# Short-term magnesium deficiency results in decreased levels of serum sphingomyelin, lipid peroxidation, and apoptosis in cardiovascular tissues

Burton M. Altura,<sup>1,2,4</sup> Nilank C. Shah,<sup>1</sup> Xian-Cheng Jiang,<sup>3</sup> Zhiqiang Li,<sup>3</sup> José Luis Perez-Albela,<sup>5</sup> Anthony C. Sica,<sup>1</sup> and Bella T. Altura<sup>1,4</sup>

Departments of <sup>1</sup>Physiology and Pharmacology, <sup>2</sup>Medicine, and <sup>3</sup>Anatomy and Cell Biology, and <sup>4</sup>The Center for Cardiovascular and Muscle Research, State University of New York, Downstate Medical Center, Brooklyn, New York; and <sup>5</sup>Instituto Bien de Salud, Lima, Peru

Submitted 3 November 2008; accepted in final form 1 May 2009

Altura BM, Shah NC, Jiang X, Li Z, Perez-Albela JL, Sica AC, Altura BT. Short-term magnesium deficiency results in decreased levels of serum sphingomyelin, lipid peroxidation, and apoptosis in cardiovascular tissues. Am J Physiol Heart Circ Physiol 297: H86-H92, 2009. First published May 8, 2009; doi:10.1152/ajpheart.01154.2008 .- The present study tested the hypothesis that short-term dietary deficiency of magnesium (Mg) (21 days) in rats would I) result in decreased serum(s) [the present study tested the levels of Mg, sphingomyelin (SM), and phosphatidylcholine (PC)]; 2) promote DNA fragmentation, lipid peroxidation (LP), and activation of caspase-3 in cardiac (ventricular and atrial) and vascular(aortic) muscle; and 3) low levels of Mg2+ added to drinking water would either prevent or greatly ameliorate these manifestations. The data indicate that short-term Mg deficiency (10% normal dietary intake) resulted in profound reductions in serum-ionized Mg and total Mg with an elevation in serum-ionized calcium (Ca<sup>2+</sup>), significant lowering of serum SM and serum PC, with concomitant LP, DNA fragmentation, and activation of caspase-3 in ventricular (right and left chambers), atrial (right and left chambers) and abdominal aortic smooth muscle. The greater the reduction in serum-ionized Mg, the greater the effects on DNA fragmentation, LP, and caspase-3 activity. The intake of water-borne Mg2+ at all levels greatly attenuated or inhibited the reductions in serum SM and serum PC, activation of LP, DNA fragmentation, and the activation of caspase-3; even very low levels of Mg<sup>2+</sup> in drinking water (i.e., 15 parts million<sup>-1</sup> day<sup>-1</sup>) were cardio- and vascular protective. In addition, we demonstrate that short-term dietary deficiency of Mg probably results in a downregulation of SM synthase and a decreased synthesis of PC.

calcium; phosphatidylcholine; caspase-3; deoxyribonucleic acid fragmentation

IT HAS BEEN SHOWN RECENTLY in primary cerebral and peripheral vascular smooth muscle cells, in culture, that a variation in free magnesium ions  $(Mg^{2+})$  causes sustained changes in membrane phospholipids and second messengers (33), as well as activation of apoptotic pathways (27), membrane oxidation (5, 32), and truncation of membrane fatty acids (32). Decreases in extracellular free Mg ions  $(Mg_o^{2+})$  produced a fall in membrane sphingomyelin (SM), whereas increases in  $Mg_o^{2+}$  resulted in increases in SM and phosphatidylcholine (PC) (33). Intracellular ceramide formation was inversely proportional to  $Mg_o^{2+}$ . Ceramide, released as a consequence of sphingomyelinase (SMase) acting on SM, is now thought to play important roles in fundamental processes such as cell proliferation, membrane-receptor functions, angiogenesis, microcirculatory functions,

immune inflammatory responses, cell adhesion, atherogenesis, and programmed cell death (4, 10, 17, 18, 23, 35, 36, 49, 51, 53, 55).

Low Mg content in drinking water found in areas of soft water and Mg-poor soil is associated with high incidences of ischemic heart disease, coronary vasospasm, and sudden cardiac death (6, 12, 14, 22, 26, 29, 42–44). At present, the average dietary intake of Mg has declined from about 450-485 mg/day in 1900 to about 185-235 mg/day for large segments of the North American population (2, 15). Both animal and human studies have shown an inverse relationship between dietary intake of Mg and atherosclerosis (1, 2, 8, 26, 32, 39, 44). The myocardial level of Mg has consistently been observed to be lower in subjects dying from ischemic heart disease and sudden cardiac death in soft-water areas than those living in hard-water areas (12, 14, 30).

We designed experiments to determine 1)whether a shortterm Mg deficiency in rats would lead to reductions in serum SM and PC, as well as DNA fragmentation, lipid peroxidation, and activation of caspase-3 in cardiac and vascular smooth muscles and 2) whether imbibing low levels of a water-soluble Mg salt in drinking water would inhibit or reverse these predicted effects of dietary deficiency of Mg.

# MATERIALS AND METHODS

Animals, diets, sera, and organ-tissue collections. Mature male and female Wistar rats (200  $\pm$  65 g) were used for all experiments. All experiments were approved by the Animal Use and Care Committee at SUNY Downstate Medical Center. Equal numbers of paired male and female animals were used for all experiments. Control [600 parts/million (ppm) Mg] and Mg-deficient (MgD, 60 ppm Mg) pellet diets were obtained from DYETS (Bethlehem, PA) (AIN-93G diets). Additional controls were used and given standard Purina rat chow diet pellets (1,000 ppm Mg, Purina). All animals were given their respective diets for 21 days. The MgD animals were allowed to drink triply distilled water (Mg<sup>2+</sup>  $\leq 10^{-6}$  M) containing one of four different levels of Mg aspartate-HCl (0, 15, 40, or 100 mg/l; Verla Pharm, Tutzing, Germany). All control animals received a normal Mgcontaining diet (either 600 or 1,000 ppm) and the triply distilled water to drink. On the 22nd day, sera and tissues (left and right ventricle, left and right atria, and abdominal aorta between superior mesenteric arteries and renal arteries cleaned of all connective tissues) were collected quickly after anesthesia and euthanasia (pentobarbital sodium, 45 mg/kg im). The tissues were stored rapidly under liquid nitrogen (-85°C) until use. Whole blood was collected under anaerobic conditions in red-stoppered (no anticoagulant present) tubes, allowed to clot under anaerobic conditions, and then centrifuged under anaerobic conditions in capped vacutainer tubes. The sera were then collected into additional red-stoppered vacutainer tubes under anaerobic conditions for processing shortly thereafter (7). Serum samples

Address for reprint requests and other correspondence: B. M. Altura, Box 31, SUNY Downstate Medical Ctr., 450 Clarkson Ave., Brooklyn, NY 11203-2098 (e-mail: baltura@downstate.edu).

Group	Day (ml consumed)								
	4	8	11	13	15	18	21		
Controls MgD MgD + 15 MgD + 40 MgD + 100	22.5±3.0 19.7±1.0 27.7±3.5 34.3±3.3 22.6±3.1	$23.5 \pm 2.0 \\ 24.3 \pm 2.5 \\ 29.1 \pm 3.0 \\ 32.2 \pm 2.5 \\ 29.0 \pm 4.1$	$31.1 \pm 3.0 \\ 36.6 \pm 1.6 \\ 36.0 \pm 3.8 \\ 36.8 \pm 5.2 \\ 42.8 \pm 5.2$	$36.1 \pm 1.7 \\ 42.0 \pm 2.5 \\ 35.5 \pm 3.7 \\ 31.8 \pm 2.3 \\ 44.2 \pm 5.7$	32.4±3.5 41.0±2.2 41.2±5.6 32.7±1.9 41.0±3.9	33.8±1.6 39.1±1.8 42.9±3.8 30.8±1.5 45.5±3.5	$35.1\pm1.2$ $38.6\pm2.1$ $41.6\pm3.6$ $35.4\pm3.5$ $45.4\pm3.5$		

Table 1. Water consumption in each group per rat per day of experiment

Values are means  $\pm$  SE; N = 18-28 animals/group. MgD, Mg-deficient controls; MgD + 15, MgD + 15 mg Mg/l in drinking water (DW); MgD + 40, MgD + 40 mg Mg/l in DW; MgD + 100, MgD + 100 mg Mg/l in DW.

were analyzed within 2 h after collection. Total Mg levels were measured by standard techniques in our laboratory (Kodak DT-60 Analyzer, Ektachem Colorimetric Instruments, Rochester, NY) (7). This method compares favorably with atomic absorption techniques for total Mg (7). A Mg<sup>2+</sup> ion-selective electrode (ISE) with a neutral carrier-based membrane and a Ca<sup>2+</sup>-ISE (NOVA 8 Analyzer, NOVA Biomedical Instruments, Waltham, MA) were used to measure these free divalent cations in the sera (7). The ISEs were used in accordance with established procedures developed in our laboratory, having an accuracy and precision of ~3% (7).

Biochemical tissue analyses. The tissues were analyzed quantitatively for DNA fragmentation by the diphenylamine method (11, 31), lipid peroxidation using malondialdehyde as the analyte (5, 38) and for caspase-3 activity (46). The caspase-3 activities were assayed using highly specific kits (Assay Design). Serum measurements of SM levels were carried out by a four-step enzymatic procedure as detailed elsewhere (20). Serum PC concentrations were determined by subtracting SM from the total phospholipid concentration (20). Here, the total choline-containing phospholipids in serum were obtained by an enzymatic method (Wako Pure Chemical Industries).

Statistical analyses. Where appropriate, means and means  $\pm$  SE were calculated. Differences between means were assessed for statistical significance by Student's *t*-tests and ANOVA, followed by a Newman-Keuls test. A P value < 0.05 was considered significant.

#### RESULTS

Influence of diet on water consumption and food intake. The data presented in Tables 1 and 2 indicate that there were no significant differences in either water consumption or food intake between the diverse groups or within the subgroups (P > 0.05, ANOVA).

Serum total Mg, ionized Mg, and ionized Ca levels. Table 3 indicates that the normal serum total Mg in Wistar rats fed either a synthetic AIN-93G pellet diet or a normal Purina rat chow pellet is  $\sim 1.08$  mM/l. Twenty-one days of feeding a diet that contains only 10% normal Mg of the AIN-93G diet resulted in almost a 70% reduction in the serum Mg level. Despite this marked drop in serum Mg, the animals showed no loss in body weight, no change in fur appearance, no change in gait, or any other outward signs of pathology or behavior. Feeding the MgD animals various levels of Mg in their drinking water resulted in concentration-dependent rises in the serum total Mg to where 100 mg/l of  $Mg^{2+}$  prevented the profound reduction in serum total Mg seen in the MgD animals. Interestingly, a level of  $Mg^{2+}$  corresponding to 40 mg/l, or approximately only 40 ppm, raised the serum total Mg to 50% of normal.

As seen from Table 3, the serum level of ionized Mg in the normal control diet (i.e., either 600 or 1,000 ppm) is  $\sim 0.63$  mM/l. The MgD animals exhibited an  $\sim 50\%$  reduction in serum-ionized Mg. Feeding the MgD animals various levels of Mg in their drinking water, like with the total serum Mg, resulted in similar concentration-dependent rises in the serum-ionized Mg levels.

With respect to the serum-ionized Ca, feeding animals MgD diets with normal Ca content resulted in a significant rise (P < 0.05) in the level of this divalent, whereas feeding these MgD animals various levels of Mg<sup>2+</sup> in their drinking water resulted in a significant, concentration-dependent fall in the serum-ionized Ca toward normality (Table 3, ANOVA, P < 0.05).

Serum SM and PC levels. Figure 1 indicates that serum in Wistar rats exhibits a SM level of about 80 mg/ml, whereas animals placed on a MgD diet for only 21 days show a 25% reduction in the SM level (P < 0.01). Feeding these MgD animals a level of Mg<sup>2+</sup> in their drinking water equivalent to only 15 mg/l (i.e., 15 ppm) almost prevents this marked reduction in serum SM observed in the MgD animals. Although the higher levels of Mg<sup>2+</sup> in the drinking water restored the SM levels to normal (P > 0.05), there is no significant difference from that observed with the 15 mg/l Mg<sup>2+</sup> level (ANOVA, P > 0.05).

With respect to serum PC levels, feeding animals diets with only 60 ppm (MgD) results in a 25% reduction in PC, whereas addition of various, low levels of Mg<sup>2+</sup> to the drinking water surprisingly and completely prevented the fall in serum PC seen in the MgD animals (Table 3, P < 0.05).

Table 2. Food consumption in each group per rat per day of experiment

Group	Day (g)								
	4	8	11	13	15	18	21		
Controls	$15.9 \pm 1.2$	19.3±1.6	$20.2 \pm 1.8$	18.2±1.6	19.6±1.8	19.0±1.2	18.7±1.5		
MgD	$15.8 \pm 1.2$	$18.5 \pm 1.6$	$24.7 \pm 2.3$	$17.2 \pm 1.6$	$18.5 \pm 1.8$	$18.5 \pm 1.4$	19.9±1.7		
MgD + 15	$16.5 \pm 1.4$	$16.0 \pm 1.8$	$18.6 \pm 2.0$	$17.7 \pm 1.5$	19.8±1.6	$20.6 \pm 1.6$	$18.4 \pm 1.4$		
MgD + 40	$18.7 \pm 1.8$	$19.6 \pm 1.6$	$21.5 \pm 2.2$	$18.8 \pm 1.6$	$18.1 \pm 1.7$	$21.4 \pm 1.8$	18.6±2.2		
MgD + 100	$15.3 \pm 2.1$	$18.9 \pm 1.7$	$21.4 \pm 2.3$	$17.6 \pm 1.8$	$20.4 \pm 1.6$	$19.8 \pm 1.6$	$18.0 \pm 1.8$		

Values are means  $\pm$  SE; N = 18-28 animals/group. Designations for groups are identical to Table 1.

# Mg DEFICIENCY, RISK FACTORS, AND DRINKING WATER

Table 3. Serum total Mg, ionized Mg, ionized calcium, and ionized Ca-to-Mg ratios in normal and MgD rats with and without Mg added to the drinking water

Total Mg	Ionized Mg	Ionized Ca	Ionized Ca-to-Mg Ratio	
14±0.06	$0.63 \pm 0.005$	$1.45 \pm 0.02$	$2.33 \pm 0.04$	
53±0.06*	$0.31 \pm 0.02 \dagger$	$1.58 \pm 0.01 \dagger$	$5.19 \pm 0.26 \ddagger$	
$00 \pm 0.14*$	$0.39 \pm 0.02 \dagger$	$1.52 \pm 0.02 \dagger$	$4.49 \pm 0.25*$	
21±0.08*	$0.41 \pm 0.14 \dagger$	$1.51 \pm 0.01 \dagger$	3.33±0.15*	
35±0.13	$0.58 \pm 0.02$	1.39±0.03	$2.54 \pm 0.08$	
	Fotal Mg $44 \pm 0.06$ $63 \pm 0.06*$ $00 \pm 0.14*$ $21 \pm 0.08*$ $35 \pm 0.13$	Total Mg         Ionized Mg $14\pm0.06$ $0.63\pm0.005$ $53\pm0.06^*$ $0.31\pm0.02\dagger$ $00\pm0.14^*$ $0.39\pm0.02\dagger$ $21\pm0.08^*$ $0.41\pm0.14\dagger$ $35\pm0.13$ $0.58\pm0.02$	Total MgIonized MgIonized Ca $14\pm0.06$ $0.63\pm0.005$ $1.45\pm0.02$ $33\pm0.06*$ $0.31\pm0.02+$ $1.58\pm0.01+$ $10\pm0.14*$ $0.39\pm0.02+$ $1.52\pm0.02+$ $21\pm0.08*$ $0.41\pm0.14+$ $1.51\pm0.01+$ $35\pm0.13$ $0.58\pm0.02$ $1.39\pm0.03$	

Values are means  $\pm$  SE; N = 18-28 animals/group. \*P < 0.001, significantly different from all other groups (ANOVA);  $\dagger P < 0.01$ , significantly different from controls and MgD + 100 (ANOVA);  $\ddagger P < 0.01$ , significantly different from all groups except MgD + 15 (ANOVA). Total Mg is given in mg/l, whereas all the other mean values are given as mM/l.

Lipid peroxidation in cardiac and vascular muscle obtained from MgD animals. Figure 2 indicates that placing rats on diets of 10% Mg intake for 21 days results in almost fivefold increases in lipid peroxidation of right and left ventricular muscle, atrial muscle, and aortic smooth muscle. All of the levels of Mg<sup>2+</sup> added to the drinking water inhibited most of the observed lipid peroxidation (P < 0.01) seen in the MgD animals; our experiments suggest that a level of only 15 mg/l of Mg<sup>2+</sup> is needed to prevent the lipid peroxidation.

DNA fragmentation in cardiac and vascular muscle obtained from MgD animals. Figure 3 indicates that dietary deficiency of Mg for 21 days resulted in four to sixfold elevations in the degree of observed DNA fragmentation, with the ventricular muscles showing the highest degrees of DNA fragmentation (P < 0.01, ANOVA). Like that observed in Lipid peroxidation in cardiac and vascular muscle obtained from MgD animals, only low levels of Mg<sup>2+</sup> (i.e., 15 mg/l) are needed to prevent the DNA fragmentation observed in the MgD animals (P < 0.05).

Caspase-3 activity in cardiac and vascular muscle obtained from MgD animals. Figure 4 indicates that dietary deficiency of Mg for 21 days resulted in two to threefold elevations in caspase-3 activities, with the ventricular muscles exhibiting the highest degrees of caspase-3 activity (P < 0.05). Like that seen with DNA fragmentation, a very low concentration of  $Mg^{2+}$  (i.e., 15 mg/l) added to the drinking water was all that was needed to inhibit completely the rise in caspase-3 activities in all of the tissues examined.

## DISCUSSION

Several distinct metabolic interrelationships exist in the body between PC, SM, and 1,2-diacylglycerol and ceramide (17, 18, 23, 53). SM synthesis, which is catalyzed by SM synthase, requires the transfer of a choline phosphate moiety from PC to ceramide, generating 1,2-diacylglycerol. Conversely, SMase regenerates ceramide and choline phosphate. SM and PC are the major phospholipids in very low-density lipoproteins (21). More than 25 years ago, it was first reported that, in humans, a decrease in the plasma PC-to-free cholesterol ratio connotes a higher correlation with ischemic vascular disease than either plasma cholesterol or high-density lipoprotein (HDL) (25). PC is important in decreasing the lymphatic absorption of cholesterol and is the major phospholipid in HDL cholesterol (21). PC is also known to play an important role in plasma cholesterol esterification-the less the PC, often the less cholesterol esterification, and thus a potential risk factor for atherogenesis, cardiovascular disease, and cerebral vascular accidents (for review, see Ref. 21).

The results reported herein are the first demonstration that short-term dietary deficiency of Mg in an intact mammal results in decreased serum levels of both PC and SM. These results thus extend previous findings that low levels of  $Mg^{2+}$  in primary cultured peripheral and cerebral vascular smooth muscle cells result in lowered intracellular levels of both PC and SM—the lower the external level of  $Mg^{2+}$ , the lower were the concentrations of both PC and SM (33). This latter study also showed that these molecular biochemical changes in low  $Mg^{2+}$ environments led to the release (synthesis) of ceramide via activation of SMase. Although the present study did not measure either serum or cellular levels of ceramide, we hypothesize that the dietary deficiency of Mg produces an activation of SMase (leading to reduction in serum SM found herein) and increased plasma levels of ceramide.

Fig. 1. Serum sphingomyelin and serum phosphatidylcholine in normal and Mg-deficient (MgD) rats with and without Mg added to the drinking water (DW). Concentrations of Mg<sup>2+</sup> per liter added to the DW are as follows: 15 mg/l (+15), 40 mg/l (+40), and 100 mg/l (+100). All values are means  $\pm$  SE. MgD mean value is significantly different from controls (P < 0.01); N, numbers of animals per group.



AJP-Heart Circ Physiol . VOL 297 . JULY 2009 . www.ajpheart.org

# H88



Fig. 2. Lipid peroxidation [%change in malondialdehyde (MDA)] in abdominal aortic smooth muscle, right ventricular (RV) muscle, left ventricular (LV) muscle, and atrial muscle in normal and MgD rats with and without Mg added to the DW. Designations and numbers of animals per group are identical to those in Fig. 1. Mean value for MgD animals is significantly different from all other groups (P < 0.001, ANOVA).

One of the important roles of ceramide is in apoptosis. Ceramides have been found in a number of cells and tissues following treatment with ionizing radiation, UV light, interleukin-1,  $\gamma$ -interferon, TNF- $\alpha$ , chemotherapeutic agents, endotoxins, and oxidative stresses (23, 24, 35, 45, 49, 53). We have recently found that several different ceramides (e.g., C2-, C6and  $C_{16}$ -ceramides) can acutely induce apoptosis in primary cultured canine cerebral vascular and rat peripheral (i.e., mesenteric arterial and aorta) vascular smooth muscle cells as verified by several types of assays (i.e., terminal transferasemediated dUTP nick end labeling, acridine orange, propidium iodide, annexin V, and caspase-3) (unpublished findings). Low Mg<sup>2+</sup> environments exacerbated both the rapidity and degree of programmed cell death in these cells (e.g., see Ref. 27). Very recently, a report appeared in which male Sprague-Dawley rats, fed a 9% MgD diet for 3 wk, were used and the results indicated that some of the excised whole hearts showed DNA fragmentation and cleaved poly(ADP-ribose)polymerase (which suggested increased caspase-3 activity) and thus signs that Mg deficiency can induce apoptosis in cardiac muscle (47). These studies, when taken together with in vitro work on perfused working rat hearts obtained from such MgD animals (50) and in vitro studies from our laboratory on perfused working rat hearts (6, 52), clearly demonstrate that even short-term Mg deficiency results in reductions in a variety of hemodynamic cardiac functions. Collectively, these in vitro hemodynamic studies on perfused rat hearts demonstrate that short-term Mg deficiency results in falls in cardiac output, coronary flow, stroke volume, developed pressures, and ischemia concomitant with a lowering of cellular high-energy phosphates. Such a compromise of cardiac hemodynamics could very well form a milieu for activation of SMase and increased plasma levels of ceramide and programmed cell death. Our new findings on MgD-induced oxidative stress add to and strengthen the growing experimental results of others, who have demonstrated that Mg deficiency may promote cardiovascular and muscle damage via lipid peroxidation and the formation of free radicals (2, 5, 24, 28, 39, 50, 51). Whether or not Mg deficiency-induced oxidative stress and release of reactive oxygen and/or reactive nitrogen species are responsible for the observed breakdown of PC, loss of SM, DNA fragmentation, and apoptosis observed herein in ventricular, atrial, and vas-



Fig. 3. Percent DNA fragmentation in abdominal aortic smooth muscle, RV muscle, LV muscle, and atrial muscle in normal and MgD rats with and without Mg added to the DW. Designations and numbers of animals per group are identical to those in Fig. 1. Mean value for MgD animals is significantly different from all other groups (P < 0.001, ANOVA).





H90

Fig. 4. Caspase-3 activity in abdominal aortic smooth muscle, RV muscle, LV muscle, and atrial muscle in normal and MgD rats with and without Mg added to the DW. Designations and numbers of animals per group are identical to those in Fig. 1. Mean value for MgD group is significantly different from all other groups (P < 0.001, ANOVA).

cular smooth muscle remains to be rigorously tested. Alternatively, it may be, as suggested above, that the formation of ceramides is the trigger for MgD-induced oxidative stress. It is likely that the generation of ceramides from the activation of SMase is playing an important role in the programmed cellular death of the tissues. The observed reduced levels of PC may also be a contributing factor in these latter events.

Using weanling rats pair-fed with MgD diets for 8 days, Tongyai et al. (48) have reported a 15% increase in fluidity of erythrocyte membranes, a reduced ratio of SM to PC, and a reduced ratio of cholesterol to phospholipid, but no change in PC content of these young MgD animals. These workers speculated that the lowered erythrocyte membrane levels of SM may be due to a lipid exchange mechanism. Although such a mechanism may be food for thought, it is important to point out that these results are from nonnucleated cells and that erythrocytes have little in the way of metabolic capacity (other than the liberation of energy via glucose metabolism), and such cells cannot be compared with heart and vascular smooth muscle cells. Moreover, our previous work on primary cultured canine and rat vascular smooth muscle cells clearly demonstrates that lowering of Mg<sup>2+</sup> in the medium results in rapid formation and the release of ceramides concomitant with a marked reduction in both PC and SM content (32, 33).

It has been reported that serum lipids of MgD rats and rabbits have dramatic changes in lipoproteins (8, 40); these lipoproteins contain SM and PC. These studies in rats and rabbits demonstrate significant elevations in serum/plasma triglycerides (8, 40) along with increases in phospholipids (16). It is thus possible that the significant changes in SM/PC containing lipoproteins, and the current report of lowered serum SM and PC concomitant with lipid peroxidation, could be due, in some measure, to a redistribution of SM and PC in the changing lipoprotein fractions independent of SMase activity.

There are now several lines of convincing evidence to implicate a diminished PC synthesis in the induction of apoptosis in several cell types (see Ref. 49 for a recent review). Ramos et al. (36) were the first to demonstrate that the inhibition of PC synthesis is one of the primary events by which C2-ceramide triggers apoptosis in cerebral neuronal granules. Others showed, using PC12 cells, that the reduction in PC levels (54) was associated with concomitant decreased levels of SM and increased levels of ceramide. Previously, using primary cultured cerebral and peripheral vascular smooth muscle cells, two of us demonstrated that when these cells were exposed to low external Mg<sup>2+</sup> environments, there was a cellular loss of PC and SM with a concomitant generation of ceramides (32, 33). On the basis of these and the present findings, we propose that dietary MgD-induced membrane oxidation leads to the generation of ceramides (via the activation of SMase) and diminished PC synthesis, which act in concert to help to induce programmed cell death in ventricular, atrial, and vascular smooth muscle cells in the intact animal. A number of previous studies suggest that ceramide accumulation precedes caspase-3 activation and apoptosis (19, 37, 45). From our present results and previous observations on primary cultured vascular muscle cells, we propose that Mg deficiency should be added to the well-known extracellular stimuli (e.g., vitamin D, TNF- $\alpha$ , FasL, IL-1 $\beta$ ,  $\gamma$ -radiation, UV radiation chemotherapeutic agents, oxidative stress, and others), which cause the activation of SMase and hence the SM-ceramide pathway.

Considerable evidence, both experimentally and epidemiologically, as well as clinically, suggests a linkage between dietary deficiency of Mg and diverse types of cardiovascular problems, e.g., high blood pressure, ischemic heart disease, coronary artery disease, congestive heart failure, atherogenesis, vasospasm, peripheral arterial disease, myocardial infarction, irregular heart rhythms, sudden cardiac death, free radical generation, and stroke (1-3, 5, 8, 9, 12-14, 50-52, 55). More than 50 years ago, it was shown in an epidemiologic study that when the hardness of drinking water was elevated, the rate of death from cardiovascular diseases decreased (22). In the intervening years to the present, this has been borne out by numerous studies around the globe (for recent reviews, see Refs. 13, 15, 42); that is, cardiovascular death rates are lower in hard-water areas than in soft-water areas. Although the hardness of water is due, primarily, to the concentrations of Ca and/or Mg, the overwhelming evidence, to date, supports the idea that it is the Mg content which is responsible for most of the protective effects of hard water (13, 29, 42). Although it has been hypothesized on the basis of several epidemiologic human studies from around the world that as little as 6-30 ppm Mg (or 6-30 mg/l) in drinking water should be cardioprotective and ameliorate vascular changes (26, 29), the present study is the very first one to clearly demonstrate that as little as 15  $mg \cdot l^{-1} \cdot day^{-1}$  of water-borne, bioavailable Mg can either

prevent or ameliorate cardiovascular organ changes induced by a short-term Mg-deficiency state in a well-controlled experimental animal model. This low concentration of water-borne Mg<sup>2+</sup> was effective in preventing DNA fragmentation, oxidative stress, and activation of programmed cell death in ventricular, atrial, and vascular smooth muscle obtained from MgD animals. Some mention should be noted here that although higher levels of Mg<sup>2+</sup> in the drinking water (e.g., 40 and 100 mg/l) were required to raise the lowered serum Mg levels (both total and ionized) toward normal, only 15 mg/l of Mg2+ were required to prevent DNA fragmentation, lipid peroxidation, and elevation of caspase-3 in our rat study. These results suggest that normal serum levels of Mg, at least in rats for a short term, may not be required to maintain normal cardiovascular functions. To our knowledge, such an insight has not been possible to make from any previous study or studies, animal or human.

This discussion could not be complete unless we attempted to address the implications of short-term Mg deficiency on Ca metabolism. From our new findings, it would seem that even a short-term deficiency of Mg exerts significant effects on Ca metabolism, at least in rats. Our data, as indicated above, demonstrate that short-term MgD results in a significant elevation in serum-ionized Ca and the serum-ionized Ca-to-Mg ratio. Previously, it has been shown that such elevations in the Ca<sup>2+</sup>-to-Mg<sup>2+</sup> ratio produces a gradient for increased levels of free Ca ions in both cardiac and vascular smooth muscles with concomitant rises in arterial blood pressure, peripheral vasoconstriction, and reduced microcirculatory (and capillary) blood flows (1-3, 5, 9). But, unlike that seen with the effects of Mg-supplemented water on PC and SM levels, tissue DNA fragmentation, lipid peroxidation, and apoptosis, 15 mg/l of Mg<sup>2+</sup> in the drinking water were not able to restore Ca balance, suggesting that even though the former cardiovascular risk factors could be beneficially ameliorated, higher levels of Mg<sup>2+</sup> in the drinking water were needed to suppress the potential high Ca and high Ca<sup>2+</sup>/Mg<sup>2+</sup> risk factors. Whether this speculation will prove to be correct will have to await further studies.

At the very least, this study would seem to support the hypothesis, suggested more than 25 years ago, that water intake (e.g., from tap water, bottled waters, and beverages using tap water) in humans varying between 1 and 2 l/day, with Mg<sup>2+</sup> intakes varying from <5 mg to higher than 100 mg/l, may represent an excellent way to overcome and control the marginal intakes of Mg obtained with most Western diets (13, 29).

#### ACKNOWLEDGMENTS

We appreciate the gratis supply of magnesium aspartate-HCl that was provided to us by Dr. Angela Weigert of Verla Pharm (Tutzing, Germany).

#### GRANTS

This study was supported, in part, by a research grant from Regalware Worldwide (to B. M. Altura).

#### REFERENCES

- Altura BM, Altura BT. Magnesium and cardiovascular biology: an important link between cardiovascular risk factors and atherogenesis. *Cell Mol Biol Res* 41: 347–359, 1995.
- Altura BM, Altura BT. Magnesium: forgotten mineral in cárdiovascular biology and atherogenesis. In: New Perspectives in Magnesium Research,

edited by Nishizawa N, Morii H, and Durlach J. New York: Springer, 2007, p. 239-260.

- Altura BM, Altura BT, Gebrewold A, Ising H, Gunther T. Magnesium deficiency and hypertension: correlation between magnesium deficient diets and microcirculatory changes in situ. Science 223: 1315–1317, 1984.
- Altura BM, Gebrewold A, Zheng T, Altura BT. Sphingomyelinase and ceramide analogs induce vasoconstriction and leukocyte-endothelial interactions in cerebral venules in the intact rat brain: insight into mechanisms and possible relation to brain injury and stroke. *Brain Res Bull* 58: 271–278, 2002.
- 5. Altura BM, Kostellow AB, Zhang A, Li W, Morill GA, Gupta RK, Altura BT. Expression of the nuclear factor-κB and proto-oncogenes c-fos and c-jun are induced by low extracellular Mg<sup>2+</sup> in aortic and cerebral vascular smooth muscle cells: possible links to hypertension, atherogenesis and stroke. Am J Hypertens 16: 701-707, 2003.
- Altura BM, Barbour RL, Dowd TL, Wu F, Altura BT, Gupta RK. Low extracellular magnesium induces intracellular free Mg deficits, depletion of high-energy phosphates and cardiac failure in intact working rat hearts: a <sup>31</sup>P-NMR study. *Biochim Biophys Acta* 1182: 329-332, 1993.
- Altura BT, Altura BM. Measurement of ionized magnesium in whole blood, plasma and serum with a new ion-selective electrode in healthy and diseased human subjects. *Magnes Trace Elem* 10: 90-98, 1991.
- Altura BT, Brust M, Barbour RL, Stempak J, Altura BM. Magnesium dietary intake modulates blood lipid levels and atherogenesis. *Proc Natl* Acad Sci USA 87: 1840–1844, 1990.
- Altura BT, Memon ZI, Zhang A, Cracco RQ, Altura BM. Low levels of serum ionized magnesium are found in patients early after stroke which result in rapid elevation in cytosolic free calcium and spasm in cerebral vascular smooth muscle cells. *Neurosci Lett* 230: 37-40, 1997.
- Bischoff A, Czyborra P, Fetscher C, Meyer Zu Heringdorf D, Jakobs KH, Michel MC. Sphingosine-1-phosphate and sphingosylphosphorylcholine constrict renal and mesenteric microvessels in vitro. Br J Pharmacol 130: 1871-1877, 2000.
- Burton K. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* 62: 315-323, 1956.
- Chipperfield B, Chipperfield JR. Relation of myocardial metal concentrations to water hardness and death from ischemic heart disease. *Lancet* 2: 709-712, 1979.
- Durlach J, Bara M, Guiet-Bara A. Magnesium level in drinking water and cardiovascular risk factor: a hypothesis. *Magnesium* 4: 5–15, 1985.
- Eisenberg MJ. Magnesium deficiency and sudden death. Am Heart J 124: 544-549, 1992.
- Ford ES, Mokdad AH. Dietary magnesium intake in a national sample of US adults. J Nutr 133: 2879–2882, 2003.
- Gueux E, Mazor A, Cardot P, Rayssiguier Y. Magnesium deficiency affects plasma lipoprotein composition in rats. J Nutr 121: 1222–1227, 1991.
- Hannun YA. The sphingomyelin cycle and the second messenger function of ceramide. J Biol Chem 269: 3125-3128, 1994.
- Hannun YA. Functions of ceramide in coordinating response to stress. Science 274: 1855–1859, 1996.
- Jaffrezou JP, Maestre N, de Mas-Mansat V, Bezombes C, Levade T, Laurent G. Positive feedback control of sphingomyelinase activity by ceramide. FASEB J 12: 999-1006, 1998.
- Jiang XC, Paultre F, Pearson TA, Reed RG, Francis CK, Lin Berglund L, Tall AR. Plasma sphingomyelin level as a risk factor for coronary artery disease. Arterioscler Thromb Vasc Biol 20: 2614-2618, 2000.
- Kaplan LA, Pesce AJ, Kazmierczak SC. Clinical Chemistry. Theory, Analysis, Correlation (4th ed.). St. Louis: Mosby, 2003.
- Kobayashi J. On geographical relationship between the chemical nature of river water and death from apoplexy. Berichte des Ohara Inst fur Landwirtsch Biol 11: 12-21, 1957.
- Kolesnick R. Signal transduction through the sphingomyelin pathway. Mol Chem Neuropathol 21: 287-297, 1994.
- Kramer JH, Misík V, Weglicki WB. Magnesium-deficiency potentiates free radical production associated with postischemic injury in rat hearts: vitamin E affords protection. *Free Radic Biol Med* 16: 713–723, 1994.
- Kuksis A, Roberts A, Thompson JS, Myher JJ, Geher K. Plasma phosphatidylcholine/free cholesterol ratio as an indicator for atherosclerosis. Arteriosclerosis 3: 389-397, 1983.

### Mg DEFICIENCY, RISK FACTORS, AND DRINKING WATER

- Leary WP. Content of magnesium in drinking water and deaths from ischemic heart disease in white South Africans. *Magnesium* 5: 150-153, 1986.
- 27. Li J, Li W, Altura BT, Altura BM. Peroxynitrite induces apoptosis and decline of intracellular free Mg with concomitant elevation in [Ca<sup>2+</sup>]i in rat aortic smooth muscle cells: possible roles of extracellular and intracellular magnesium ions in peroxynitrite-induced cell death. Drug Metab Lett 1: 85-89, 2007.
- Mahfouz MM, Zhou Q, Kummerow FA. Cholesterol oxides in plasma and lipoproteins of magnesium-deficient rabbits and effects of their lipoproteins on endothelial barrier function. *Magnes Res* 7: 207–222, 1994.
- Marier JR, Neri LC. Quantifying the role of magnesium in the interrelationship between human mortality/morbidity and water hardness. *Magnesium* 4: 53-59, 1985.
- Marx A, Neutra RR. Magnesium in drinking water and deaths from ischemic heart disease. *Epidemiol Rev* 19: 258-272, 1997.
- Matassov D, Kagan T, Leblanc J, Sihorska M, Zakari Z. Measurement of apoptosis by DNA fragmentation. In: *Apoptosis Methods and Protocols*, edited by Brady HJ. Totowa: Humana, 2004, p. 1–17.
- Morrill GA, Gupta RK, Kostellow AB, Ma GY, Zhang A, Altura BT, Altura BM. Mg<sup>2+</sup> modulates membrane lipids in vascular smooth muscle: a link to atherogenesis. *FEBS Lett* 408: 191–194, 1997.
- Morrill GA, Gupta RK, Kostellow AB, Ma GY, Zhang A, Altura BT, Altura BM. Mg<sup>2+</sup> modulates membrane sphingolipids and lipid second messengers levels in vascular smooth muscle cells. *FEBS Lett* 440: 167-171, 1998.
- Obeid LM, Linardic CM, Karolak LA, Hannun YA. Programmed cell death induced by ceramide. *Science* 259: 1769–1771, 1993.
- Quillet J, Kilkus J, McShan CL, Gottschalk AR, Dawson G. Ceramide mediates the apoptotic response of WEHI 231 cells to anti-immunoglobulin, corticosteroids and irradiation. *Biochem Biophys Res Commun* 202: 710-714, 1994.
- Ramos B, Salido GM, Campo ML, Claro E. Inhibition of phosphatidylcholine synthesis preceding apoptosis induced by C<sub>2</sub>-ceramide: protection by exogenous phosphatidylcholine. *Neuroreport* 11: 3103–3108, 2000.
- Ravid T, Tsaba A, Gee P, Rasooly R, Medina EA, Goldkorn T. Ceramide accumulation precedes caspase-3 activation during apoptosis of A549 human lung adenocarcinoma cells. Am J Physiol Lung Cell Mol Physiol 284: L1082-L1092, 2003.
- Ray SD, Balasubramanian G, Bagchi D, Reddy CS. Ca<sup>2+</sup> calmodulin antagonist chlorpromazine and poly(ADP-ribose) polymerase modulators 4-aminobenzamide and nicotinamide influence hepatic expression of BCL-XL and P53 and protect against acetaminophen-induced programmed and unprogrammed cell death in mice. *Free Radic Biol Med* 31: 277-291, 2001.
- Rayssiguier Y, Gueux E. Magnesium and lipids in cardiovascular disease. J Am Coll Nutr 5: 507-519, 1986.
- Rayssiguier Y, Gueux E, Bussiere L, Durlach J, Mazur A. Dietary magnesium affects susceptibility of lipoproteins and tissues to peroxidation in rats. J Am Coll Nutr 12: 133-137, 1993.

- Rock E, Astier C, Lab C, Vignon X, Gueux E, Motta C, Rayssiguier Y. Dietary magnesium deficiency in rats enhances free radical production in skeletal muscle. J Nutr 125 1205–1210, 1995.
- Rubenowitz E, Molin I, Axelsson G, Rylander R. Magnesium in drinking water in relation to morbidity and mortality from acute myocardial infarction. *Epidemiology* 11: 416-421, 2003.
- Schroeder HA. Relations between hardness of drinking water and deaths from certain chronic and degenerative diseases in the United States. J Chronic Dis 12: 586-591, 1960.
- 44. Singh RB. Magnesium status and risk of coronary artery disease in rural and urban populations with variable magnesium consumption. *Magnes Res* 1: 205-213, 1997.
- 45. Smyth MJ, Perry DK, Zhang J, Poirler GG, Hannun YA, Obeid LM. prICE: a downstream target for ceramide-induced apoptosis and for the inhibitory action of Bcl-2. *Biochem J* 316: 25–28, 1996.
- Starcevic SL, Elferink C, Novak RF. Progressive resistance to apoptosis in a cell lineage model of human proliferative breast disease. J Natl Cancer Inst 93: 776-782, 2001.
- Tejero-Taido MI, Chmielinska JJ, Weglicki WB. Chronic dietary Mg<sup>2+</sup> deficiency induces cardiac apoptosis in the rat heart. *Magnes Res* 20: 208-212, 2007.
- Tongyai S, Rayssiguier Y, Motta C, Gueux E, Maurois P, Heaton FW. Mechanism of increased erythrocyte membrane fluidity during magnesium deficiency in weanling rats. Am J Physiol Cell Physiol 257: C270–C276, 1989.
- Vaandrager AB, Houweling M. Effect of ceramides on phospholipid biosynthesis and its implications for apoptosis. In: *Phospholipid Metabolism in Apoptosis*, edited by Quinn PJ and Kagan VE. New York: Kluwer Academic, 2002, p. 207-227.
- 50. Weglicki WB, Mak IT, Dickens BF, Stafford RE, Komarov AM, Gibson B, Cassidy MM, Phillips TM, Kramer JH. Neuropeptides, free radical stress and antioxidants in models of Mg-deficient cardiomyopathy. In: Magnesium: Current Status and New Developments, edited by Theophanides Y and Anastassopolou J. London: Kluwer Academic, 1997, p. 169-178.
- Weglicki WB, Phillips TM, Freedman AM, Cassidy MM, Dickens BF. Magnesium-deficiency elevates circulating levels of inflammatory cytokines and endothelin. *Mol Cell Biochem* 118: 105–111, 1992.
- Wu F, Altura BT, Gao J, Barbour RL, Altura BM. Ferrylmyoglobin formation induced by acute magnesium deficiency in perfused rat heart causes cardiac failure. *Biochim Biophys Acta* 1225: 158-164, 1994.
- Wymann MP, Schneiter R. Lipid signaling in disease. Nat Rev Mol Cell Biol 9: 162-176, 2008.
- Yen CL, Mar MH, Ziesel SH. Choline-deficiency-induced apoptosis in PC12 cells is associated with diminished phosphatidylcholine and sphingomyelin, accumulation of ceramide and diacylglycerol, and activation of a caspase. FASEB J 13: 135-142, 1999.
- 55. Zheng T, Li W, Wang J, Altura BT, Altura BM. Sphingomyelinase and ceramide analogs induce contraction and rises in [Ca<sup>2+</sup>]<sub>i</sub> in canine cerebral vascular muscle. Am J Physiol Heart Circ Physiol 278: H1421-H1428, 2000.

H92