

Chronic dietary Mg²⁺ deficiency induces cardiac apoptosis in the rat heart

M. Isabel Tejero-Taldo¹, Joanna J. Chmielinska¹, William B. Weglicki^{1,2}

¹ Division of Experimental Medicine, Department of Biochemistry & Molecular Biology; ² Department of Medicine, The George Washington University Medical Center, 2300 I ST, NW, Washington, D.C. 20037, USA

Correspondence: M. Isabel Tejero-Taldo, Department of Biochemistry and Molecular Biology, The George Washington University Medical Center, Ross Hall Room 439-A, 2300 I St, NW, Washington, DC, 20037, USA. Tel.: 202 994 9921; fax: 202 994 3553 <phymit@gwumc.edu>

Abstract. Severe Mg²⁺ deficiency provokes pro-oxidative and pro-inflammatory changes, and also has been shown to be pro-apoptotic in thymus and certain cell cultures. In this study we examined the extent that chronic severe dietary Mg²⁺ deficiency induces apoptosis in the heart. Sprague-Dawley rats were fed during three weeks with normal (25 mM, Mg-control) or magnesium deficient (2.25 mM, Mg-deficient) diets, after which, hearts were harvested and frozen. DNA fragmentation was examined in heart tissue sections, and while < 1% of nuclei were positive for apoptosis in Mg-control rat tissue, over 32% of nuclei gave positive for Klenow fragments in hearts from Mg-deficient rats. Caspase 3 activity measurements in heart homogenates showed a 3.9-fold increase in enzyme activity in Mg-deficient rat hearts compared to Mg-controls ($p < 0.002$); and furthermore, western blot analysis of cleaved PARP (caspase 3 substrate), showed a 4.6-fold increase of cleaved PARP in Mg-deficient rat hearts ($p < 0.002$). In summary, our data indicate that chronic Mg²⁺ deficiency induces apoptosis of myocardium *in vivo*.

Key words: magnesium, apoptosis, heart

Hypomagnesemia is a common finding among patients suffering from diverse chronic diseases such as diabetes, rheumatoid arthritis or alcoholism [1]. Likewise, low plasma Mg²⁺ concentration is found frequently in patients with cardiovascular diseases (hypertension, congestive heart failure, arrhythmia, and myocardial infarction) [1], and in patients with HIV or cancer [2], where the use of Mg-wasting therapies may greatly increase the hypomagnesemia [3].

Magnesium is a mostly intracellular ion necessary for normal cellular function [4], and its deficiency results in multi-organ inflammation seen as early as in 5-7 days [5], and can also induce a chronic inflammatory response [6]. Results from our earlier work

suggest a key role for the neuropeptide substance P (SP) both in the early (days) and late (weeks) stages of the systemic response to low Mg²⁺ [5, 6]. Furthermore, *in situ* blockade of SP receptors in the heart [7], or inhibition of SP release at neuronal level by blocking the NMDA receptor [8], ameliorated most of the associated cardiovascular pathology during severe dietary Mg²⁺ restriction.

cDNA array studies indicated increased translation of pro-apoptotic genes in thymocytes and neutrophils [9, 10], and lung tissue from Mg-deficient animals [11]. Others have shown pro-apoptotic effects of Mg²⁺ deficiency in the retina [12], or, *in vitro*, in primary cultures of rat hepatocytes [13], although data regarding other organs are still missing. Thus, the objective of this study was to examine the effect of severe Mg²⁺ deficiency on cardiac apoptosis, and test the hypothesis that severe Mg²⁺ deficiency would induce cardiomyocyte apoptosis.

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Materials and methods

Experimental protocol

All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (publication No. 85-23, revised 1996). Ten male Sprague-Dawley outbred rats (Harlan Dawley Co, Indianapolis, IN, USA) about 7 weeks old were housed under 12-hour light/dark cycle, with free access to bi-distilled water and food. Animals were separated into two diet groups: Mg-control, receiving the usual Mg²⁺ content in rat chow (25 mM); and Mg-deficient, which animals were fed only 2.25 mM Mg²⁺. Although plasma Mg²⁺ levels were not measured during this study, Mg²⁺ plasma concentration in our animal model has been characterized in the past [14]. Treatment lasted three weeks, when the animals were sacrificed and hearts were excised and cleaned. Ventricles were cut with half snap-frozen in liquid nitrogen and half embedded in OCT compound and frozen in methylbutane cooled in dry ice (tissue sections). All tissue samples were stored at -70°C until further use.

Visualization of DNA fragments on tissue samples

The Klenow FragEL™ DNA Fragmentation Detection Kit (EMD Biosciences, San Diego, CA, USA), was used to detect nuclear DNA fragments on heart tissue samples as indicated by the included protocol. Tissue sections were fixed in 4% formaldehyde and permeabilized with proteinase K (1:100 in 10 mM Tris pH = 8) after rinsing with tris-buffered saline (TBS). Sections were incubated with 3% H₂O₂ for endogenous peroxidases inactivation, equilibrated in Klenow Equilibration Buffer at room temperature (RT) and then labeled with Klenow Labeling Reaction Mixture at 37°C. Labeling was terminated with stop buffer, the sections rinsed in TBS, and then incubated in blocking buffer, followed by DAB (3,3'-Diaminobenzidine) at RT. Sections were counterstained with methyl green, dehydrated with alcohols and xylene, and mounted using Permount mounting medium (Fisher Scientific, Hampton, NH, USA). Positive and negative controls were included as part of the assay. Apoptotic and normal nuclei were counted on microphotographs (6-8 per section) taken at 40X magnification and ratios of apoptotic: normal nuclei were calculated. Hematoxylin and eosin (H&E) staining of corresponding tissue sections was used to visualize cardiac morphology and inflammation.

Caspase 3 activity assay

Caspase 3 activity was measured in ventricle homogenates using the Colorimetric Caspase 3 Assay Kit (Sigma, St. Louis, MO, USA), based on the ability of caspase 3 to hydrolyze Ac-DEVD-pNA releasing pNA. The assay was run according the protocol included with the kit. Sample duplicates were incubated overnight at 37°C in a buffer mixture containing caspase 3 substrate (Ac-DEVD-pNA) with or without the caspase 3 inhibitor Ac-DEVD-CHO. Light absorbance was measured at 405 nm wavelength. pNA concentration in samples was extrapolated from a standard created with absorbances of sequential pNA concentrations. Both positive and negative controls were included in the assay design.

Western Blot analyses of ventricular tissue

Snap-frozen heart tissue (n = 5 per group) was homogenized at 4°C with a tissue homogenizer in 5 volumes of radioimmunoprecipitation assay (RIPA) buffer, centrifuged at 33 000 g and the pellets discarded. Supernatant samples containing 50 µg of protein were separated by SDS-PAGE electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane as previously described [8]. Membranes were probed overnight with specific antibodies against PARP and actin (1:300; rabbit polyclonal; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit (1:18 000; Amersham Bio-sciences Inc., Piscataway, NJ, USA). Membranes were developed using enhanced chemoluminescence (ECL plus, Amersham Biosciences Inc., Piscataway, NJ, USA), and exposed to X-ray film. Optical densitometric evaluation was performed using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA, USA) and computerized analysis system (ImageQuant v 5.2, Molecular Dynamics, 1999). Arbitrary units calculated by the software for each band were normalized against background.

Statistical analyses

Data are expressed as means ± S.E.M. All statistical analyses were performed using GraphPad Prism® (v. 3.03; GraphPad Software Inc., San Diego, CA, USA, 2002) Differences among treatment groups were analyzed with Unpaired t-test, with statistical significance set at p < 0.05.

Results

Changes in nuclear DNA fragmentation in the Mg-deficient rat heart

One of the most apparent signs of advanced apoptotic cell death is DNA fragmentation. We used a modified TUNEL assay to highlight apoptotic nuclei in ventricular tissue sections. All tissue sections were assayed at the same time and analyzed. Less than 1% of nuclei in heart sections from control animals were positive for apoptosis. Severe chronic Mg^{2+} deficiency significantly increased the presence of apoptotic nuclei in ventricles, amounting to $32 \pm 3\%$ of total nuclei ($p < 0.0001$; *figure 1*). Analysis of cardiac morphology with H&E staining showed increased cellular infiltrates present in Mg-deficient hearts, increased inflammatory cellular infiltrates that were not present in control hearts (*figure 1*).

Caspase 3 activity in the Mg-deficient rat heart

Caspase 3 activation is one of the hallmarks of apoptotic cell death. In this study we measured the enzyme activity in ventricular homogenates. Using a synthetic substrate, we found caspase 3 activity in Mg-control rat hearts to be 7.1 ± 0.9 nmol pNA/mg protein. Three weeks of severe dietary Mg^{2+} deficiency significantly elevated caspase 3 activity to 27.6 ± 2 nmol pNA/mg protein ($p < 0.002$; *figure 2*). We further characterized changes in caspase 3 activity by western blot analysis of its natural substrate, the poly (ADP-ribose) polymerase (PARP). Caspase 3 cleaves a 24 kD fragment from PARP, and a second band corresponding to the 89 kD fragment appears in the western blot. Tissue homogenates from Mg-control hearts displayed a $15.4 \pm 4\%$ PARP protein cleavage. Three weeks of Mg^{2+} deficiency increased PARP cleavage 4.6-fold to $70.7 \pm 6\%$ ($p < 0.001$; *figure 3*).

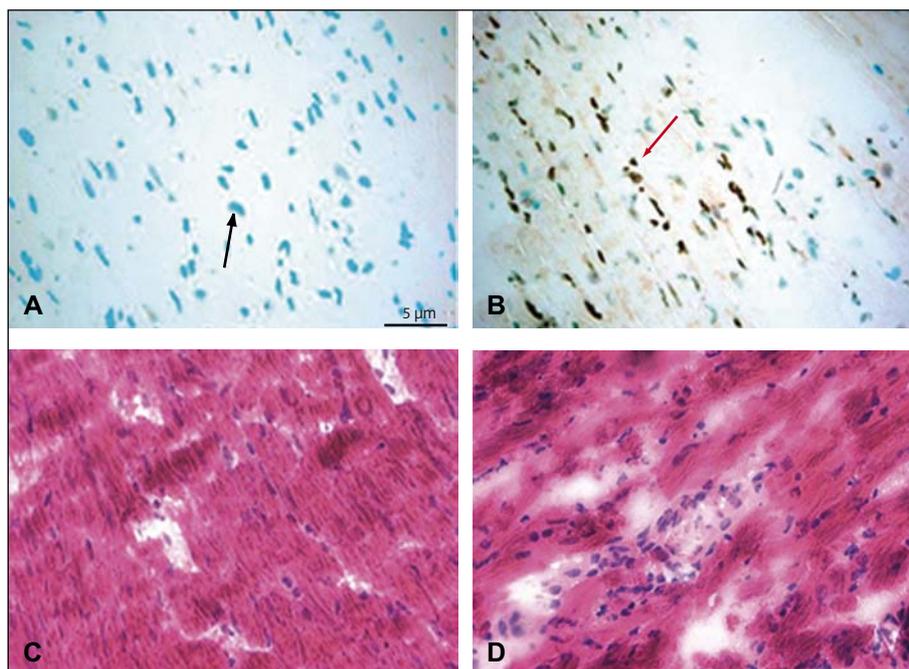


Figure 1. Apoptotic nuclei presence in ventricle of Mg-deficient rats, correlation with inflammatory infiltrates. Klenow fragments in heart ventricle sections were visualized by a modified TUNEL technique using a commercially available kit (see Methods for full description). Shown are micrographs representative of hearts from rats fed control (A, 25 mM Mg^{2+}), or Mg-deficient diet (B, 2.25 mM Mg^{2+}). Hematoxylin and eosin staining of ventricular tissue of Mg-control (C) and Mg-deficient rat hearts (D) was used to visualize cellular infiltrates within cardiac tissue. Magnification 40X. Normal nucleus (black arrow); Apoptotic nucleus (red arrow).

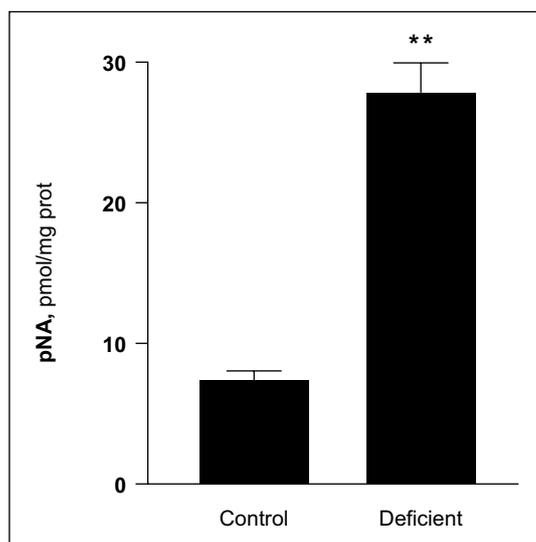


Figure 2. Cardiac Caspase 3 activity. Enzyme activity was measured using a commercially available kit (see Methods) in heart tissue homogenate. Animals were fed diet containing normal (Mg-control, 25 mM) or low (Mg-deficient, 2.25 mM) Mg²⁺ during three weeks as described in the experimental protocol section. *: $p < 0.002$ versus Mg-control.

Discussion

In this study we report that Mg²⁺ deficiency induces apoptosis in the rat heart. We used a TUNEL-like histochemical approach to visualize *in situ* the presence of apoptotic nuclei along with the analysis of caspase 3 enzymatic activity directly, and by western blot analysis of cleaved PARP in heart tissue homogenates.

The TUNEL-like histochemical analysis highlights the typical findings in apoptosis of DNA fragmentation and chromatin condensation within intact nuclei. More importantly, it localizes Mg²⁺ deficiency-induced apoptotic changes to cardiac muscle cells. Although morphological analysis of myocardium indicated the presence of inflammatory infiltrates in Mg-deficient rat hearts, we could not find clear apoptotic signs among the infiltrated areas, and whether inflammation has a role in myocardial apoptosis is unclear. Our findings agree with previous reports of *in vivo* Mg²⁺ deficiency-induced increased expression of pro-apoptotic genes [9, 11, 15] and enhanced apoptotic involution of thymus [16] and retina [12]. Furthermore, we found

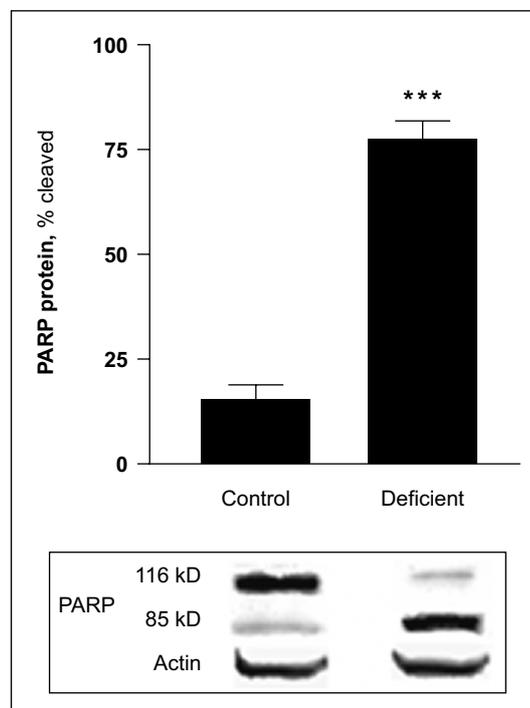


Figure 3. Poly (ADP-ribose) polymerase (PARP) cleavage in the Mg-deficient rat heart. Proteins in heart tissue homogenate (50 μ g protein/sample) were separated by SDS-page electrophoresis and then transferred to a PVDF membrane, which was then immuno-probed for PARP protein, stripped and re-probed for actin to ascertain comparable lane loading. Data is presented as mean \pm S.E.M; original data represents cleaved PARP (85 kD) as percent of total PARP calculated per each sample. Rats were fed Mg²⁺ control diet (Mg-control, 25 mM) or low Mg²⁺ diet (Mg-deficient, 2.25 mM). ***: $p < 0.001$ versus Mg-control. Below graph, a representative western blot of each treatment group is included for PARP (116 kD and 85 kD) and actin.

increased *in vitro* caspase 3 activity in the Mg-deficient rat heart. Activation of caspase 3 is a key step in apoptosis. Our findings in heart agree with the Martin *et al.* report of increased caspase 3 activation and elevated apoptotic markers, in a cell culture model of Mg²⁺ deficiency [13]. Caspase 3 cleaves PARP *in vivo*, and changes in this protein size directly correlate with enzymatic activity [17]. Mg-deficient hearts had a significantly higher amount of cleaved PARP than controls, confirming increased caspase 3 activity *in vivo*.

Conclusion

This brief study is the first report of myocardial apoptosis induced by severe Mg^{2+} deficiency in an “*in vivo*” animal model. Further studies are needed to characterize the underlying pathology and to understand what roles, if any, cardiac inflammation and neuropeptide release secondary to Mg^{2+} deficiency have in the induction of myocardium apoptosis.

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