

ORIGINAL RESEARCH

Magnesium Deficiency Causes a Reversible, Metabolic, Diastolic Cardiomyopathy

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BACKGROUND: Dietary Mg intake is associated with a decreased risk of developing heart failure, whereas low circulating Mg level is associated with increased cardiovascular mortality. We investigated whether Mg deficiency alone could cause cardiomyopathy.

METHODS AND RESULTS: C57BL/6J mice were fed with a low Mg (low-Mg, 15–30 mg/kg Mg) or a normal Mg (nl-Mg, 600 mg/kg Mg) diet for 6 weeks. To test reversibility, half of the low-Mg mice were fed then with nl-Mg diet for another 6 weeks. Low-Mg diet significantly decreased mouse serum Mg (0.38 ± 0.03 versus 1.14 ± 0.03 mmol/L for nl-Mg; $P < 0.0001$) with a reciprocal increase in serum Ca, K, and Na. Low-Mg mice exhibited impaired cardiac relaxation (ratio between mitral peak early filling velocity E and longitudinal tissue velocity of the mitral anterior annulus e, 21.1 ± 1.1 versus 15.4 ± 0.4 for nl-Mg; $P = 0.011$). Cellular ATP was decreased significantly in low-Mg hearts. The changes were accompanied by mitochondrial dysfunction with mitochondrial reactive oxygen species overproduction and membrane depolarization. cMyBPC (cardiac myosin-binding protein C) was S-glutathionylated in low-Mg mouse hearts. All these changes were normalized with Mg repletion. In vivo (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride treatment during low-Mg diet improved cardiac relaxation, increased ATP levels, and reduced S-glutathionylated cMyBPC.

CONCLUSIONS: Mg deficiency caused a reversible diastolic cardiomyopathy associated with mitochondrial dysfunction and oxidative modification of cMyBPC. In deficiency states, Mg supplementation may represent a novel treatment for diastolic heart failure.

Key Words: Ca handling ■ diastolic dysfunction ■ hypomagnesemia ■ mitochondrial dysfunction

In the United States, about 6.5 million people have heart failure, and the number is increasing yearly.¹ Half of the heart failure is heart failure with preserved ejection fraction (EF), and diastolic dysfunction is thought to be essential to the pathology. Cardiac diastolic dysfunction is characterized by the reduced ability of the left ventricle to relax and fill with blood adequately. Because of poor understanding of the underlying pathophysiology, there are no specific treatments currently for diastolic dysfunction.^{2–4} Previously, our group has studied hypertensive and diabetic mouse models of isolated diastolic dysfunction and heart failure with preserved EF and confirmed that

cardiac mitochondrial oxidative stress is central to diastolic dysfunction, the relaxation defect being caused by increased S-glutathionylation of cMyBPC (cardiac myosin-binding protein C).^{5–7}

Hypomagnesemia is increasingly common. As the fourth most abundant mineral and the second most abundant intracellular divalent cation, Mg is an essential element for cell functions, such as ATP production and protein synthesis.^{8,9} Mg has been reported to play critical roles in heart rhythm,^{10–12} muscle contraction,^{13,14} blood pressure,^{15,16} insulin/glucose metabolism,^{17,18} and bone integrity.^{19,20} Increased consumption of processed food, filtered/deionized drinking water, and crops grown

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CLINICAL PERSPECTIVE

What Is New?

- Hypomagnesemia is common in diabetes mellitus, and is associated with worsening heart failure symptoms; Mg supplementation can improve these symptoms.
- Herein, we show that severe hypomagnesemia caused a reversible diastolic cardiomyopathy.
- The diastolic cardiomyopathy was associated with mitochondrial dysfunction and responded to mitochondrial antioxidant therapy.

What Are the Clinical Implications?

- The causative relationship between hypomagnesemia and diastolic dysfunction may help explain previous epidemiological data linking heart failure and Mg.
- Mg repletion may help treat diastolic heart failure by improving mitochondrial function and reducing oxidative stress.

Nonstandard Abbreviations and Acronyms

cMyBPC	cardiac myosin-binding protein C
E/e'	ratio between mitral peak early filling velocity E and longitudinal tissue velocity of the mitral anterior annulus e
low-Mg	mice fed with low-Mg diet
low→nl-Mg	mice fed with low-Mg diet for 6 weeks and then normal Mg diet for another 6 weeks
mitoTEMPO	(2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride
nl-Mg	control mice fed with normal Mg diet
ROS	reactive oxygen species

in Mg-deficient soil has led to a significant decline of Mg intake in developed countries.^{21,22} Most of the North American population consumes 185 to 235 mg/d Mg, compared with 450 to 485 mg/d in ≈1900.²³ Dietary Mg intake is inversely correlated with the occurrence of metabolic diseases,^{24,25} such as diabetes mellitus (types 1, 2, and 3)^{26–28} and hypertension,^{29,30} that are high risk factors for diastolic dysfunction. Moreover, chronic diseases and medication can further decrease Mg absorption levels and cause hypomagnesemia (serum Mg concentration <0.8 mmol/L).

Mg deficiency is commonly observed in heart failure.^{31–33} Low serum Mg is a predictor for

cardiovascular and all-cause mortality³⁴ and is associated with unstable cardiac repolarization, contributing to sudden cardiac death.^{34–36} Mg supplementation has been shown to improve heart function and contribute to a decreased risk of developing heart failure.^{37,38} On the basis of these facts and our previous observation that Mg supplementation could improve cardiac relaxation in diabetes mellitus, we speculated that these clinical observations might be explained, in part, if Mg deficiency caused diastolic dysfunction and if this pathology were reversible with Mg.

METHODS

Any supporting data not available within the article are available from the corresponding author on reasonable request. Full description of methods is in Data S1.

Reagents

Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO), except as stated otherwise.

Study Approval

Animal care and interventions were undertaken in accordance with the National Institutes of Health *Guide for the Care and Use of Experimental Animals*, and the animal protocol (IACUC-2003-37940A) was approved by the Institutional Animal Care and Use Committees of the University of Minnesota.

Animal Groups

C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were randomly assigned to different groups at 10 weeks of age: (1) nl-Mg: control mice fed with a normal Mg diet (TD.94253, containing 600 mg/kg Mg; Envigo Teklad Diets, Madison, WI) for 6 weeks; (2) low-Mg: mice fed with a low-Mg diet (TD.93106, containing ≈15–30 mg/kg Mg; Envigo Teklad Diets) for 6 weeks; (3) low→nl-Mg: mice fed with the low-Mg diet for 6 weeks and then given the normal Mg diet for another 6 weeks. Distilled and deionized H₂O was given to all the mice to control any possible Mg intake from drinking water. In different experiments, different animal numbers (3–17 mice) were used for each group. The exact numbers are shown in figures or figure legends and Table in parentheses. The low-Mg diet was chosen on the basis of a previous study by Rude et al, showing that this diet caused significant hypomagnesemia.²⁰ Six of the low-Mg mice were also treated with (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (mitoTEMPO, IP injection, 1 mg/kg per day)

Table. Characteristics and Parameters of Mg Deficiency–Induced Changes in Low-Mg Mouse Hearts and Cells: Reintroduction of Mg Normalized the Impairments Caused by Mg Deficiency

Parameter	nl-Mg	Low-Mg	Low→nl-Mg
Cardiac function			
EF, %	52.0±1.7 (13)	42.3±1.4 (21)*	52.1±1.4 (18) [†]
E/e'	15.4±0.4 (5)	21.1±1.1 (17) [†]	15.3±0.7 (12) [†]
Volume, diastolic, μ L	66.0±3.2 (11)	45.5±4.8 (12)*	63.3±4.1 (6) [§]
LV mass Cor, mg	94.6±3.0 (5)	84.6±3.6 (6)	91.2±3.5 (10)
LVPW, diastolic, mm	0.77±0.05 (9)	0.77±0.03 (6)	0.81±0.02 (10)
LVPW, systolic, mm	1.02±0.04 (9)	0.95±0.03 (6)	1.10±0.04 (10)
LVAW, diastolic, mm	0.86±0.03 (5)	0.77±0.04 (6)	0.87±0.04 (10)
LVAW, systolic, mm	1.12±0.06 (5)	0.95±0.04 (6) [†]	1.21±0.06 (10) [†]
QTc, ms	46.0±1.2 (8)	55.1±0.8 (6)*	49.4±1.7 (8) [§]
Serum chemistry			
Mg, mmol/L	1.14±0.03 (13)	0.38±0.03 (12)*	1.10±0.04 (13) [†]
Ca, mmol/L	2.29±0.02 (13)	2.73±0.10 (12)*	2.36±0.03 (13) [†]
K, mmol/L	4.37±0.14 (13)	4.87±0.12 (12) [†]	4.11±0.12 (12) [†]
Na, mmol/L	144.3±3.7 (7)	152.8±0.9 (8) [†]	146.4±2.3 (8) [§]
Cellular parameters			
Mg, F/F ₀	6.57±0.08 (13)	2.90±0.02 (12)*	6.46±0.09 (13) [†]
Diastolic Ca, F/F ₀	1.28±0.03 (42)	1.19±0.04 (42)	1.25±0.09 (59)
ATP, μ mol/g heart tissue	2.7±0.2 (7)	1.2±0.2 (9)*	3.2±0.2 (8) [†]
Mitochondrial ROS, Δ MFI	232.4±17.3 (29)	432.2±24.8 (67)*	207.1±6.3 (118) [†]
Mitochondrial $\Delta\psi_m$, JC-1 red/green Δ MFI	0.76±0.04 (49)	0.37±0.01 (79)*	0.61±0.03 (70)

Data represent the mean±SEM values. Numbers in parentheses indicate the mouse or cell numbers tested for each group. The 2-tailed Student *t* test and 1-way ANOVA with Bonferroni post hoc tests for multiple group comparisons were used. E/e' indicates ratio between mitral peak early filling velocity E/longitudinal tissue velocity of the mitral anterior annulus e'; EF, ejection fraction; F/F₀, ratio of cell fluorescent intensity F/background intensity F₀; Low-Mg, mice fed with low-Mg diet; Low→nl-Mg, mice fed with low-Mg diet for 6 weeks and then normal Mg diet for another 6 weeks; LV Mass Cor, left ventricular mass corrected; LVAW, left ventricular anterior wall thickness; LVPW, left ventricular posterior wall thickness; nl-Mg, control mice fed with normal Mg diet; QTc, corrected intervals between the Q and T waves of the ECG; ROS, reactive oxygen species; $\Delta\psi_m$, mitochondrial membrane potential; and Δ MFI, difference of mean fluorescent intensity between cells and background in the same images.

**P*<0.01 vs nl-Mg.

[†]*P*<0.01 vs low-Mg.

[‡]*P*<0.05 vs nl-Mg.

[§]*P*<0.05 vs low-Mg.

for 2 weeks after 6-week low-Mg diet. Under general anesthesia with 2% isoflurane, the mouse heart was excised as the final step, and the heart was used for ventricular myocyte isolation, ATP tests, and protein lysates.

Echocardiographic Assessment

Noninvasive echocardiography was performed with a Vevo 2100 ultrasound system (VisualSonics, Toronto, Canada), as we have done previously.⁵ The ratio between mitral peak early filling velocity E/longitudinal tissue velocity of the mitral anterior annulus e' (E/e') was used to evaluate the diastolic function. Cardiac EF was used to evaluate the systolic function.

ECG Recording

One minute of ECG signals from limb lead II were averaged for the measurement of corrected intervals

between the Q and T waves of the ECG using the Mitchell formula.³⁹

Telemetry

Cardiac rhythm was monitored using telemetry devices. Briefly, mice were implanted with ETA-F10 transmitter (Data Science International, St. Paul, MN), as done previously.⁴⁰

Serum Ion Levels and Urine Mg levels

Ions were measured with the Beckman Coulter AU480 Chemistry analyzer (Veterinary Medical Center, Clinical Pathology Lab, University of Minnesota, St. Paul, MN) within 24 hours of sample collection.

Cellular ATP Measurements

ATP was measured with the EnzyLight ATP Assay Kit (BioAssay Systems, Hayward, CA) by following the

manufacturer's instructions with ≈ 20 to 30 mg frozen ventricles.

Isolation of Ventricular Cardiomyocytes

Ventricular cardiomyocytes were isolated, as described before,^{5,41,42} and suspended in standard Tyrode solution for experiments.

Cytosol Mg Levels

The cytosol level of free Mg was measured using a specific Mg probe, Mag-fluo-4 AM (10 $\mu\text{mol/L}$; Thermo Fisher Scientific, Eugene, OR) by fluorescence microscopy (Zeiss Axio Inverted Observer.Z1; Zeiss, Thornwood, NY), as described previously.⁵

Mitochondrial Reactive Oxygen Species and Mitochondrial Membrane Potential

Mitochondrial reactive oxygen species (ROS) and mitochondrial membrane potential were measured by confocal imaging with isolated cardiomyocytes, as described before.^{5,41–43}

Cellular Ca Changes

The mechanical properties of cardiomyocytes were assessed using an IonOptix system (IonOptix LLC, Milton, MA), and cellular Ca^{2+} levels and transients were monitored by Indo-1 fluorescence (2 $\mu\text{mol/L}$; Thermo Fisher Scientific), as done previously.^{5–7}

Western Blotting for Protein Levels

Heart ventricles were collected and processed for Western blotting. The mouse cMyBPC antibody was purchased from Santa Cruz Biotechnology (sc-137180; Dallas, TX), and the anti-glutathione antibody was purchased from Virogen (101-A100; Watertown, MA). GAPDH and β -actin (Abcam, ab9484 and ab8277, Cambridge, MA) were used as loading controls.

Statistical Analysis

Data are presented as mean \pm SEM. For the dot plots, the lines indicated the mean values, and the error bars indicated SEM values. GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA) was used for statistical analysis. The 2-tailed Student *t* test and 1-way ANOVA with Bonferroni post hoc tests for multiple group comparisons were used where appropriate. Figure S1 used the Kaplan-Meier plot for the survival analysis of mice with the nl-Mg and low-Mg diet, and the log-rank (Mantel-Cox) test was applied for comparison between the 2 groups. The 2-tailed paired Student *t* test was used when the same animals were tested before and after the mitoTEMPO treatment. $P < 0.05$ was considered statistically significant.

RESULTS

Changes of Serum and Urine Mg Under Low-Mg Diet and Mg Repletion

After 6 weeks of low-Mg diet, 31 of 48 male (64.6%) and 0 of 40 (0.0%) female mice survived. The Kaplan-Meier survival curve of the mice on the normal and low-Mg diet is shown in Figure S2. The log-rank (Mantel-Cox) test gave a $P < 0.0001$. It is unknown if a shorter treatment time would have been equally deleterious. On the basis of observation and ECG telemetry, the mice died of seizures, a known complication of hypomagnesemia.⁴⁴ We monitored the serum and urine Mg levels during the low-Mg diet and after Mg repletion (for 2 weeks). As shown in Figure 1A, significantly decreased serum Mg was observed after 1 week of low-Mg diet and reached a nadir after 2 weeks. The urine Mg levels reached a nadir after 1 week of low-Mg diet (Figure 1B). The full recovery of serum and urine Mg levels after Mg repletion with the nl-Mg diet took 1 and 2 weeks, respectively. Similar fast recovery of Mg levels has been observed in humans.⁴⁵

Mg Deficiency–Induced Cardiomyopathies

Echocardiography showed that low-Mg mice had impaired relaxation with decreased ratio between the early and late diastole tissue velocity of the mitral valve anterior annulus e' and a' (as shown in Figure 2A) and increased E/e' (Figure 2B and Table). In addition, decreased contractile function was observed in low-Mg mice (EF, $42.3 \pm 1.4\%$ versus $52.0 \pm 1.7\%$ of nl-Mg mice; $P = 0.0001$; Table). ECGs showed prolongation of the corrected intervals between the Q and T waves of the ECG (Figure 2C). The low \rightarrow nl-Mg mice showed fully recovered heart function (Table). This implied that the Mg deficiency–induced cardiomyopathy was reversible by Mg repletion.

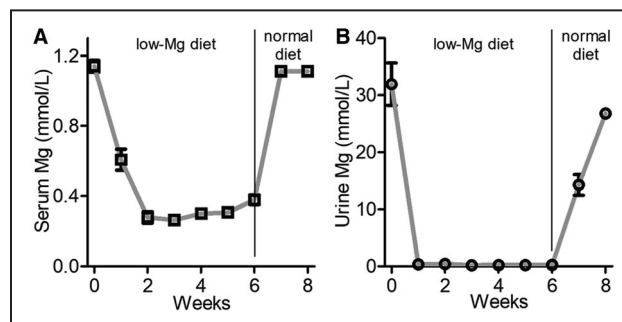


Figure 1. The time courses of Mg changes in mouse serum and urine under different diets.

The Mg levels in mouse serum (A) and urine (B) were decreased during 6 weeks of the low-Mg diet and recovered within 2 weeks of the normal Mg diet. A total of 3 to 13 mice were tested for each time point.

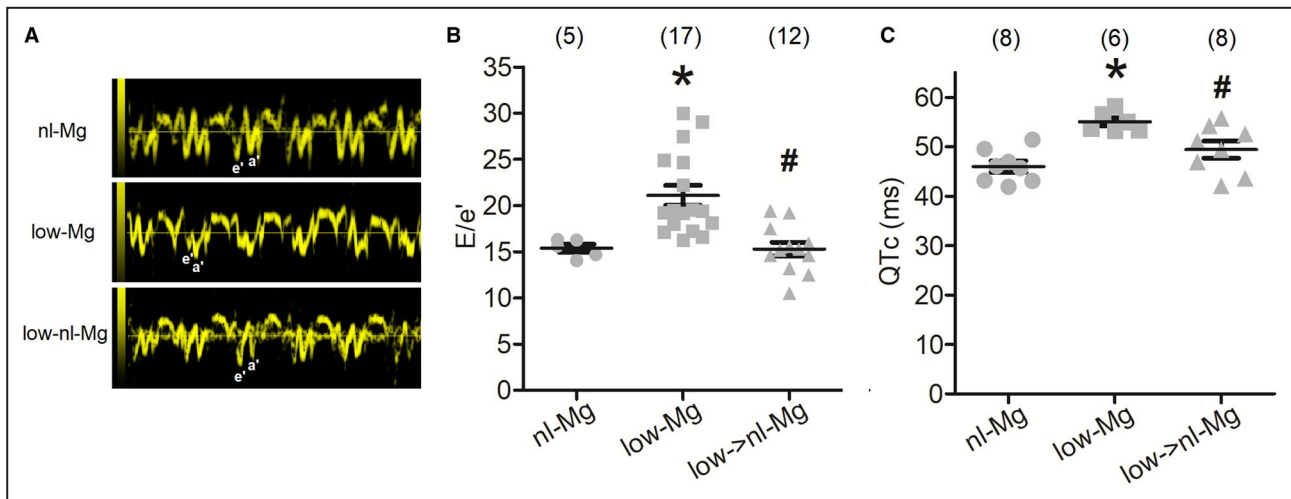


Figure 2. Mg deficiency impaired the cardiac functions with decreased ratio between the early and late diastole tissue velocity of the mitral valve anterior annulus e' and a' (e'/a') ratio (A), increased ratio between mitral peak early filling velocity E/longitudinal tissue velocity of the mitral anterior annulus e' (E/e') (B), and prolonged corrected intervals between the Q and T waves of the ECG (QTc interval) (C).

Mg repletion in mice fed with low-Mg diet for 6 weeks and then normal Mg diet for another 6 weeks (low->nl-Mg) normalized these changes. **A**, Representative images of tissue Doppler echocardiography showed decreased e'/a' ratio in low-Mg mice compared with normal Mg (nl-Mg) and low->nl-Mg mice. The mean and SEM (error bars; see Table) and the exact numbers of mice tested are shown in parentheses in each figure. The 2-tailed Student *t* test and 1-way ANOVA with Bonferroni post hoc tests for multiple group comparisons were used. **P*<0.05 vs nl-Mg; #*P*<0.05 vs low-Mg.

Serum and Cellular Ion Changes in Mg Deficiency

Compared with nl-Mg mice, serum Mg levels were significantly decreased in low-Mg mice (Table and Figure 3A), accompanied by increased serum Ca, K, and Na levels (Figure 3B through D). After 6-week low-Mg diet, the serum Mg level decreased to 0.38 ± 0.03 mmol/L, compared with 1.14 ± 0.03 mmol/L of nl-Mg mice. This serum Mg level corresponds to severe hypomagnesemia in humans, which is <0.5 mmol/L, and has been associated with use of proton pump inhibitors and chemotherapeutic regimens.^{46–48} A correlation between serum Mg and E/e' is shown in Figure S2. The linear regression fitting gave the following results: $r^2=0.3870$, $P=0.0077$, and the slope= -7.0 ± 2.3 per mmol/L serum Mg. The cellular Mg was also decreased in low-Mg mice ($P<0.0001$ versus nl-Mg; Table and Figure 3E). These parameters were fully restored to normal in low->nl-Mg mice. As we have observed before with diastolic dysfunction, the cellular diastolic Ca level was not affected by low-Mg diet (Figure 3F).⁵ The Ca transient peak time (the time from the start of Ca transient to the peak) and decay time (the time from peak to 10% of the baseline) were unaltered in low-Mg myocytes (Figure S3). Consistent with the systolic dysfunction (decreased EF), the cellular Ca transient amplitude was decreased, together with reduced sarcoplasmic reticulum Ca load and fractional release in low-Mg myocytes (Figure S3).

Cellular ATP and Mitochondrial Function

As shown in Figure 4 and Table, the cellular ATP level was significantly decreased in low-Mg mouse hearts (1.2 ± 0.2 versus 2.7 ± 0.2 $\mu\text{mol/g}$ heart tissue of nl-Mg; $P=0.0002$), indicating a dysregulated cellular energy metabolism. At the mitochondrial level, we observed significantly increased mitochondrial ROS and depolarized mitochondrial membrane potential in low-Mg mouse cardiomyocytes. Cellular ATP and mitochondrial ROS were fully recovered in low->nl-Mg myocytes, and mitochondrial membrane potential was partially repolarized. This change in mitochondrial ROS production was not secondary to antioxidant manganese superoxide dismutase or catalase, which were unaltered in low-Mg hearts (1.07 ± 0.14 -fold and 0.96 ± 0.09 -fold of nl-Mg, respectively; $P>0.05$ versus nl-Mg; Figure S4).

Possible Mechanisms for the Mg Deficiency-Induced Diastolic Dysfunction

Mg deficiency-induced diastolic dysfunction is associated with impaired sarcomere relaxation and S-glutathionylated cMyBPC.^{6,7} Herein, we observed significant elevation of S-glutathionylated cMyBPC in low-Mg heart tissue (1.4 ± 0.2 -fold; $P=0.020$ versus nl-Mg; Figure 5), consistent with increased oxidative stress and diastolic dysfunction in low-Mg mice (Figure 2). These protein changes were reversed in low->nl-Mg mouse hearts ($P=0.038$ versus low-Mg).

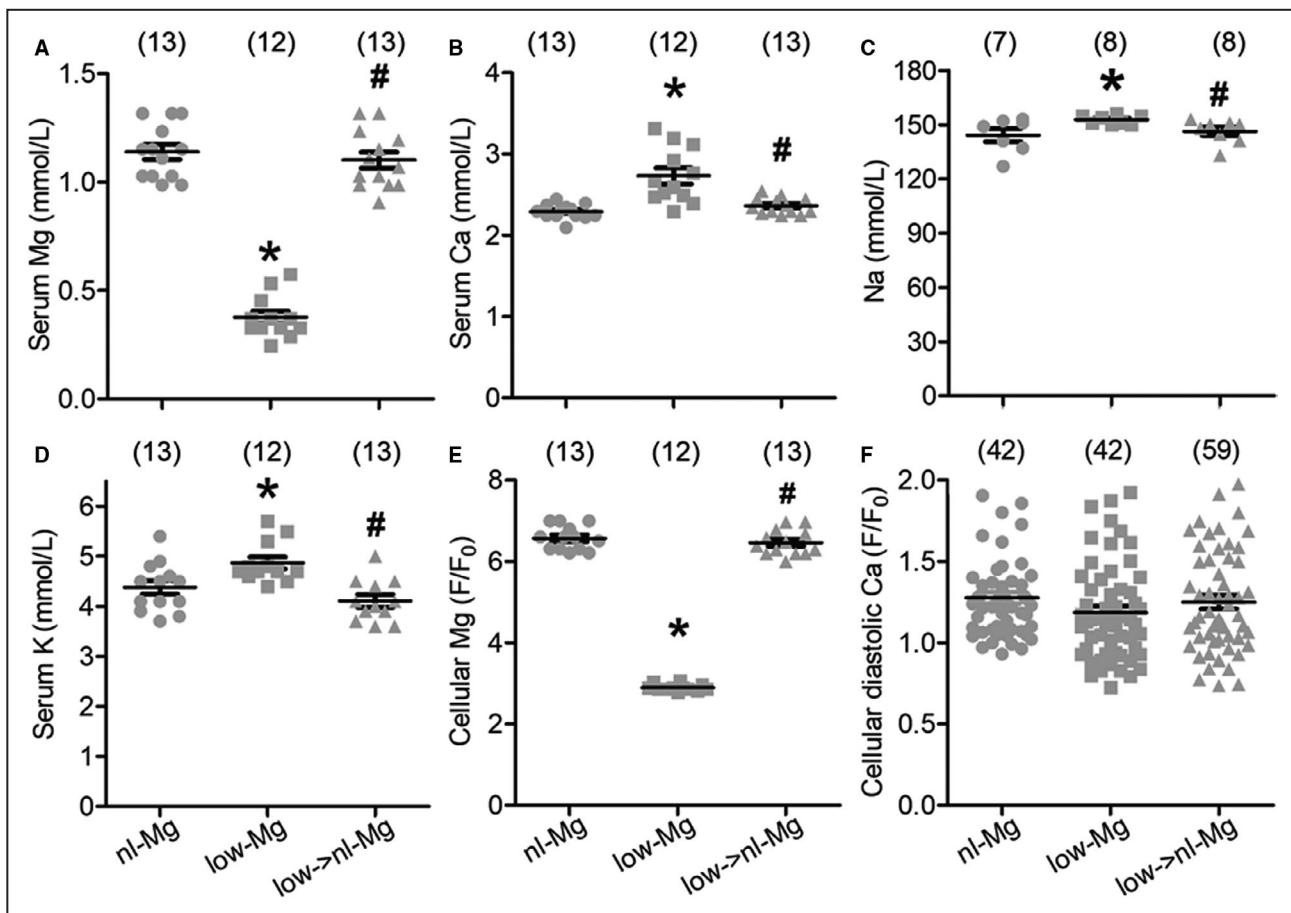


Figure 3. Serum ion levels and the cellular Mg level were altered by Mg deficiency.

The serum chemistry (A through D: serum Mg, Ca, Na, and K) and cellular Mg (E) levels were altered by Mg deficiency in low-Mg mice and normalized by Mg replenition in mice fed with low-Mg diet for 6 weeks and then normal Mg diet for another 6 weeks (low->nl-Mg). F, The cellular diastolic Ca levels were unaltered under these conditions. The mean and SEM (error bars; see Table) and the exact numbers of mice or cardiomyocytes tested are shown in parentheses in each figure. The 2-tailed Student *t* test and 1-way ANOVA with Bonferroni post hoc tests for multiple group comparisons were used. **P*<0.05 vs normal Mg (nl-Mg); #*P*<0.05 vs low-Mg. F/F₀ indicates ratio of cell fluorescent intensity F/background intensity F₀.

mitoTEMPO Improved Cardiac Diastolic Function

Previously, we have reported that a mitochondrial-specific ROS scavenger, mitoTEMPO, improves cardiac diastolic function.⁶ Herein, we treated low-Mg mice with mitoTEMPO (IP injection, 1 mg/kg per day) for 2 weeks after a 6-week low-Mg diet and observed significant improvement in diastolic function. Figure 6A showed representative tissue Doppler traces with *e'* and *a'* waves. Figure 6B showed decreased *E/e'* ratio after mitoTEMPO treatment (16.7±2.0 after treatment versus 23.2±2.0 before treatment; *P*=0.046) determined by echocardiography. Cellular ATP levels were significantly increased by mitoTEMPO treatment (4.4±0.6 μmol/g heart weight versus 1.2±0.2 μmol/g heart weight of low-Mg mouse hearts; *P*<0.0001; Figure 6C). The increased S-glutathionylated cMyBPC in low-Mg mouse hearts was decreased by mitoTEMPO

treatment from 1.6±0.1-fold of the nl-Mg group to 1.2±0.1-fold (*P*=0.007 versus low-Mg and *P*=0.25 versus nl-Mg), as shown in Figure 6D and E.

DISCUSSION

Hypomagnesemia has been associated with heart failure, but this has generally been assumed to be the result of a systolic cardiomyopathy.⁴⁹ Nevertheless, we have shown that Mg supplementation can reverse diastolic heart failure caused by diabetes mellitus.⁵ Therefore, we tested whether hypomagnesemia alone could cause diastolic dysfunction that might contribute to the clinical syndrome of heart failure. After 6 weeks of low-Mg diet, mice had severe hypomagnesemia and showed cardiac diastolic dysfunction, accompanied by systolic dysfunction, disturbed cellular Mg and Ca homeostasis, mitochondrial dysfunction,

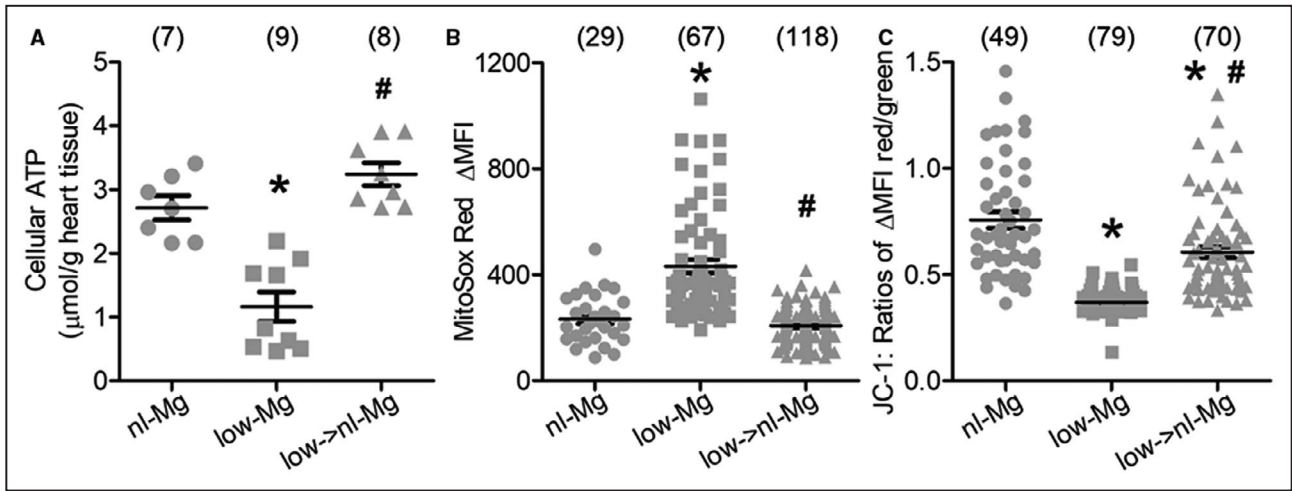


Figure 4. The mitochondrial function was altered in low-Mg hearts, as shown in decreased cellular ATP levels (A), increased mitochondrial reactive oxygen species production (B), and depolarized mitochondrial membrane potential (C).

All 3 parameters were normalized by Mg repletion in mice fed with low-Mg diet for 6 weeks and then normal Mg diet for another 6 weeks (low→nl-Mg). The mean and SEM (error bars; see Table) and the exact numbers of mice (A) or cardiomyocytes (B and C) tested are shown in parentheses in each figure. The 2-tailed Student *t* test and 1-way ANOVA with Bonferroni post hoc tests for multiple group comparisons were used. **P*<0.01 vs normal Mg (nl-Mg); #*P*<0.001 vs low-Mg. ΔMFI indicates difference of mean fluorescent intensity between cells and background in the same images.

altered cellular energy metabolism, and increased S-glutathionylated cMyBPC. Mg repletion was able to reverse these changes and improve diastolic function, suggesting that cardiac structural changes were unlikely to explain the cardiomyopathy. In agreement with our previous study,⁶ the diastolic myopathy was reversible by inhibition of mitochondrial oxidative stress. A scheme of these mechanism changes is shown in Figure 7. The effect of low Mg on female heart function

was prevented by premature death and remains to be determined.

Mg deficiency has been associated with oxidative stress in diabetes mellitus, hypertension, Alzheimer disease, and Parkinson disease.^{5,50–52} In cardiomyocytes, intracellular Mg concentration is normally tightly maintained (free ionized Mg, 0.8–1.0 mmol/L).^{53,54} In our study, the cellular Mg level was significantly decreased in cardiomyocytes isolated from low-Mg

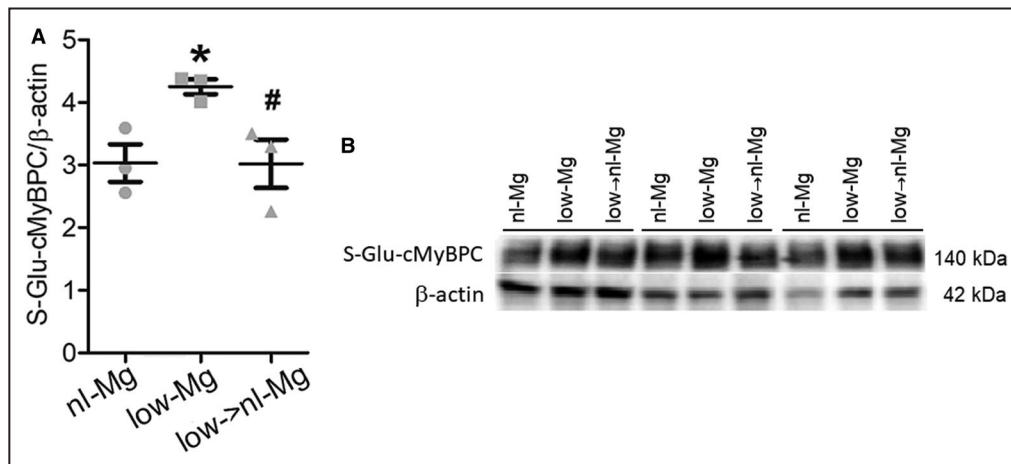


Figure 5. Mg deficiency increased the S-glutathionylation of cMyBPC (cardiac myosin-binding protein C) (S-Glu-cMyBPC), a marker protein of diastolic function, which was normalized in mice fed with low-Mg diet for 6 weeks and then normal Mg diet for another 6 weeks (low→nl-Mg) mouse hearts.

A, The protein levels of S-Glu-cMyBPC were normalized by the loading control protein, β-actin. B, Images of protein bands were obtained with 3 hearts from each group by Western blotting. The mean and SEM (error bars) values are shown. The 2-tailed Student *t* test and 1-way ANOVA with Bonferroni post hoc tests for multiple group comparisons were used. **P*=0.020 vs normal Mg (nl-Mg); #*P*=0.038 vs low-Mg.

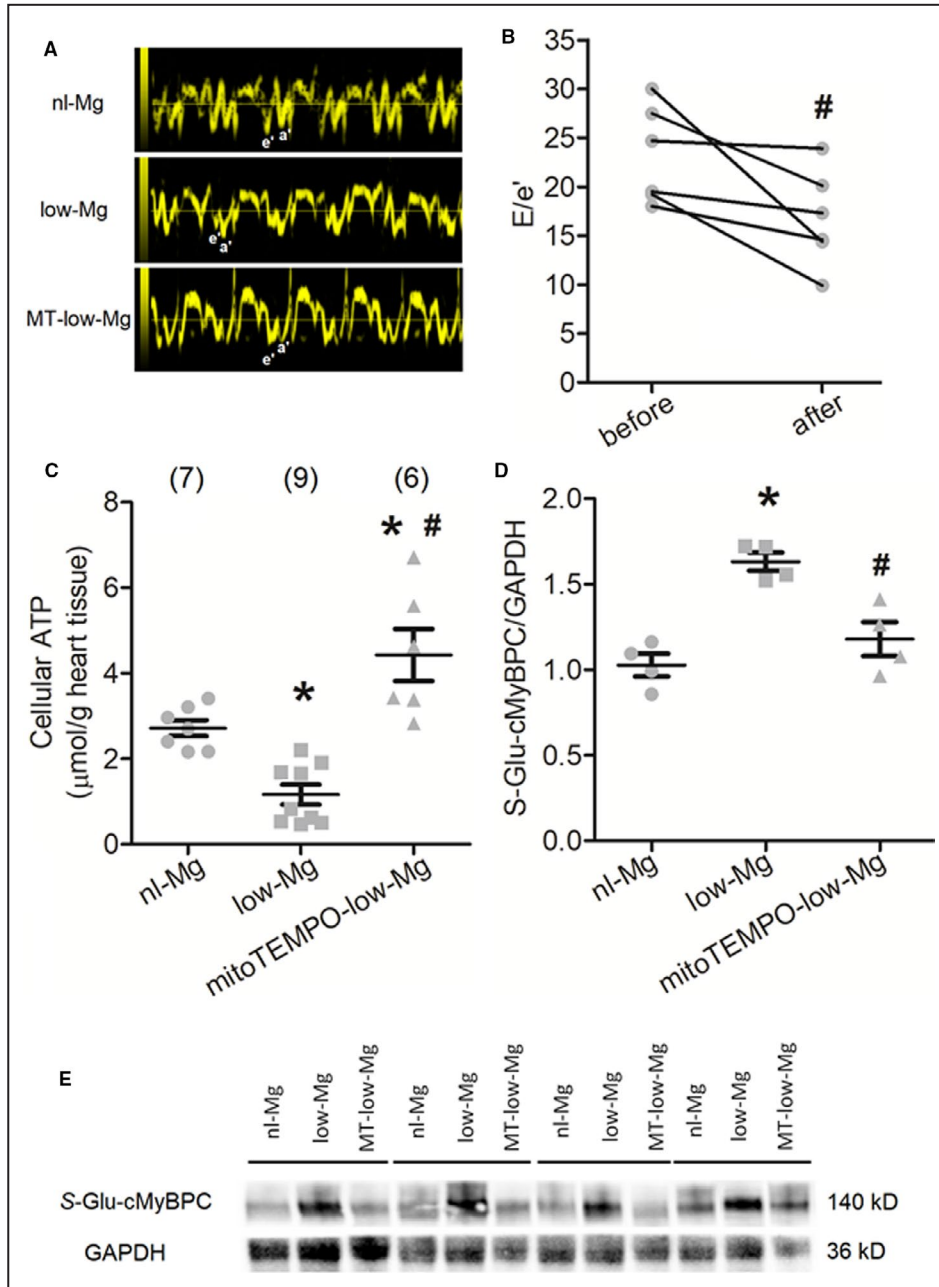


Figure 6. mitoTEMPO (2-[2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino]-2-oxoethyl) triphenylphosphonium chloride; MT) treatment (1 mg/kg per day, IP injection for 2 weeks) of low-Mg mice improved the cardiac diastolic function with increased ratio between the early and late diastole tissue velocity of the mitral valve anterior annulus e' and a' (e'/a') (A), decreased ratio between mitral peak early filling velocity E/longitudinal tissue velocity of the mitral anterior annulus e' (E/e') (B), increased cellular ATP levels (C), and decreased S-glutathionylation of cMyBPC (cardiac myosin-binding protein C) (S-Glu-cMyBPC) protein levels (D).

A, Representative images of tissue Doppler echocardiography showed decreased e'/a' ratio in low-Mg mice, which was reversed by MT treatment (MT-low-Mg). **E,** Images of protein bands were obtained with 4 hearts from each group by Western blotting. Protein levels were normalized by the loading control protein GAPDH. The mean and SEM (error bars) values and the tested mouse numbers (in parentheses) are shown in **C** and **D**. The 2-tailed Student *t* test and 1-way ANOVA with Bonferroni post hoc tests for multiple group comparisons were used. **P*<0.05 vs normal Mg (nl-Mg); #*P*<0.05 vs low-Mg or before MT treatment. In **B**, “before,” low-Mg mice before MT treatment; “after,” same low-Mg mice after MT treatment. The 2-tailed paired Student *t* test was used in **(B)** and *P*=0.034.

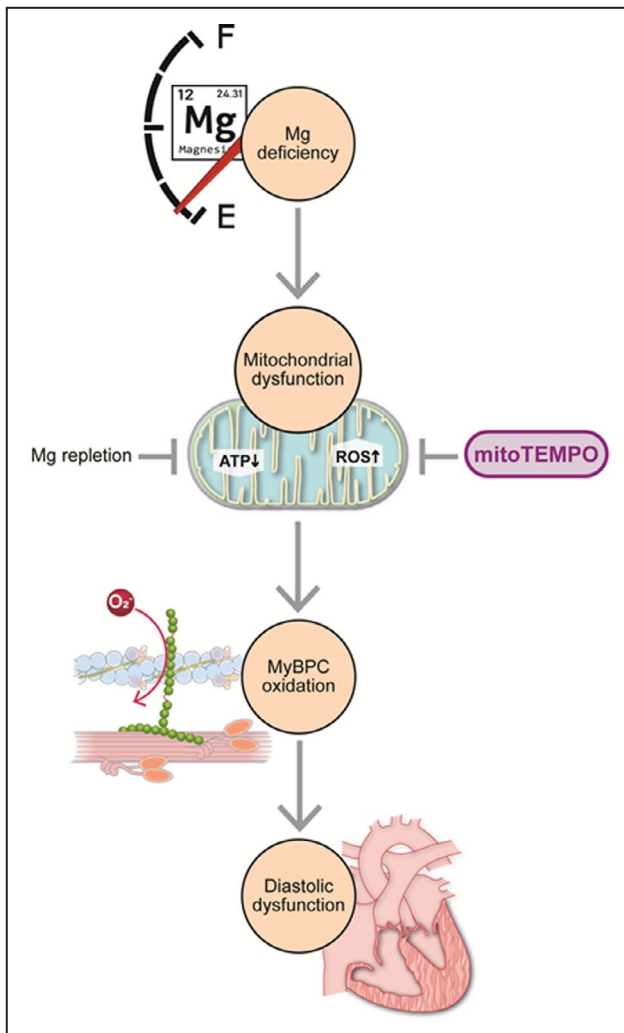


Figure 7. A summarized scheme of Mg deficiency–induced cardiac diastolic dysfunction that can be reversed by Mg repletion or mitoTEMPO (2-[2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino]-2-oxoethyl)triphenylphosphonium chloride) treatment.

MyBPC indicates myosin-binding protein C.

mouse hearts, likely resulting from hypomagnesemia-induced Mg efflux. Such an intracellular Mg deficiency has been shown to disrupt mitochondrial function by altering coupled respiration^{55,56} and increasing mitochondrial ROS production.^{5,57} Mitochondrial dysfunction and accompanying oxidative stress have been seen in diastolic cardiomyopathies.^{6,58} The degree of hypomagnesemia seen in our study is rare but not unreported.⁴⁶ Lesser degrees of hypomagnesemia would likely show a less prominent phenotype. Nevertheless, our previous study has shown that mild hypomagnesemia (plasma Mg, 0.80 ± 0.04 mmol/L of diabetic mice versus 1.00 ± 0.03 mmol/L of healthy control mice; $P=0.0003$) induced by diabetes mellitus was associated with diastolic dysfunction.⁵ Therefore, it seems likely that even milder hypomagnesemia will be pathogenic.

Mg intake has shown beneficial effects on inhibiting mitochondrial ROS production in heart, liver, and carotid artery stenosis.^{5,59} Herein, we showed directly that Mg deficiency can cause diastolic dysfunction by inducing mitochondrial dysfunction. This was evidenced by reduced cellular ATP levels, increased mitochondrial ROS, and depolarized mitochondrial membrane potential in low-Mg mice. Mg repletion successfully reversed these changes, suggesting that Mg deficiency–induced mitochondrial dysfunction underlies diastolic cardiomyopathy. The amounts of antioxidant proteins were unaltered in low-Mg hearts, suggesting that the increased mitochondrial oxidative stress likely resulted from ROS overproduction from the mitochondrial electron transport chain.⁵⁵

In vivo treatment with a mitochondria-targeted ROS scavenger, mitoTEMPO, has shown significant improvement in 2 animal models of diastolic dysfunction induced by hypertension and diabetes mellitus, respectively.^{6,7} Herein, we showed that this drug also improved Mg deficiency–induced diastolic dysfunction, suggesting that mitochondrial oxidative stress is a common cause of many forms of diastolic dysfunction. Nevertheless, we cannot rule out other sources of oxidative stress as important in the pathology and have not shown that S-glutathionylation of cMyBPC is the only possible mechanism for generation of diastolic dysfunction. For example, we cannot rule out changes in the ADP/ATP ratio as contributing to alterations in the cross-bridge kinetics.

Our previous studies on cardiac diastolic dysfunction have shown no changes in the expression levels or phosphorylation of major myofilament proteins, such as troponin I, myosin light chain 2, and cMyBPC, that correlate with the presence of diastolic dysfunction.^{7,60} On the other hand, the S-glutathionylation of cMyBPC, an oxidative modification, is significantly increased in cardiac diastolic dysfunction in different animal models and can be reversed by antioxidants, such as mitoTEMPO and tetrahydrobiopterin, that prevent or treat diastolic dysfunction.^{6,7,60} Furthermore, this modification alters myofilament properties in a manner that can explain diastolic dysfunction.^{7,60} Therefore, we used the protein levels of S-glutathionylated cMyBPC as a marker of diastolic dysfunction in this study while recognizing that other yet to be elucidated modifications may also contribute to diastolic dysfunction. Moreover, the diastolic dysfunction was reversed by Mg repletion and mitoTEMPO, both of which suppressed mitochondrial ROS overproduction, improved mitochondrial function, and reduced levels of the oxidized myofilament protein. These results suggest that Mg deficiency alone is sufficient to cause diastolic dysfunction and that Mg repletion and mitoTEMPO have protective effects on diastolic function.

In addition to diastolic dysfunction, hypomagnesemia induced a reversible systolic dysfunction. As expected, this systolic dysfunction was associated with alterations in Ca handling. Although the origin of the systolic cardiomyopathy is unclear, the reversal with Mg repletion argues for a physiological change, rather than cell death or another structural alteration.

Female mice appeared more susceptible to seizures induced by hypomagnesemia than did male mice. This occurred despite similar serum Mg levels and similar loss of Mg in the urine. Mg deficiency has been shown to cause irritability of the nervous system, leading to epileptic seizures in clinical and experimental studies,⁶¹ and Ca leak induced by gain-of-function changes of ryanodine receptor 2 has been linked to sudden unexpected death from epilepsy.^{62,63} Nevertheless, it remains unclear why a sexual dimorphism occurred, but it appears to be independent of effects on the heart.

In summary, Mg deficiency independently caused diastolic cardiomyopathy associated with mitochondrial dysfunction. Even with severe hypomagnesemia, the systolic function was only mildly affected, suggesting that diastolic dysfunction may help explain the association of hypomagnesemia and heart failure in patients. Mg repletion could be an effective therapy for heart failure associated with hypomagnesemia.

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Disclosures

None.

Supplementary Material

Data S1
Figure S1–S5

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SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Reagents. Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO), except as stated otherwise.

Animal groups. C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, MA). Mice were randomly assigned to different groups at 10 weeks of age: (1) nl-Mg: control mice fed with normal (nl) chow (TD.94253, 600 mg/kg Mg, Envigo Teklad Diets, Madison, WI) for 6 weeks; (2) low-Mg: mice fed with low-Mg diet (TD.93106, containing ~15-30 mg/kg Mg, Envigo Teklad Diets) for 6 weeks; (3) low→nl-Mg: mice fed with low-Mg diet for 6 weeks and then given normal chow for another 6 weeks. Distilled and deionized H₂O was given to the mice to control any possible Mg intake from drinking water.

Echocardiographic assessment. Noninvasive echocardiography was performed with a Vevo 2100 ultrasound system (VisualSonics, Toronto, Canada) as we have done previously ⁵. During the image acquisition, mice were anesthetized with 1% isoflurane and a heart rate ~400 beats/min was maintained. M-mode images in the parasternal long axis and the left ventricular (LV) short-axis views at the mid-papillary level were taken. The ratio between mitral peak early filling velocity (E) and longitudinal tissue velocity of the mitral anterior annulus (e'), E/e', was assessed in the subcostal four-chamber view by pulse-wave and tissue Doppler imaging to evaluate diastolic relaxation function ⁷. Measurements were averaged from five consecutive beats during expiration. The images for each mouse were recorded for at least 5 s (30–40 cardiac cycles). For the baseline evaluation, the following LV diastolic and systolic dimensions were measured: the posterior wall thickness in diastole and in systole, the anterior wall thickness in diastole and in systole, and the LV end diastolic (LVEDD) and end systolic dimensions (LVESD). From these

dimensions, the main functional parameter, percent LV ejection fraction (EF%) was calculated as $100 \times (7/2.4 + LVEDD) \times LVEDD^3 - (7/2.4 + LVESD) \times LVESD^3 / (7/2.4 + LVEDD) \times LVEDD^3$.

Surface ECG recording and programmed ventricular stimulation. Under general anesthesia with 1% isoflurane, a standard limb ECG was recorded at sampling rate of 4000 Hz using 25-gauge needle electrodes connected to an amplifier (ADInstruments, Colorado Spring, CO). One minute of ECG signals from limb lead II were averaged for the measurement of corrected intervals between the Q and T waves of the ECG (QTc interval) using Mitchell's formula³⁹.

Telemetry. Cardiac rhythm was monitored using telemetry devices. Briefly, mice were implanted with ETA-F10 transmitter (Data Science International, St. Paul, MN) as we have done previously⁴⁰. Under 1% isoflurane anesthesia, a skin incision was made at the dorsal neck area and a transmitter was inserted subcutaneously. The two electrocardiogram (ECG) leads were tunneled under skin and positioned to generate a lead II ECG configuration. Continuous ECG recording was initiated immediately after transmitter implantation using Dataquest ART software (Version 4.1, DSI), and lasted until the occurrence of animal death or the end of 6-week low-Mg diet. The ECG prior to the animal death was analyzed to determine the cause of death.

Serum ions and urine Mg. Mice were anesthetized with 2% isoflurane and blood was collected in plain red tubes (BD Microtainer Capillary) with no additive and allowed to clot at room temperature for 15-30 min. Serum was obtained by centrifugation at $1,700 \times g$ for 10 min at 4°C. Urine was collected with autoclaved Eppendorf. Serum ions and urine Mg were measured with the Beckman Coulter AU480 Chemistry analyzer (Veterinary Medical Center, Clinical pathology Lab, University of Minnesota, St. Paul, MN) within 20 hours of collection. Twelve to 13 mice were tested for each group.

Cellular ATP measurements. ATP was measured with the EnzyLight™ ATP assay (BioAssay Systems, Hayward, CA) by following the manufacturer's instructions. Ventricles were excised when mice were under 2% isoflurane anesthesia, washed briefly with cold phosphate buffer solution, and quickly frozen in liquid nitrogen. About ~20-30 mg frozen ventricle tissue was homogenized in cold phosphate buffer solution on ice. Suspensions were spun down at 12,000 × g for 5 min at 4°C to obtain the supernatant for cellular ATP measurement. In a Greiner CELLSTAR 96 well plate, 10 µL of heart lysate supernatant or ATP standards (0-9 µmol/L) were mixed with 90 µL of reconstituted reagent (1 µL fresh D-luciferin reaction substrate, 1 µL ATP enzyme, and 95 µL assay buffer). Luminescence was read immediately with a Cytation 3 plate reader (BioTek, Winooski, VT). Cellular ATP was measured as µmol/g heart tissue.

Isolation of ventricular cardiomyocytes. Mouse ventricular cardiomyocytes were isolated as described before ^{5,41,42}. Briefly, hearts were excised under 2% isoflurane anesthesia and perfused with buffer (mmol/L: 113 NaCl, 4.7 KCl, 0.6 Na₂HPO₄, 0.6 KH₂PO₄, 1.2 MgSO₄, 0.032 Phenol Red, 12 NaHCO₃, 10 KHCO₃, 10 HEPES, 30 Taurine, 10 2-3-butanedione monoxime) for 7 min at 3 mL/min flow rate. Then, hearts were perfused with collagenase II (0.8 mg/mL, Worthington Biochemical Co. Lakewood, NJ) for 10 min at 37°C using a temperature controlled Langendorff perfusion system. The atria were removed. Following suspension with serially increasing Ca²⁺ concentrations (0.2, 0.5, and 1 mmol/L), ventricular cardiomyocytes were suspended immediately in standard Tyrode's solution, containing (in mmol/L) 140 NaCl, 5.4 KCl, 1 MgCl₂, 10 HEPES, 1.8 CaCl₂, and 5.5 glucose (pH 7.4).

Cytosol Mg levels. The cytosol level of free Mg was measured using a specific Mg probe, Mag-fluo-4 AM (10 µmol/L, Thermo Fisher Scientific, Eugene, OR) by fluorescence microscopy (Zeiss Axio Inverted Observer.Z1, Zeiss, Thornwood, NY) as described previously ⁵. Isolated

cardiomyocytes were loaded with Mag-fluo-4 AM for 40 min followed by washing three times with Tyrode's solution containing no Mg. This was followed by de-esterification of intracellular the acetoxymethyl esters at room temperature for 30 min. Fluorescent intensity of cells (F) and background (F₀) were recorded at excitation/emission of 488/520 nm at different extracellular Mg concentrations (0-1.5 mmol/L) as titration tests with 3 mice for each group. Thirty-two to 82 cardiomyocytes were measured for each extracellular Mg concentration. The fluorescent signals F/F₀ obtained with ImageJ-FIJI software were used to indicate cytosol Mg levels.

Mitochondrial ROS and mitochondrial membrane potential. Mitochondrial reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\psi_m$) were measured by confocal imaging as described before ^{5,41-43}. Briefly, freshly isolated ventricular cardiomyocytes were plated on glass-bottom MatTek 35-mm dishes (1.5 glass bottom, 0.16-0.19 mm, Ashland, MA) and recovered in an incubator for 1 h. Then, cells were treated with MitoSOX Red (1 μ mol/L, Thermo Fisher Scientific) for mitochondrial ROS or 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide (JC-1, 2 μ mol/L, Thermo Fisher Scientific) for $\Delta\psi_m$ in an incubator for 15 min. Cells were washed three times with standard Tyrode's solution to remove unbound fluorescent dyes. The cell dish was put onto a microscope stage with humidified 95%/5% O₂/CO₂ at 37°C. Confocal imaging was obtained with a Nikon A1R FLIM confocal microscope (Nikon Inc., Melville, NY) at excitation/emission of 488/560 nm for MitoSOX Red and 488/529 and 488/560 for JC-1. For MitoSOX Red, the difference of mean fluorescent intensity between cells and background in the same images (Δ MFI) was obtained from the subtraction between the mean fluorescent intensity of the cells and the background of the same images with ImageJ-FIJI. For JC-1, when mitochondria are polarized electrically, JC-1 forms J-aggregates that emit orange-red fluorescence with a maximum at 595 nm. J-monomers, indicating

depolarized mitochondria, emit green fluorescence with a maximum at 530 nm. Mitochondrial $\Delta\psi_m$ was calculated by a ratio of red ΔMFI to green ΔMFI . A smaller ratio indicated mitochondrial membrane depolarization.

Cellular Ca^{2+} transient and sarcoplasmic reticulum Ca load measurements. As we have done previously ⁵, cellular Ca^{2+} transients were monitored by loading cardiomyocytes with Indo-1 (2 $\mu\text{mol/L}$, 40 min, Thermo Fisher Scientific), followed by washing 3 times with standard Tyrode's solution. De-esterification was allowed to occur for 20 min at room temperature. Cardiomyocytes were paced with 15-20 V, 4 ms square wave pulses at 0.5 Hz. Indo-1 was excited at 405 nm, and emitted fluorescence was collected at 470-490 nm (Olympus IX81, Japan). Intensity of Indo-1 was sampled at a rate of 1 kHz by an IonOptix system with a real-time fluorescence microscope (IonOptix LLC). Cellular Ca^{2+} transient amplitude was calculated as the ratio of cell fluorescent intensity F normalized by the background fluorescence F_0 , F/F_0 . Three consecutive traces were averaged for each myocyte. The sarcoplasmic reticulum (SR) Ca load was measured as the amplitude of cellular Ca transient induced by application of caffeine (10 mmol/L). The SR Ca fractional release was calculated as the ratio between the amplitudes of the stimulation-induced and caffeine-induced Ca transient. Three to six mice were used for each group, and 9-20 myocytes from each mouse were measured. Data analysis was performed using IonWizard 11 (IonOptix LLC).

Western blotting for protein levels. Heart ventricles of three mice from each group were collected and processed for Western blotting. Protein lysates prepared from mouse ventricles were solubilized in a non-reducing 2X Laemmli buffer (Bio-Rad). Without adding reducing agent and sample heating, proteins were separated on SDS-PAGE gels and transferred onto methanol-activated polyvinylidene difluoride membrane for 2 h at 55 mV and 4°C. Following 5% nonfat dry

milk in Tris-buffered saline, 0.1% Tween 20 blocking for 1 h, the membranes were incubated with the primary antibodies overnight at 4°C. Primary antibodies for cardiac myosin binding protein C (cMyBPC) and *S*-glutathionylation of cMyBPC (*S*-Glu-cMyBPC, with anti-Glutathione monoclonal antibody) were purchased from Santa Cruz Biotechnology (Dallas, TX, sc-137180, 1:500) and Virogen (Watertown, MA, 101-A100, 1:1000 dilution), respectively. GAPDH and β -actin (Abcam, ab9484 and ab8277, 1:4000, Cambridge, MA) were used as loading controls. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG secondary antibodies were used with dilution of 1:5000 for 1 h at room temperature. Optical density of the bands was measured with ChemiDoc MP system (Bio-Rad, Hercules, CA) and analyzed with Image Lab 6.0.0 software (Bio-Rad).

Figure S1. The Kaplan Meier survival curve of the mice on normal and low-Mg diet. The Log-rank (Mantel-Cox) test gave a $P < 0.0001$. The number of mice are 18 on the normal diet (half for each sex) and 88 (48 female and 40 male) on the low-Mg diet.

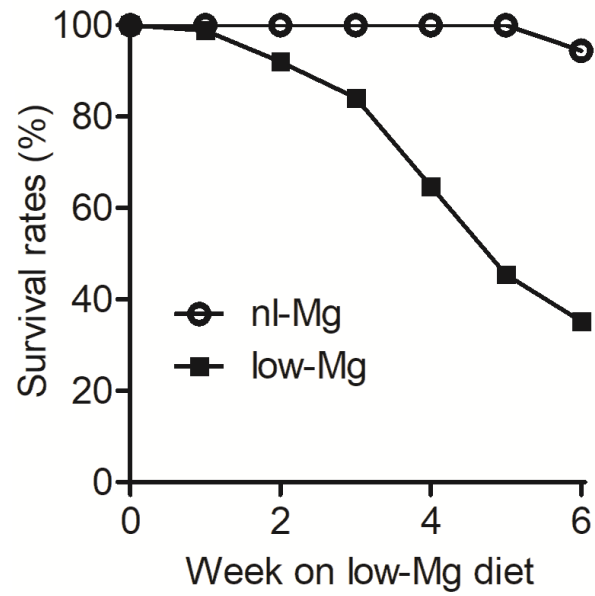


Figure S2. The correlation of serum Mg levels and cardiac E/e' on echocardiography for mice under the normal and low-Mg diet. Linear regression fitting results are $r^2=0.3870$, $P=0.0077$, and the slope = -7.0 ± 2.3 per mM serum Mg. Data were obtained from 5 nl-Mg mice and 12 low-Mg mice.

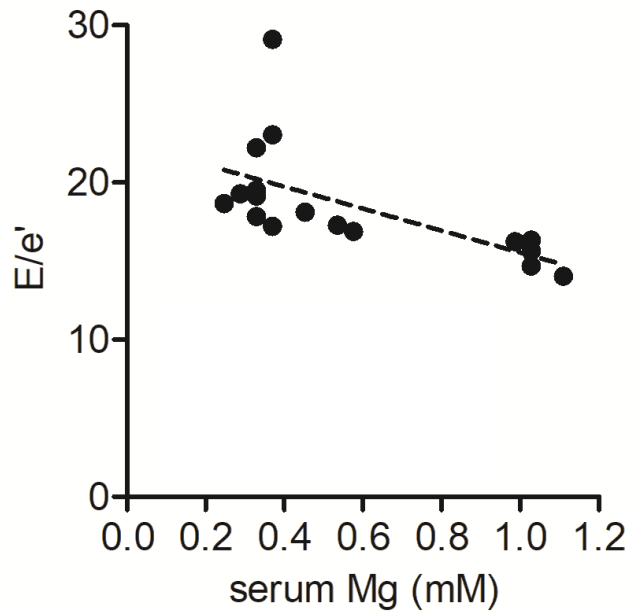


Figure S3. Mg deficiency decreased (a) Ca transient amplitude, (b) SR Ca load, and (c) SR Ca fractional release. Mg repletion reversed these changes. (d) Representative traces of Ca transient before and after caffeine treatment. (e) The Ca transient peak time (the time from the start of Ca transient to the peak) and (f) decay time (the time from peak to 10% of the baseline) were unaltered in low-Mg myocytes or low→nl-Mg myocytes, compared with nl-Mg myocytes. Results were qualitatively similar when low-Mg cardiomyocytes were tested in a 0.38 mM MgCl₂ containing solution. The mean and SEM (error bars) and the exact numbers of cardiomyocytes tested are shown in parentheses in each figure. The 2-tailed Student's t test and 1-way ANOVA with Bonferroni post-hoc tests for multiple group comparisons were used. *P<0.05 vs. nl-Mg; #P<0.05 vs. low-Mg.

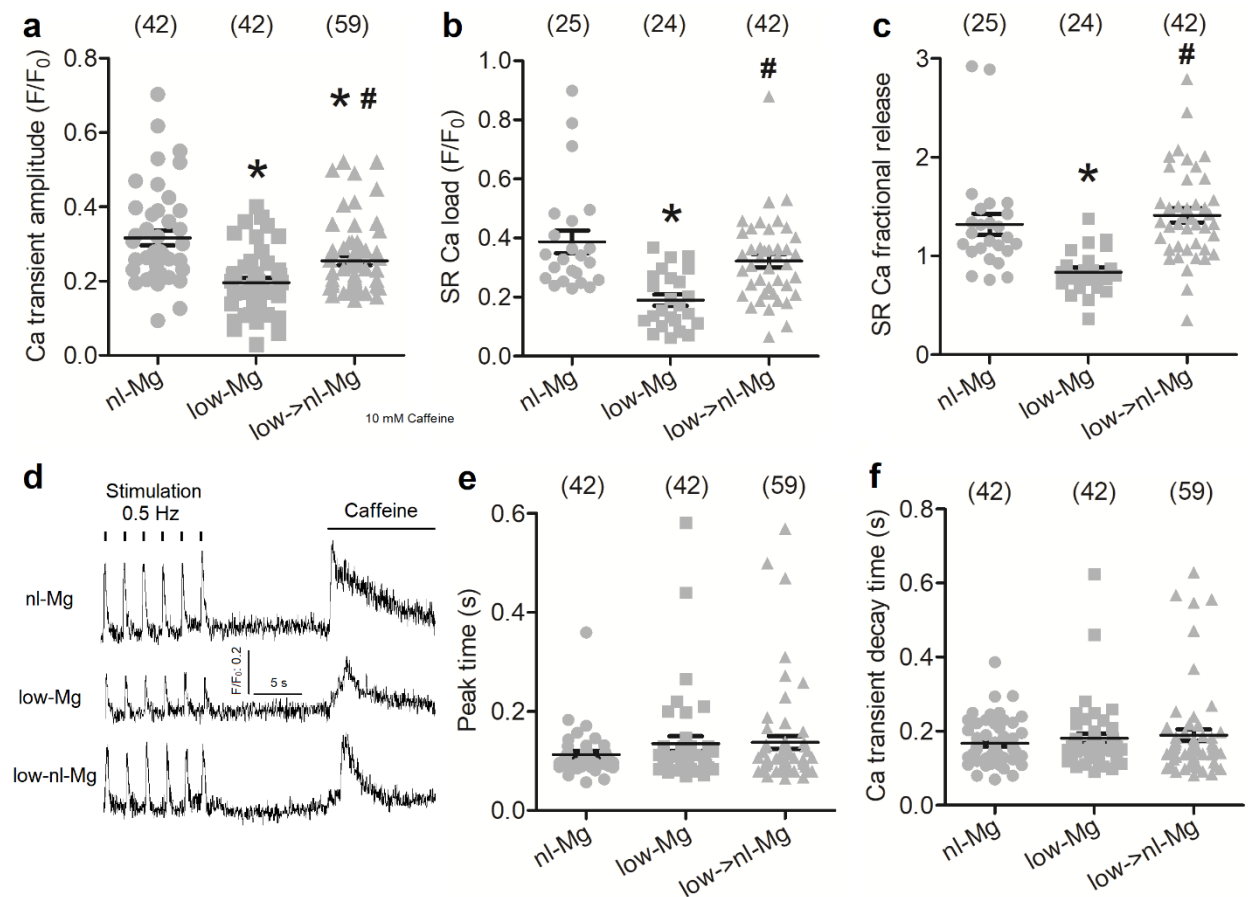


Figure S5. Mg deficiency affected (a and b) sarcomere lengthen and (a and c) sarcomere shortening. Mg repletion in low→nl-Mg mice normalized these changes. The mean and SEM (error bars) values are shown. The exact number of cardiomyocytes tested is shown in parentheses in each figure. The 2-tailed Student's t test and 1-way ANOVA with Bonferroni post-hoc tests for multiple group comparisons were used. *P<0.05 vs. nl-Mg; #P<0.05 vs. low-Mg.

