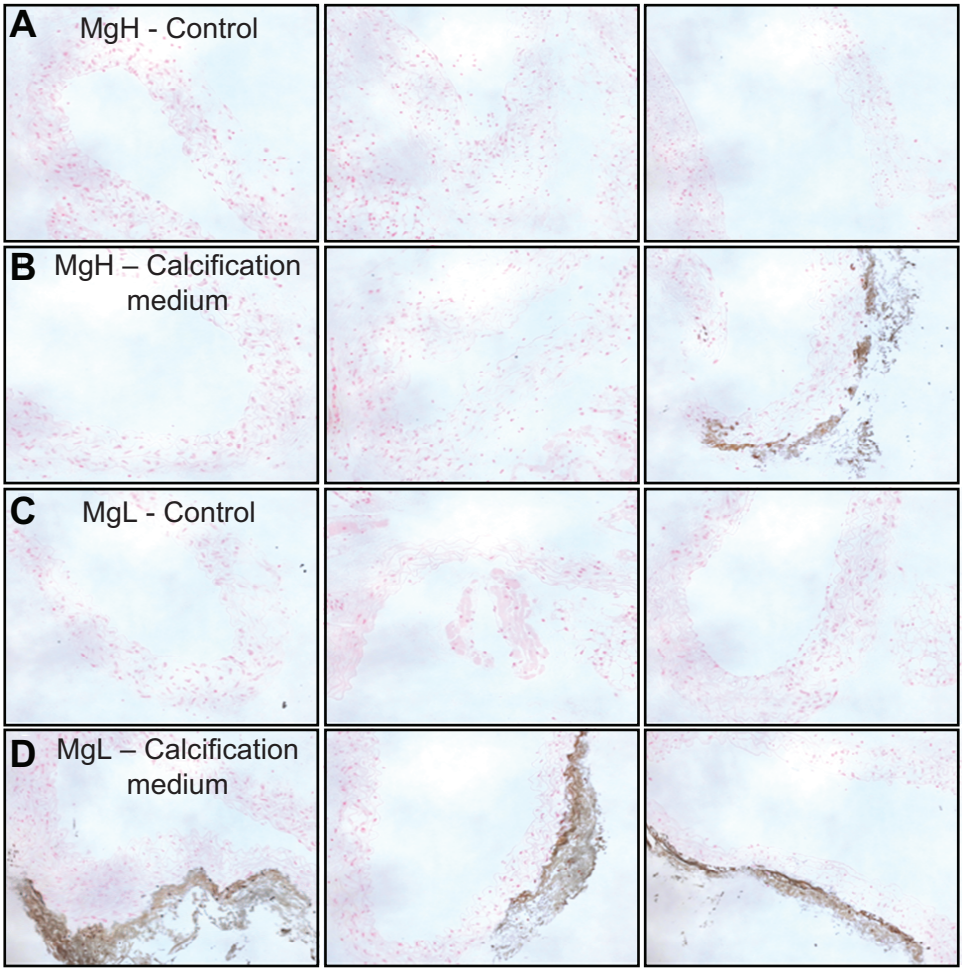

Figure 8. Effects of calcification medium on aortic sections from MgH and MgL mice. Aortas from MgH and MgL mice were extracted, and von Kossa staining was performed. Examples of aortas from 3 MgH (A) and 3 MgL (C) exposed to control medium or calcification medium (B, MgH; D, MgL) for 10 days are shown. Ca^{2+} deposits were observed in all the aortas from MgL mice exposed to calcification medium (6 of 6). Only 1 of 5 aortas from MgH was positive for Ca^{2+} deposits. Original magnification: ×100.

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Disclosures

None.

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Supplemental Material

Vascular Smooth Muscle Cell Differentiation to an Osteogenic Phenotype Involves TRPM7 - Modulation by Magnesium.

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Short title: Vascular calcification, TRPM7 and magnesium

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Methods

Cell Culture

This study was approved by the Animal Ethics Committee of the University of Ottawa and performed according to the recommendations of the Canadian Council for Animal Care. VSMCs derived from adult male WKY rats (16 weeks old) were studied. In addition VSMCs from inbred mice (16-20 weeks old) selected for normal-high (MgH) or low (MgL) intracellular magnesium levels were investigated (1,2). Mesenteric arteries were isolated and characterized as described in detail previously (3). Briefly, mesenteric beds were cleaned of adipose and connective tissue; VSMCs were dissociated by enzymatic digestion of vascular arcades for 60 minutes at 37°C. Cell suspension was centrifuged and resuspended in Dulbecco modified Eagle medium containing 10% fetal calf serum, 2 mmol/L glutamine, 20 mmol/L HEPES (pH 7.4), and antibiotics.

In vitro Calcification

Calcification of VSMCs was induced by high phosphate- and high calcium-containing medium (4). When confluent, DMEM was changed to calcification medium, comprising DMEM (high glucose, 4.5 g/l) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), 1.8 mmol/l $CaCl_2$, 1 mmol/l sodium pyruvate, 2 mmol/L of inorganic phosphate. The medium was replaced with fresh medium every 2 days for a total of 10 days. In a different set of experiments, cells were exposed to the calcification or control medium enriched with different concentrations of Mg^{2+}: 2.0, 2.5 and 3.0 mmol/L for 10 days. In some experiments VSMCs were exposed to the TRPM7 inhibitor 2-aminoethoxy-diphenylborate (2-APB) (10^{-6}, 10^{-5} mol/L). 2-APB was added to the medium during the last 3 days of incubation with control medium and the calcification medium containing 3.0 mmol/L of Mg^{2+}.

Von Kossa staining

VSMC seeded on coverslips or deparaffinized paraffin sections were used. After rinsing in several changes of distilled water, sections were incubated with 1% silver nitrate solution in clear glass placed under ultraviolet light for 20 minutes. Coverslips and/or slides were again rinsed in several changes of distilled water. Unreacted silver was removed with 5% sodium thiosulfate for 5 minutes. After washing with several changes of distilled water, coverslips and/or slides were counterstained with nuclear fast red for 5 minutes and prepared for microscopy (4,5).

Western Blotting

Proteins were extracted from VSMCs, separated by electrophoresis on a 10% polyacrylamide gel, and transferred onto a nitrocellulose membrane as previously described (6). Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween for 1 hour at 24°C. Membranes were then incubated with specific antibodies (1:1000) overnight at 4°C. Antibodies were as follows: anti-osteocalcin (Santa Cruz), anti-osteopontin (OPN) (Santa Cruz), anti-BMP-2 (Santa Cruz), anti-BMP-4 (Santa Cruz), anti-BMP-7 (Santa Cruz), matrix gla protein (Sant Cruz), Pit-I (Santa Cruz), anti-Bcl-2 (Santa Cruz), anti-Bax (Cell Signaling), anti-caspase 3/anti-cleaved caspase 3 (Cell Signaling) and anti-TRPM7 (from F. van Leeuwen Radboud University). After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography, and quantified densitometrically. Results

were normalized by the total protein and expressed as percentage of vehicle used in the experimental protocols.

Ex vivo vascular calcification

Under sterile conditions, aortas from MgH (5 animals) and MgL (6 animals) mice were gently stripped of excess adventitia and cut into 1-mm rings, as previously described (7). The aorta rings were placed in serum-free DMEM and incubated at 37°C in a 5% CO2 atmosphere with medium changes every 2 days. Two rings of each aorta from each animal were used. From those two rings, one ring was exposed to control medium (DMEM) and the other aorta ring was exposed to the calcification medium (DMEM supplemented with 1.8 mmol/l $CaCl_2$, 1 mmol/l sodium pyruvate, 2 mmol/L of inorganic phosphate). Aorta were divided in 4 groups: MgH exposed to control medium, MgH exposed to calcification medium, MgL exposed to control medium and MgL exposed to calcification medium. Aorta rings were incubated for 10 d in these culture media for all experiments. Calcification was detected by Von Kossa staining.

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Figure S1

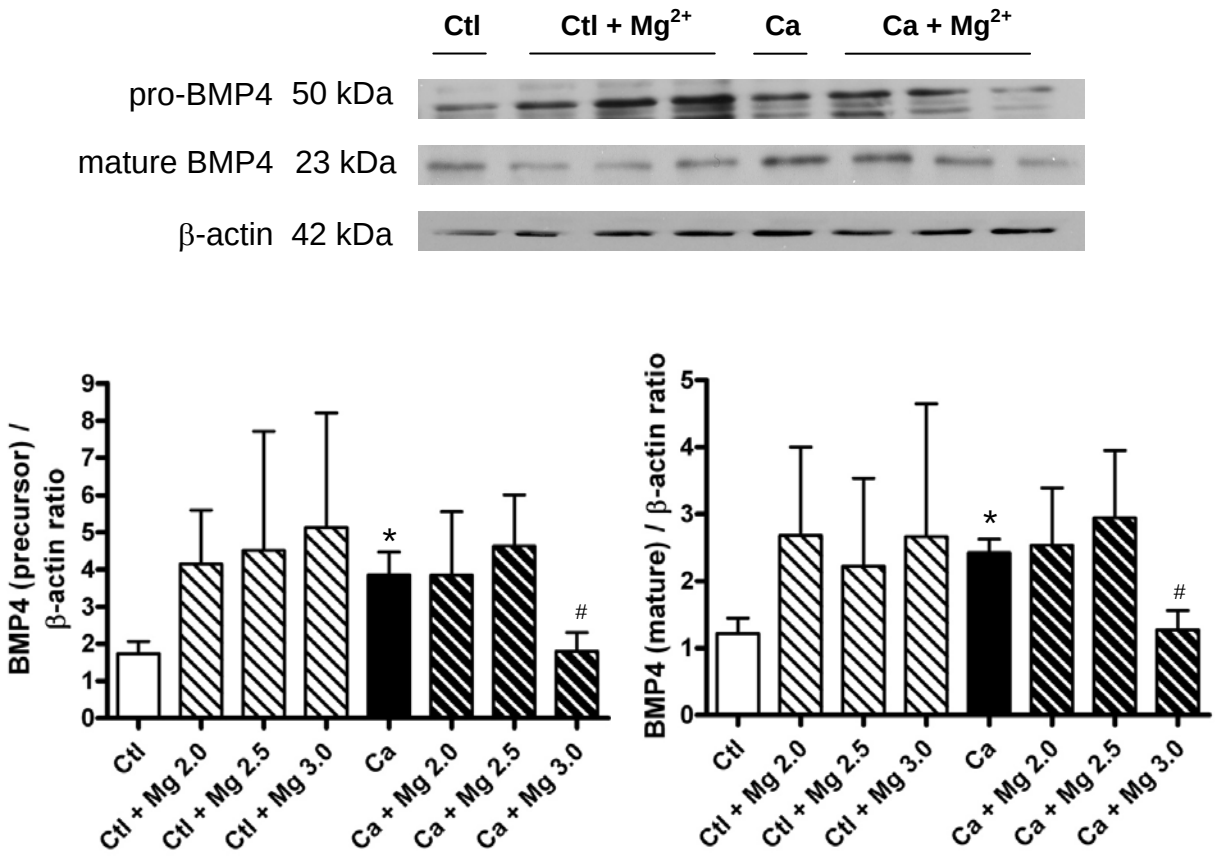

Figure S1. Effects of magnesium on expression of BMP-4 in VSMCs exposed to calcification medium. VSMCs were exposed to control and calcification medium in the absence and presence of increasing concentrations of magnesium. High phosphate and high calcium medium increased expression of BMP-4 (pre-cursor and mature forms). Open bars represent VSMCs exposed to control medium and Mg^{2+}-enriched control medium (hashed lines), whereas closed bars represent VSMCs exposed to calcification medium and Mg^{2+}-enriched calcification medium (hashed lines). Data are presented as BMP:β-actin ratio. Results are means ± SEM of 6-8 experiments. *$p<0.05$ vs control. # $p<0.05$ vs calcification group without magnesium.

Figure S2

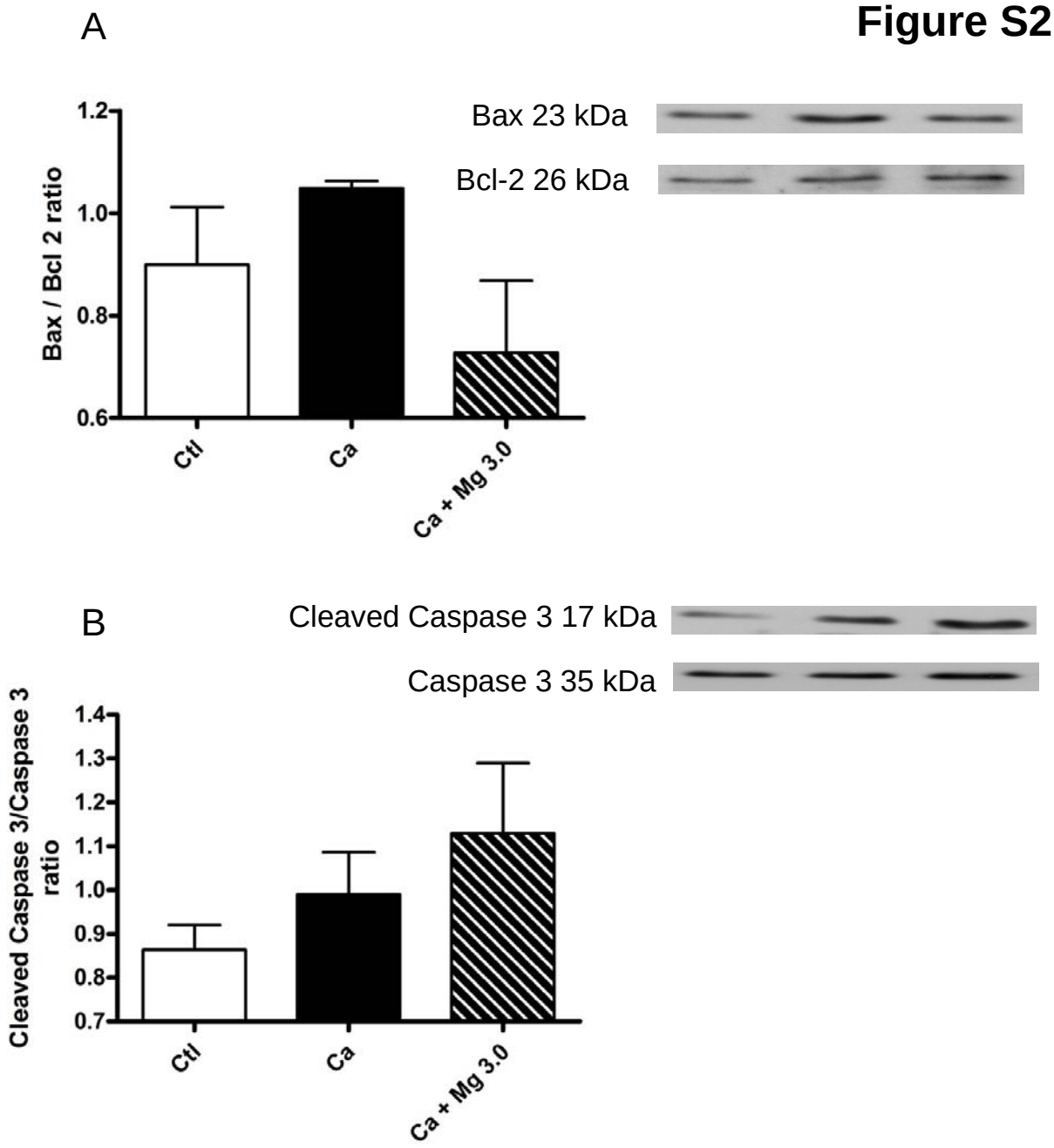

Figure S2 – Effects of calcification medium on VSMC apoptosis. Bax/Bcl-2 and cleaved caspase-3/caspase 3 ratios in VSMCs exposed to control, calcification and Mg^{2+}-enriched calcification medium. Calcification medium tended to increase Bax/Bcl-2 and cleaved caspase 3/caspase 3 ratios, but significance was not achieved. Mg^{2+} treatment tended to decrease Bax/Bcl-2, but not cleaved caspase 3/caspase 3 ratio. Open bars represent VSMCs exposed to control medium, closed bars represent VSMCs exposed to calcification medium and Mg^{2+}-enriched calcification medium (hashed lines). Data are representative immunoblots, with corresponding bar graphs from 4-6 experiments. Results are means ± SEM.

Figure S3

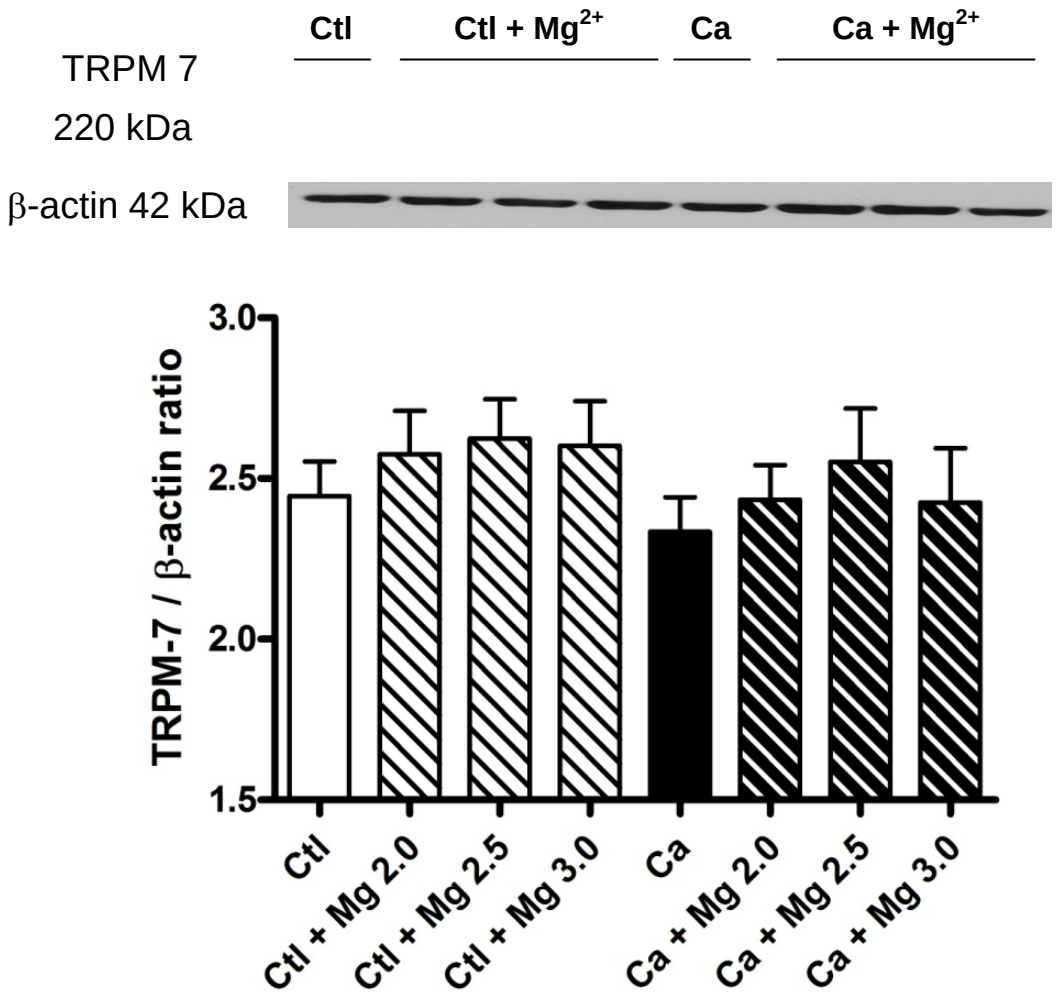

Figure S3. TRPM7 expression in VSMCs from WKY rats. TRPM7 expression in WKY VSMCs exposed to calcification medium in the absence and presence of increasing concentrations of magnesium. Calcification medium tended to decrease TRPM7 expression, but significance was not achieved. Open bars represent VSMCs exposed to control medium and Mg^{2+}-enriched control medium (hashed lines), whereas closed bars represent VSMCs exposed to calcification medium and Mg^{2+}-enriched calcification medium (hashed lines). Data are representative immunoblots, with corresponding bar graphs from 4-6 experiments. Results are means ± SEM.

Figure S4

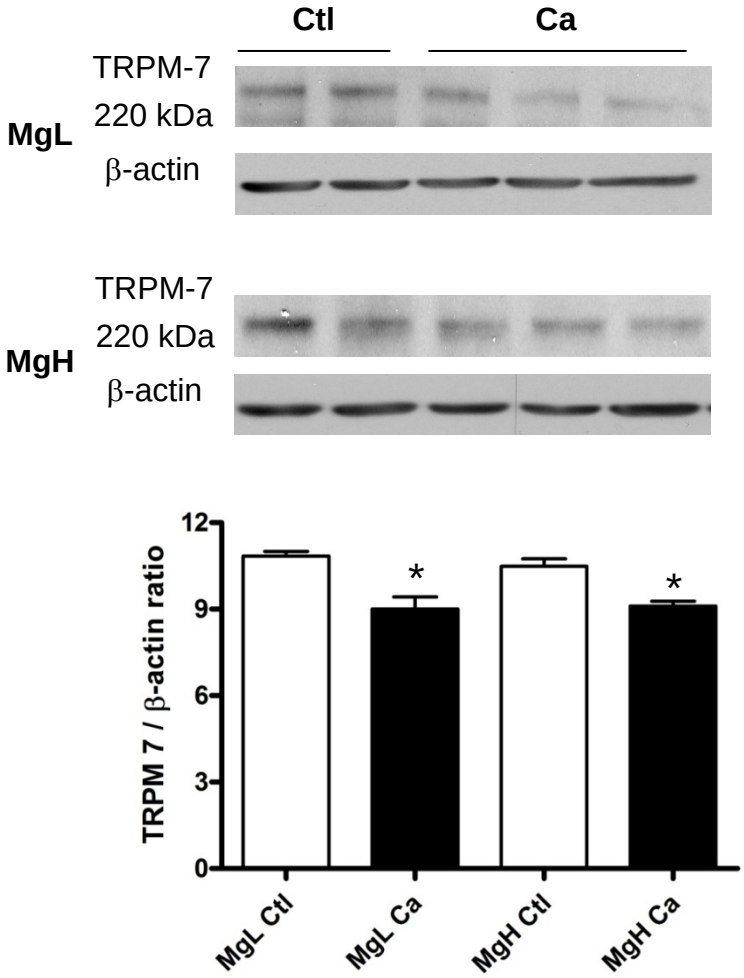

Figure S4. TRPM7 expression in VSMCs from MgH and MgL mice. TRPM7 content, evaluated in whole cell lysate, was reduced by calcification medium in VSMCs from MgL mice and MgH mice. Open bars represent VSMCs exposed to control medium and closed bars represent VSMCs exposed to calcification medium. Data are representative immunoblots, with corresponding bar graphs from 4-6 experiments. *$p<0.05$ vs Control (Ctl) groups.

Figure S5

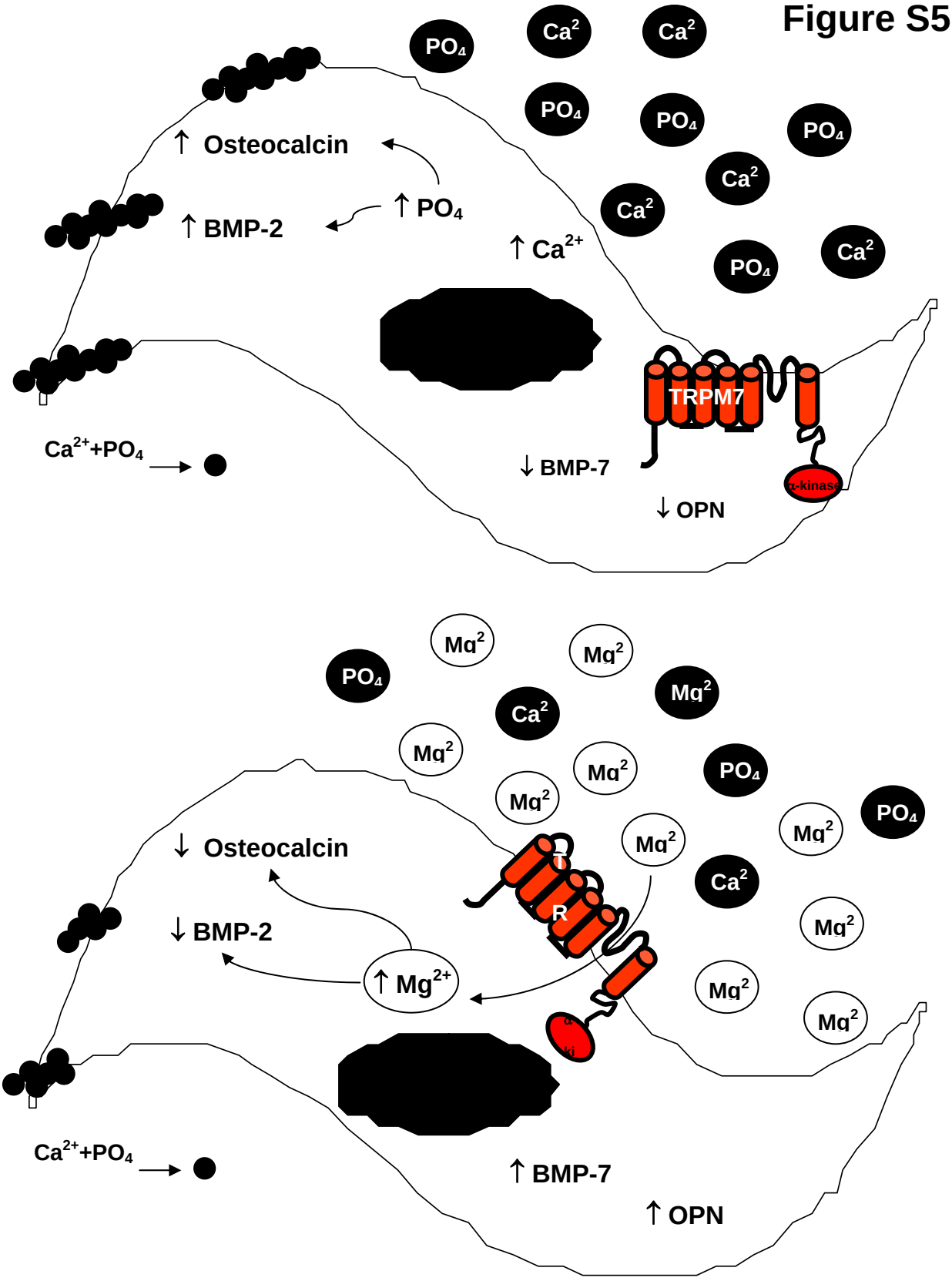

Figure S5. Possible mechanisms whereby magnesium and TRPM7 influence calcification and osteogenic transformation of VSMCs. In the presence of a high phosphate/calcium milieu, VSMC undergo calcification and exhibit an osteoblast-like phenotype, characterized by increased expression of osteocalcin and BMP-2 and decreased expression of osteopontin and BMP-7. These phenomena are coupled to decreased activity of TRPM7. In the presence of increased extracellular magnesium, TRPM7 activity is restored. This is associated with decreased calcification, reduced expression of osteogenic proteins and increased osteopontin (OPN) content.