Dysregulation of Vascular TRPM7 and Annexin-1 Is Associated With Endothelial Dysfunction in Inherited Hypomagnesemia

Tamara M. Paravicini, Alvaro Yogi, Andrzej Mazur, Rhian M. Touyz

Abstract—Inadequate magnesium intake and hypomagnesemia may contribute to chronic diseases, such as hypertension. The novel magnesium transporter TRPM7 is a critical regulator of magnesium homeostasis in vascular cells, but its role in pathophysiology is unclear. In a model of hypomagnesemia, we examined microvascular structure and function, TRPM7 expression, and vascular inflammatory status using inbred mice selected for normal-high intracellular magnesium levels or low intracellular magnesium levels (MgLs). Blood pressure was significantly increased in MgLs compared with normal-high intracellular magnesium levels. Pressurized myography of mesenteric resistance arteries showed that MgLs had significantly impaired endothelial function together with decreased plasma nitrate levels and endothelial NO synthase expression when compared with normal-high intracellular magnesium levels. Significant differences in vascular structure were also evident in both mesenteric arteries and aortas from MgLs. Aortas from MgLs had increased medial cross-sectional areas, whereas mesenteric arteries from MgLs had increased lumen diameters with increased medial cross-sectional areas, indicating outward hypertrophic remodeling. Expression of the magnesium transporter TRPM7 was significantly elevated in the vasculature of MgLs, whereas expression of a TRPM7 downstream target, the anti-inflammatory molecule annexin-1, was reduced. MgLs had increased expression of vascular cell adhesion molecule-1 and plasminogen activator inhibitor-1, indicating vascular inflammation. Taken together, these data demonstrate that the inherited magnesium status of MgLs and normal-high intracellular magnesium levels mice affects magnesium transporter expression, endothelial function, vascular structure, and inflammation. Our findings suggest a potential regulatory role for TRPM7 signaling in the maintenance of vascular integrity. Alterations in magnesium status and/or TRPM7 signaling may contribute to vascular injury in conditions associated with hypomagnesemia. (Hypertension. 2009;53[part 2]:423-429.)

Key Words: magnesium ■ TRPM7 ■ endothelial function ■ remodeling ■ hypertrophy

Magnesium, the second most abundant intracellular cation, is involved in many physiological processes regulating cardiovascular function. Magnesium influences vascular tone, vascular smooth muscle cell growth, inflammation, ion channel activity, and the production of vasoactive agents. Under normal physiological conditions, magnesium levels in serum are maintained within a narrow range (0.7 to 1.1 mmol/L), and whole body magnesium balance is tightly controlled by regulating gastrointestinal absorption and renal excretion. On the other hand, in pathological conditions, hypomagnesemia and decreased tissue content of magnesium have been reported in various chronic diseases, such as type 2 diabetes mellitus and hypertension. Several studies demonstrated that, in various experimental models of hypertension, magnesium supplementation attenuates the increase in blood pressure and ameliorates vascular damage. Epidemiological data indicate an inverse association between dietary magnesium intake and blood pressure in humans, whereas data from the National Health and Nutrition Examination study suggests that many American adults have an inadequate dietary magnesium intake.

Although magnesium is an abundant cytosolic cation important in many biological processes, little is known about the transport mechanisms that regulate its homeostasis, especially in vascular cells. Transporters and exchangers that have been implicated include the Na+/Mg2+ exchanger, Mg2+/Ca2+ exchanger, and, more recently, 2 novel ion channels of the transient receptor potential (TRP) cation channel superfamily, namely, TRPM6 and TRPM7, were identified as critical regulators of vertebrate intracellular magnesium levels. TRPM6 and TRPM7 are members of the newly described “chanzyme” family, composed of both a magnesium-

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permeable ion channel pore and an unique carboxy-terminal α-kinase domain, which may signal independently of the ion channel through its recently identified substrates annexin-1, calpain, and myosin II heavy chain. TRPM6 and TRPM7 are functionally nonredundant and have differing tissue distribution patterns. TRPM6 is expressed primarily in the cecum and kidney, specifically, the apical membrane of distal convoluted tubule cells, where it regulates transepithelial magnesium reabsorption. TRPM6 is considered to be crucial for magnesium homeostasis, with mutations in TRPM6 being the causative mutations in patients with autosomal recessive hypomagnesemia with secondary hypocalemia. In contrast, TRPM7 is ubiquitously expressed, and targeted deletion of TRPM7 is lethal, indicating its vital physiological role.

We showed that, in vascular cells, magnesium influx is driven mainly through TRPM7-sensitive pathways and that an altered cellular magnesium homeostasis and abnormal vascular smooth muscle cell function in hypertension may be, in part, related to defective TRPM7 expression/activity. To further explore the pathophysiological significance of these findings, we used a model of inherited hypomagnesemia to investigate the effects of chronic magnesium deficiency on microvascular structure and function, magnesium transporter (TRPM7) expression, and vascular inflammatory status. Using bidirectional selective breeding, mice from a heterogenous population were selected for low (MgL) and normal-high (MgH) levels of erythrocyte magnesium. The MgL mice demonstrate inherited hypomagnesemia with significant reductions in plasma, bone, and kidney magnesium levels.

**Methods**

For detailed methodology, please see the online data supplement, available at http://hyper.ahajournals.org.

**Animals**

Experiments in this study were approved by the University of Ottawa Animal Ethics Committee and performed according to the recommendations of the Canadian Council for Animal Care. Mouse colonies were selectively bred for low and normal-high erythrocyte magnesium concentrations. Colonies were subsequently maintained at the University of Ottawa and allowed standard rodent chow (Teklad Global 18% protein diet, Harland) and tap water ad libitum. Animals from matched age ranges (18±2 weeks) were used for this study.

**Blood Pressure**

Systolic blood pressure was measured via tail cuff plethysmography (BP-2000, Visitech Systems). Animals were trained to the system for 7 consecutive days, and measurements were recorded for 3 days after the acclimatization period.

**Plasma and Urine Analysis**

To measure electrolyte levels, spot urine and blood samples (via cardiac puncture) were collected immediately before sacrifice. Levels of electrolytes, Mg2+, Ca2+, and creatinine were determined using an automated analyzer (Synchron CX5 PRO, Beckman). To measure plasma nitrate, samples were filtered through 10 000 molecular weight cutoff filters before analyzing with a commercially available kit (Total Nitric Oxide, Assay Designs). Albuminuria (albumin/creatinine ratio) was measured with commercially available kits (Albuwell M and Creatinine Companion, Exocell).

**Myography**

Pressurized myography was used to measure microvascular function (contractility and endothelial function), structure (lumen diameter and medial cross-sectional area), and mechanics (distensibility). Second-order branches of mesenteric arteries (corresponding with resistance arteries) from MgLs and MgHs were cleaned of connective tissue and mounted in a pressurized myograph at 45 mm Hg. Vessel contractility was assessed by cumulative concentration-response curves to norepinephrine. Endothelium-dependent and -independent relaxations were assessed using acetylcholine and sodium nitroprusside, respectively. To measure microvascular structure and mechanics, vessels were superfused with Ca2+-free physiological salt solution containing 1 mmol/L of EGTA to remove intrinsic tone. In response to increasing intraluminal pressure (3 to 140 mm Hg), lumen diameter and vessel thickness were measured at 3 points along the vessel and medial cross-sectional area (CSA), and distensibility, circumferential stress, and strain were calculated as described previously.

**Histology**

Medial CSA, media:lumen ratio, and collagen deposition were examined in aortas from MgLs and MgHs. Aortic segments were fixed in methacarn (60% methanol, 30% chloroform, and 10% acetic acid) for 6 hours before paraffin embedding and sectioning (5 μm). Slides were stained with Sirius red and hematoxylin for the determination of collagen content and measurement of the media:lumen ratio and CSA.

**Analysis of TRPM7 Expression**

Quantitative real-time PCR (Taqman, Applied Biosystems) was used to measure the expression of TRPM7 mRNA in aortas from MgLs and MgHs. The expression of TRPM7 in the samples was interpolated from a standard curve (constructed from an independent sample of mouse kidney cDNA) and expressed relative to the housekeeping gene 18S.

**Cell Culture**

Endothelial cells (ECs) were isolated and cultured from MgL and MgH aortas to measure the expression of endothelial NO synthase (eNOS).

**Western Blotting**

Expression of eNOS, annexin-1, calpain, plasminogen activator inhibitor (PAI-1), and vascular cell adhesion molecule (VCAM) was measured by Western immunoblotting. Proteins extracted from frozen aortic tissue (20 μg) and ECs (30 μg) were separated using polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by incubating in 5% skim milk in Tris-buffered saline solution with Tween before incubating at 4°C overnight with primary antibodies to eNOS, calpain, annexin-1, PAI-1, or VCAM (1:500, Santa Cruz) diluted in Tris-buffered saline solution with Tween with 3% BSA. After washing, membranes were incubated for 1 hour with secondary antibody (1:1000) diluted in 5% milk in Tris-buffered saline solution with Tween before the development with chemiluminescence (PicoSignal West, Pierce). Membranes were stripped before reprobing with an antibody for GAPDH (ECs, 1:10 000, Chemicon) or β-actin (aortic homogenates, 1:20 000, Sigma) as an internal control. Densitometric analysis of the resulting bands was performed using ScionImage (National Institutes of Health).

**Statistics**

All of the data are presented as means±SEM. Groups were compared using the unpaired t test or 2-way ANOVA as appropriate, with significance taken at P<0.05.

**Results**

Blood Pressure, Plasma, and Urine Analysis

Systolic blood pressure, measured by tail-cuff plethysmography, was increased in MgLs compared with MgHs (116±2
versus 104±1 mm Hg; n=11 to 14; P<0.05). No significant differences between MgLs and MgHs were found in plasma electrolytes. In contrast, urinary magnesium (MgH: 3.46±0.39 versus MgL: 6.00±0.72 mmol Mg2+/mmol of creatinine; n=11 to 13; P<0.05) and potassium (MgH: 53.2±3.8 versus MgL: 80.4±5.4 mmol K+ /mmol of creatinine; n=11 to 13; P<0.05) concentrations were significantly increased in MgL mice compared with MgH mice. MgL mice exhibited some microalbuminuria as evidenced by the increased albumin:creatinine ratio (MgH: 2.62±0.23 mmol/L) than in MgH mice (4.44±0.61 mmol/L).

Microvascular Function

In mesenteric arteries, maximum contractile responses and sensitivity to norepinephrine were similar in MgL and MgH mice (pEC50=7.10±0.15 in MgH versus 6.78±0.24 in MgL; maximum response=97.2±2.0% in MgH and 99.9±1.9% in MgL; n=6; P<0.05). Urinary creatinine was slightly lower in MgL mice (2.62±0.23 mmol/L) than in MgH mice (4.44±0.61 mmol/L).

In response to increases in intraluminal pressure, arteries from MgL and MgH mice showed similar deformation relationships (Figure 4A), stress-pressure (Figure 4B), and stress-strain (Figure 4C). Both strains showed similar media:lumen ratios (Figure 3C) when compared with arteries from MgH mice. Increases in both lumen diameter (Figure 3A) and medial CSA (Figure 3B) were observed between MgL and MgH mice. However, arteries from MgLs showed significant impairment of endothelial function, as determined by responses to the vasodilator acetylcholine (pEC50=6.80±0.12 in MgH versus 6.89±0.41 in MgL; maximal relaxation=78.6±8.9% in MgH versus 37.4±16.2 in MgL; n=6; P<0.05; Figure 1B). This impairment was specific for endothelium-dependent relaxation, because arteries from MgL and MgH mice showed similar responsiveness to the NO donor sodium nitroprusside (pEC50=6.85±0.30 in MgH versus 6.67±0.46 in MgL; maximal relaxation=88.9±3.3% in MgH versus 79.0±7.3% in MgL; n=6; Figure 1C).

To further investigate the underlying mechanisms of this endothelial dysfunction, we examined plasma nitrate levels and eNOS expression. MgLs had reduced plasma nitrate levels when compared with MgHs (7.30±0.59 and 5.66±0.43 μmol/L in MgH and MgL, respectively; n=6; P<0.05). eNOS expression was significantly reduced in both cultured ECs and aortic homogenates from MgL mice (P<0.05; Figure 2).

Vascular Structure

Pressurized mesenteric arteries from MgLs had significant increases in both lumen diameter (Figure 3A) and medial CSA (Figure 3B) when compared with arteries from MgH. Both strains showed similar media:lumen ratios (Figure 3C). In response to increases in intraluminal pressure, arteries from MgL and MgH mice showed similar deformation (Figure 4A), stress-pressure (Figure 4B), and stress-strain relationships (Figure 4C).

**Figure 1.** Functional responses of pressurized mesenteric arteries from MgL and MgH mice in response to cumulative additions of (A) norepinephrine, (B) acetylcholine, and (C) sodium nitroprusside. Data in A are expressed as a percentage of the maximum contraction to high-potassium physiological salt solution. In B and C, relaxations were measured in vessels precontracted to ~70% of maximum with norepinephrine and responses to the vasodilator agonist expressed as a percentage of that precontraction. N=6; *P<0.05.

**Figure 2.** eNOS expression in (A) cultured ECs and (B) aortic homogenates from MgL and MgH mice. Top, Representative immunoblots and (bottom) the corresponding bar graphs of the above data expressed as relative optical density of the target protein normalized to GAPDH (A) and β-actin (B). N=4 to 5; *P<0.05.
Major findings from the present study demonstrate that relative to a
expression was reduced in aortas from MgL mice (expressed
increased (18-fold) when compared with MgH (Figure 6A).
expression was reduced in aortas from MgL mice (expressed
This is consistent with previous reports indicating that chronic magnesium deficiency is
It has been demonstrated that magnesium supplementation attenuates blood pressure increases in various
models of hypertension, including the stroke-prone spontaneously hypertensive rat, angiotensin II–induced hyperten-
and mineralocorticoid salt hypertension. Although the effects of magnesium on blood pressure seem to
be more rapid and pronounced in disease states such as hypertension, our data indicate that chronic magnesium
deficiency also influences blood pressure in normotensive animals.
Our data that mesenteric resistance arteries from MgL exhibit increased lumen diameter and medial CSA indicate an
outward hypertrophic remodeling in these animals. This is
supported in part by findings that long-term magnesium deprivation is associated with increased CSA in various blood vessels (mesenteric resistance artery, common carotid artery, and aorta), although no changes in lumen diameter were observed in these studies. Reasons for these discrepancies are still unclear, but one may argue that differences in the model being used (inherited hypomagnesemia versus dietary magnesium deprivation of adult animals) are likely to be a contributing factor. Alternatively, the remodeling seen in MgL mice may be a secondary response, because outward hypertrophic remodeling of the resistance vasculature has been shown to occur in both aging and in response to increased blood flow. Arterial remodeling is often considered an adaptive process to normalize vascular wall stress and maintain tissue perfusion and is associated with structural changes in the vessel wall in response to various pathophysiological conditions. The outward hypertrophic remodeling seen in the MgL mice may be such an adaptive response, because the stress-pressure and stress-strain response curves are similar in MgL and MgH mice. Furthermore, in our study, there were no differences in either arterial distensibility or aortic collagen deposition between MgL and MgH mice, indicating that the remodeling is occurring without overt alterations in vascular stiffness. Although we did not investigate the molecular mechanisms underlying the outward hypertrophic remodeling in MgL mice we speculate that mitogen-activated protein kinase signaling pathways are involved in many of the processes that contribute to vascular remodeling (eg, vascular smooth muscle cell hypertrophy, hyperplasia, and migration), and we have demonstrated previously that activation of mitogen-activated protein kinases in hypertension is enhanced by magnesium deficiency.

An intriguing finding is that endothelial function is significantly impaired in MgL mice, as indicated by the impaired endothelium-dependent vasodilatation. This seems to be mediated by a reduction in eNOS expression, although at this point we cannot rule out a role for increased reactive oxygen species production, because magnesium deficiency is known to increase oxidative stress. Studies from cultured cells support a role for magnesium in modulating the NO pathway, because it has been demonstrated that magnesium supplementation increases both eNOS expression and NO production in cultured cells. Conversely, magnesium deprivation in cultured ECs inhibits cell proliferation, increases the expression of the proinflammatory markers VCAM-1 and PAI-1, and induces a senescent phenotype.

Whole body magnesium balance is determined by the combination of gastrointestinal absorption and renal excretion, and both processes are tightly regulated to maintain magnesium homeostasis. In recent years, there has been significant progress in our understanding of the molecular mechanisms underlying magnesium transport with the discovery that TRPM6 and TRPM7 are critical magnesium transporters.
transporters. The primary mechanism underlying hypomagnesemia in the MgL mouse is unclear but may relate to increased renal magnesium wasting.17 This is supported by findings from the present study where we observed higher levels of magnesium in the urine of MgL mice. The molecular mechanisms underlying this magnesium wasting in MgL are yet to be fully elucidated; however, dysregulation of TRPM6 in both kidneys and cecum may be important.33 It is also possible that magnesium wasting and blood pressure elevation may be attributed, at least in part, to renal dysfunction in MgL, because these mice exhibited microalbuminuria (as evidenced by higher urinary albumin:creatinine ratios in MgL compared with MgH mice).

When we examined the expression of the Mg transporter TRPM7 in vascular tissue, we observed that aortas from MgL mice have a striking increase in TRPM7 mRNA levels compared with MgH mice. Previously, studies from our laboratory have demonstrated that TRPM7 is a critical regulator of magnesium influx and intracellular magnesium levels in vascular smooth muscle cells.15 Furthermore, TRPM7 expression can be modulated by vasoactive agents such as angiotensin II and is involved in mediating angiotensin II–induced growth of vascular smooth muscle cells.15 The increased TRPM7 expression shown in the present study may be a compensatory mechanism in response to hypomagnesemia and may contribute to the associated vascular hypertrophy.

To date, 3 substrates for the α-kinase domain of TRPM7 have been identified: annexin-1, calpain, and myosin II heavy chain.8–10 Annexin-1 is an endogenous modulator of inflammation that was originally identified as a mediator of glucocorticoid signaling. Anti-inflammatory actions of annexin-1 include the inhibition of the phospholipase A2/arachadonic acid cascade and reduced neutrophil-endothelial interactions.34 Angiotensin II activates annexin-1 in vascular smooth muscle cells, an effect that is blunted in cells obtained from hypertensive animals.16 In the present study, we demonstrated reduced expression of annexin-1 in aortas from MgL mice together with increased expression of the proinflammatory molecules VCAM-1 and PAI-1. Interestingly, the expression of annexin-1 does not parallel the changes seen in TRPM7 levels, as we observed previously in the kidneys of aldosterone-infused mice.35 This disconnect may relate to the tissues under examination (renal versus vascular) or may reflect the complicated relationship between the ion channel and the kinase domain. This relationship is yet to be fully characterized, although it has been shown that magnesium is required for kinase activity,36 which may be a contributing factor to the reduced levels of annexin-1 seen in the MgL mice.

In conclusion, data from the present study demonstrate that, in inherited hypomagnesemia, there is an increase in blood pressure, impaired endothelial function, outward hypertrophic remodeling of arteries, and vascular inflammation. We also report increased vascular TRPM7 expression and reduced annexin-1 expression. These novel findings suggest a potential regulatory role for TRPM7 in the vasculature, together with a protective effect of magnesium on vascular function and structure.

**Perspectives**

Inadequate magnesium intake and hypomagnesemia are widely prevalent in the community and may contribute to the pathophysiology of chronic diseases, such as hypertension. Our data demonstrate that magnesium plays an important role in the maintenance of vascular integrity, with hypomagnesemia exerting detrimental effects on endothelial function, vascular structure, and inflammation. We also demonstrate that the magnesium transporter TRPM7 is upregulated in hypomagnesemia, which may represent a compensatory response. Additional work is required to fully understand the relationships between the complex systems responsible for the regulation of magnesium homeostasis, the role of TRPM7 in vascular signaling, and their impact on the cardiovascular system. Such research has important consequences for understanding and estimating the clinical implications of hypomagnesemia for cardiovascular disease.

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Disclosures

None.

References

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ONLINE METHODS

DYSREGULATION OF VASCULAR TRPM7 AND ANNEXIN-1 IS ASSOCIATED WITH ENDOTHELIAL DYSFUNCTION IN INHERITED HYPOMAGNESEMIA

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Online supplement - methods

Myography
Mesenteric beds from MgH and MgL were excised and placed into ice-cold physiological salt solution (PSS, composition in mmol/L: NaCl 118, KCl 4.65, MgSO4 1.18, KH2PO4 1.18, CaCl2 2.5, NaHCO3 25 mM; glucose 5.5 mM, EDTA 0.026 mM). Second order branches of the mesenteric artery were cleaned of connective tissue and mounted in a pressurized myograph (Living Systems Instrumentation, VT). Vessels were pressurized to 45 mmHg, checked for leaks, and allowed to equilibrate for 1 hour before testing viability using high potassium PSS containing equimolar substitution of KCl for NaCl.
Vessel contractility was assessed by cumulative concentration-response curves to norepinephrine (NE, 0.001 – 10 µM). Endothelium-dependent and –independent relaxations were assessed using acetylcholine (ACh) and sodium nitroprusside (SNP) respectively. Vessels were contracted to ~ 70% of the maximum contraction to high potassium PSS (KPSSmax) with titrated concentrations of NE prior to addition of cumulative concentrations of ACh and SNP (0.001 – 100 µmol/L). Contractile responses were expressed as a percentage of KPSSmax, whilst relaxation responses were expressed as a percent relaxation of NE-induced precontraction. Non-linear regression (Prism, version 4.0) was used to determine the pEC50 and maximum response for each vessel.
To measure microvascular structure, vessels were superfused with Ca2+-free PSS containing 1 mmol/L EGTA to remove any intrinsic tone. In response to increasing intraluminal pressure (3 – 140 mmHg), lumen diameter and vessel thickness were measured at three points along the vessel. Media cross-sectional area (CSA), distensibility, circumferential stress and strain were calculated as described previously.1

Analysis of TRPM7 expression
TRPM7 expression was measured using quantitative real-time PCR. Total RNA was extracted from frozen aorta homogenates using Trizol (Invitrogen), reverse transcribed using random hexamers (Applied Biosystems), and 50 ng of the resulting cDNA was used for real-time PCR. Specific primers and FAM-labeled probes for TRPM7 were designed using Primer Express 3.0 and sequences from the NCBI database (NM_021450; forward primer 5'-CAATCCAATGTTGTTCCATCTCA; reverse primer 5'-TGTGTATGTGCATCTGTAGGGTCCCT; probe 5'-AGCAGGGTAATCTCCCCCGGG) so as to exclude amplification of genomic DNA. Commercially available primers and VIC-labelled probes (Applied Biosystems) were used for amplification of the housekeeping gene 18S. Expression of TRPM7 in the unknown samples was interpolated from a standard curve constructed from an independent sample of mouse kidney cDNA and expressed relative to 18S.

Endothelial cell culture
Aortic endothelial cells from MgH and MgL were isolated as previously described.2 Briefly, the aortas were removed, cleaned of periadventitial fat and connective tissue, opened longitudinally and cut into small pieces (1 to 3 mm). These pieces were placed with the intima side down on Matrigel-coated six-well plates. Matrigel was diluted in Dulbecco’s minimum essential medium (DMEM) supplemented with fetal bovine serum, endothelial cell growth supplement (3 mg/100mL, Sigma), heparin (10 U/mL final), penicillin/streptomycin (100 U/mL), l-glutamine
(1x final) and minimal essential amino acids (1x final). After 24 hours more medium was added and 7-10 days later the aortic pieces were removed and the endothelial cells that had migrated from the aortic segments were allowed to grow to confluence. ECs were characterized by positive immunostaining for von Willebrand factor (an EC marker) and the absence of immunostaining for smooth muscle α-actin. Low passage ECs grown in DMEM enriched medium were used in this study (passage 4 to 7).

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