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Magnesium deficiency upregulates serine palmitoyl transferase (SPT 1 and SPT 2) in cardiovascular tissues: relationship to serum ionized Mg and cytochrome *c*

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Departments of [1]Physiology and Pharmacology, [2]Medicine, and [3]Anatomy and Cell Biology and [4]The Center for Cardiovascular and Muscle Research, State University of New York, Downstate Medical Center, Brooklyn, New York; [5]Bio-Defense Systems, Incorporated, Rockville Centre, New York; and [6]Instituto Bien de Salud, Lima, Peru

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Altura BM, Shah NC, Li Z, Jiang X, Perez-Albela JL, Altura BT. Magnesium deficiency upregulates serine palmitoyl transferase (SPT 1 and SPT 2) in cardiovascular tissues: relationship to serum ionized Mg and cytochrome *c*. *Am J Physiol Heart Circ Physiol* 299: H932–H938, 2010. First published June 25, 2010; doi:10.1152/ajpheart.01076.2009.—The present work tested the hypothesis that a short-term dietary deficiency of magnesium (Mg) (21 days) in rats would result in the upregulation of the two major subunits of serine palmitoyl-CoA-transferase, serine palmitoyl transferase (SPT 1) and SPT 2 (the rate-limiting enzymes responsible for the de novo biosynthesis of ceramides) in left ventricular, right ventricular, and atrial heart muscle and abdominal aortic smooth muscle, as well as induce a reduction in serum sphingomyelin concomitant with the release of mitochondrial cytochrome *c* (Cyto *c*) in these tissues. Our data indicate that short-term Mg deficiency (MgD) resulted in an upregulation of SPT 1 and SPT 2, concomitant with a very significant release of Cyto *c* in left ventricular, right ventricular, atrial, and abdominal aortic smooth muscle. Short-term MgD also produced a lowering of serum sphingomyelin and ionized Mg. The greater the reduction in serum ionized Mg, the greater the upregulation of SPT 1 and 2 and the more the increase in free Cyto *c*. The data suggest that MgD, most likely, causes a biosynthesis of ceramides via two pathways in cardiovascular tissues, viz., via the activation of serine palmitoyl-CoA-transferase and sphingomyelinase, which lead to apoptotic events via intrinsic (present study) and extrinsic pathways (previous studies). Low levels of drinking water Mg were cardio- and vasculoprotective.

sphingomyelin; apoptosis; ceramides; cardiac muscles; vascular smooth muscle

IT HAS BEEN SHOWN 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 (38), as well as the activation of apoptotic pathways (30), including an increased expression of the apoptotic protease factor-1 (suggestive of the activation of the intrinsic pathway of apoptosis), membrane oxidation (5, 37), and truncation of membrane fatty acids (37). 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) (38). Intracellular ceramide formation was inversely proportional to Mg^{2+}. We have also found that an incubation of primary cerebral and peripheral vascular muscle cells, in culture exposed to low Mg_o^{2+} with myriocin [an inhibitor of serine palmitoyl-CoA transferase (SPT)], rather significantly decreased the cellular levels of SM, PC, and ceramides and inhibited apoptosis (B. M. Altura, A. Zhang, W. Li, B. T. Altura, unpublished findings). Ceramides, released as a consequence of sphingomyelinase (SMase) acting on SM or as a consequence of the activation of SPT (a de novo synthetic pathway) (34), are now thought to play important roles in fundamental processes such as angiogenesis, membrane-receptor functions, cell proliferation, microcirculatory functions, cell adhesion, atherogenesis, immunoinflammatory responses, excitation-contraction coupling in smooth muscles, and programmed cell death (4, 10, 21, 22, 27, 43, 44, 55, 62, 65). Since a de novo synthesis of ceramides via the SPT pathway has been linked to the mitochondrial (intrinsic) pathway of apoptosis in certain cells, promoting the release of cytochrome *c* (Cyto *c*) (28), we wondered whether such a pathway is present in cardiovascular tissues in short-term Mg deficiency (MgD).

SPT is the rate-limiting enzyme in the biosynthesis of sphingolipids (35). More than 20 years ago, it was first demonstrated that SPT activity is increased in aortas of rabbits fed a high-cholesterol diet (60). A short time after these latter studies were published, two of us showed that dietary deficiency of Mg, in levels found in Western diets, vastly increased atherosclerotic plaques in rabbits fed high-cholesterol diets, whereas high dietary levels of Mg inhibited plaque formation (8). SPT is a heterodimer of 53-kDa SPT-1 and 63-kDa SPT-2 subunits (18, 58), both of which are bound to the endoplasmic reticulum (63). An upregulation of SPT activity has been hypothesized to play a role in apoptosis (17). We hypothesized that a short-term dietary deficiency of Mg would upregulate both SPT 1 and 2 in cardiovascular tissues.

Recently, we have demonstrated, using short-term dietary deficiency of Mg in rats, that this experimental design resulted in decreased serum levels of SM and PC as well as lipid peroxidation and apoptosis in the ventricular, atrial, and aortic smooth muscle tissues (6). In these studies, we noted that MgD resulted in a fragmentation of DNA and an activation of caspase-3. In addition, we noted that low concentrations of Mg^{2+} in the drinking water (i.e., <25 mg/l) greatly attenuated or inhibited the reductions in SM and PC, lipid peroxidation, and apoptosis (6).

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 (1–3, 11, 13, 14, 26, 29, 32, 47, 50, 51). At present, the average dietary intake of Mg has declined from about

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450–485 mg/day in 1,900 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, 29, 32, 33, 45, 51). 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 in hard water areas (11, 14, 33, 54).

We designed experiments to determine *1*) whether a short-term MgD would lead to the activation of both SPT 1 and SPT 2, concomitant with reduction in serum levels of SM and ionized Mg, as well as increased cellular levels of Cyto *c* (indicative of the intrinsic mitochondrial pathway of apoptosis) (28) and *2*) whether imbibing low levels of a water-soluble Mg salt in drinking water would inhibit or reverse these predicted effects of a dietary deficiency of Mg.

MATERIALS AND METHODS

Animals, diets, sera, and organ tissue collections. Mature male and female Wistar rats (200 ± 65 g) were used, as previously described (6), for all experiments. All experiments were approved by the Animal Use and Care Committee of State University of New York Downstate Medical Center. Equal numbers of male and female animals were used for all experiments. Control [600 parts/million (ppm)] Mg and Mg-deficient (MgD, 60 ppm) pellet diets were obtained from DYETS (AIN-93G diets; Bethlehem, PA). Additional controls were used and given standard Purina rat chow diet pellets (1,000 ppm Mg, Purina) (6). All animals were given their respective diets for 21 days. The MgD animals were allowed to drink triply distilled water ($Mg^{2+} < 10^{-6}$ M) containing one of four different levels of Mg aspartate-HCl (Mg^{2+} = 0, 15, 40, or 100 mg/l; Verla Pharm, Tutzing, Germany). All control animals received a normal Mg-containing diet (either 600 or 1,000 ppm) and the triply distilled water to drink. On the 22nd day, sera and tissues (left and right ventricles, atria, and abdominal aorta between superior mesenteric arteries and renal arteries cleaned of all connective tissues) were quickly collected after anesthesia and euthanasia (pentobarbital sodium, 45 mg/kg im). The tissues were rapidly stored under liquid nitrogen (−85°C) until use. Whole blood was collected under anaerobic conditions in red-stoppered (no anticoagulant present) vacutainer 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 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 favorably compares with atomic absorption techniques for total Mg (7). A Mg^{2+} ion-selective electrode with a neutral carrier-based membrane (NOVA 8 Analyzer, NOVA Biomedical Instruments, Waltham, MA) was used to measure this free divalent cation in the sera (7). The Mg^{2+} ion-selective electrode was used in accordance with established procedures developed in our laboratories, having an accuracy and precision of ~3% (7).

Tissue analyses for SPT 1 and 2. SDS-PAGE was performed on 4 to 15% SDS-polyacrylamide gradient gels, using tissue homogenates of the left and right ventricles and atria as well as the abdominal aortae (150 μg protein each), and the separated proteins were transferred to nitrocellulose membranes (23). Western blot analysis for SPT 1 was performed using a polyclonal anti-mouse SPT-1 antibody (BD Biosciences Pharmingen, San Diego, CA). Analysis for SPT 2 was done using polyclonal anti-mouse SPT-2 antibody generated by the Proteintech Group, according to mouse SPT-2 peptide sequence: kysrhrlvplidrpfdettyeeted (536–560 aa) (23). Horseradish peroxidase-conjugated rabbit polyclonal antibody to mouse IgG (Novus Biologicals) was used as a secondary antibody for SPT 1, and horseradish peroxidase-conjugated goat polyclonal antibody to rabbit IgG (Novus Biologicals) was used for SPT 2. The blots were developed by an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia).

Tissue analyses of Cyto c. We used a modification of previously published methods (42). Tissue sections of the left and right ventricles, atria, and abdominal aortae were mounted on slides at room temperature for about 30 min. The sections were washed with a PBS-0.2% Triton X-100 buffer mixture for 3 h at room temperature. The sections were next incubated in the latter mixture for 30 min at room temperature and then subsequently washed in the buffer mixture for another 3 h. Nonspecific binding sites were next blocked with 2% normal goat serum in the PBS-Triton X-100 buffer mixture for 60 min at room temperature. After this, the serum was removed and the sections were incubated with a primary anti-Cyto *c* mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 3% BSA-PBS-0.2% Triton X-100 for 4 h at 4°C. The sections were next placed in a FITC-conjugated goat anti-rabbit IgG antibody mixture in 1% BSA-0.2% Triton X-100 for 4–6 h at room temperature. The sections were subsequently washed in the PBS-0.2% Triton X-100 mixture for 6.5 min at room temperature. Finally, the sections were placed in contact with antifade medium. Quantitation was obtained with densitometric scanning using a laser densitometer coupled with appropriate software.

Serum analyses of SM and PC. Serum measurements of SM levels were carried out by a four-step enzymatic procedure as detailed elsewhere (25).

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

RESULTS

Influence of diet on water consumption, food intake, body weight, and overall physiological condition. As shown recently, with the use of an identical dietary regimen of Mg in controls and MgD animals (6), there were no significant differences in either water consumption or food intake between the diverse subgroups of rats (i.e., controls = 600 or 1,000 ppm Mg; MgD; MgD + 15 mg·l^{-1}·day^{-1} of Mg; MgD + 40 mg/day of Mg; or MgD + 100 mg/day of Mg) (Tables 1 and 2). None of the subgroups showed any significant differences in body weights at the end of the 21 days (Table 3, $P > 0.05$, ANOVA). All of the MgD subgroups (n = 10–18 animals per group), irrespective of the amount of Mg in the diets or in the drinking water, showed no loss in gait, fur, or any other outward signs of pathology or behavior.

Serum total and ionized Mg levels. Feeding the animals either a normal Purina rat chow pellet diet or the synthetic AIN-93G MgD diet (n = 10–18/group) resulted in a total serum Mg level of ~1.00 mM/l, whereas the animals receiving the MgD diet exhibited a serum total Mg level of about 0.4 mM/l (Table 3) ($P < 0.01$). The serum level of ionized Mg in both normal, control groups was ~0.6 mM/l, whereas in the MgD group the serum ionized level was reduced to about 0.3 mM/l ($P < 0.01$) (Table 3).

Feeding the MgD animals various levels of Mg in their drinking water (as seen previously, Ref. 6) resulted in concentration-dependent rises in both the total and ionized levels of Mg. Mg^{2+} (100 mg·l^{-1}·day^{-1}) elevated the total Mg level to normal, i.e., ~1.0 mM/l, whereas feeding 15 and 40 mg·l^{-1}·day^{-1} of Mg^{2+} in the drinking water raised the total

Table 1. *Water consumption in each group given as per gram of rat body weight per day of experiment*

Group	Day, ml consumed			
	4	*11*	*15*	*21*
Controls	0.096 ± 0.009	0.141 ± 0.014	0.140 ± 0.012	0.137 ± 0.011
MgD	0.096 ± 0.010	0.148 ± 0.012	0.144 ± 0.014	0.140 ± 0.012
MgD + 15	0.105 ± 0.009	0.140 ± 0.0.13	0.140 ± 0.016	0.146 ± 0.014
MgD + 40	0.104 ± 0.008	0.140 ± 0.012	0.130 ± 0.015	0.140 ± 0.016
MgD + 100	0.108 ± 0.011	0.158 ± 0.018	0.156 ± 0.016	0.154 ± 0.016

Values are means ± SE normalized per gram of weight of each rat; n = 10–18 animals/group. MgD, Mg-deficient controls; MgD + 15, MgD + 15 mg Mg^{2+}/l in drinking water (DW); MgD + 40, MgD + 40 mg Mg^{2+}/l in DW; MgD + 100, MgD + 100 mg Mg^{2+}/l in DW.

Mg levels to 69 and 84%, respectively, of normal (n = 10–18, $P < 0.05$). With respect to the serum ionized level, feeding 15 and 40 mg·l^{-1}·day^{-1} elevated the serum ionized Mg levels to 60 and 65%, respectively, whereas feeding the MgD animals 100 mg·l^{-1}·day^{-1} restored the level of ionized Mg to normal ($P < 0.05$) (Table 4).

Serum SM levels: relationship to serum ionized Mg. Table 5 indicates that feeding animals MgD diets for 21 days produced, approximately, a 25% reduction in serum SM, similar to that reported previously (6). The addition of only 15 mg·l^{-1}·day^{-1} of Mg^{2+} in the drinking water prevented this fall in SM. Although not shown, linear regression analyses demonstrated a strong correlation between the reduction in serum ionized Mg and SM (r = 0.89, $P < 0.05$). However, there were no or weak correlations (e.g., r = 0.35–0.45) of total serum Mg to serum SM levels.

Influence of MgD and addition of Mg^{2+} to the drinking water on SPT-1 and SPT-2 activity: relationship to serum ionized Mg. Figure 1 indicates that placing rats on diets of 10% Mg intake for 21 days results in almost fourfold increases in SPT-1 activity in left and right ventricular muscle and more than twofold increases in activity in the atrial and aortic smooth muscle tissues. All of the levels of Mg^{2+} added to the drinking water inhibited in a dose-dependent manner the observed rises in these subunit enzymatic activities, with 100 mg·l^{-1}·day^{-1} producing a complete suppression of the increases in activities (Fig. 1).

With respect to SPT 2, the MgD diets result in threefold increases in the activity of this subunit in both the left and right ventricular muscles and ~2.5-fold increases in SPT-2 activities in the atrial and aortic smooth muscles (Fig. 2). Like that seen with SPT 1, all levels of Mg^{2+} added to the drinking water inhibited in a dose-dependent fashion the observed rises in activity of SPT 2, with 100 mg·l^{-1}·day^{-1} producing a complete suppression of these increases in enzymatic activities in all of the cardiovascular tissues.

As shown in Fig. 3, we found high degrees of correlation between the falls in serum ionized Mg and the rises in the enzymatic activities of both of the SPT subunits; the lower the serum Mg^{2+}, the greater the elevation in activities of both SPT 1 and 2 in all of the cardiovascular tissues studied (r = 0.78–0.97, $P < 0.01$). Like that for the SM data, there were either no ($P > 0.05$) or weak correlations (e.g., r = 0.23–0.35) between total serum Mg and either SPT 1 or SPT 2.

Influence of MgD diets and addition of Mg^{2+} to drinking water on Cyto c in cardiovascular tissues: relationships to SPT-1 and SPT-2 activities and serum ionized Mg. Figure 4 indicates that 21 days of MgD results in very significant (i.e., 3- to 4-fold, $P < 0.001$) elevations in the levels of free (i.e., release of) Cyto c in the ventricular, atrial, and aortic smooth muscle tissues. However, the addition of different levels of Mg^{2+} to the drinking water inhibited the release of Cyto c from the mitochondrial membranes in a dose-dependent manner. As shown in Fig. 5, linear regression analyses indicated inverse correlations to the serum levels of Mg^{2+} (r = −0.94–0.98, $P < 0.01$). Like that observed for SM, SPT 1, and SPT 2, there were either weak (e.g., r = 0.38–0.45) or no significant correlations ($P > 0.05$) between total serum Mg and Cyto c levels.

Linear regression analyses of a comparison between Cyto c levels, SPT-1 and SPT-2 levels, indicated a strong correlation between the release of Cyto c and both subunits of SPT; the higher the increment in activity of either SPT 1 or SPT 2, the greater the release of Cyto c (r = 0.79–0.92, $P < 0.01$).

DISCUSSION

The results reported herein are the first demonstration that a short-term dietary deficiency of Mg in an intact mammal produces an upregulation of the activities of the two subunits of SPT, the rate-limiting enzyme in the biosynthesis of sphingolipids, in several different cardiovascular tissues. To our

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

Group	Day, g			
	4	*11*	*15*	*21*
Controls	15.5 ± 1.3	21.5 ± 2.3	19.9 ± 1.7	18.9 ± 1.5
MgD	15.9 ± 1.5	23.1 ± 2.5	19.7 ± 1.5	19.5 ± 1.9
MgD + 15	16.6 ± 1.6	20.5 ± 2.1	19.6 ± 1.8	18.8 ± 1.6
MgD + 40	17.4 ± 1.6	21.3 ± 2.5	18.8 ± 1.8	19.1 ± 2.1
MgD + 100	15.9 ± 2.3	21.5 ± 2.1	20.3 ± 1.7	18.2 ± 1.6

Values are means ± SE; n = 10–18 animals/group.

Table 3. *Body weights of the control and experimental rat groups*

Group	Day, g				
	0	*4*	*11*	*15*	*21*
Controls	207 ± 21	227 ± 22	240 ± 22	262 ± 25	275 ± 23
MgD	225 ± 23	246 ± 25	272 ± 28	292 ± 27	316 ± 36
MgD + 15	215 ± 23	239 ± 25	258 ± 22	278 ± 22	291 ± 27
MgD + 40	212 ± 28	235 ± 21	254 ± 24	267 ± 23	276 ± 25
MgD + 100	198 ± 22	216 ± 20	252 ± 24	270 ± 26	288 ± 24

Values are means ± SE; n = 10–18 animals/group.

Table 4. *Serum total Mg and ionized Mg levels in normal MgD rats and MgD rats fed different amounts of Mg in their drinking water*

Group	Total Mg	Ionized Mg
Controls	1.03 ± 0.007	0.64 ± 0.006
MgD	0.39 ± 0.03*	0.27 ± 0.01†
MgD + 15	0.70 ± 0.05‡	0.40 ± 0.02‡
MgD + 40	0.85 ± 0.06‡	0.45 ± 0.02‡
MgD + 100	0.98 ± 0.08	0.60 ± 0.03

Values (in mM/l) are means ± SE; n = 12–18 animals/group. *$P < 0.001$, significantly different from all other groups (ANOVA); †$P < 0.01$, significantly different from controls and all other groups (ANOVA); ‡$P < 0.01$, significantly different from controls, MgD, and MgD + 100 groups (ANOVA).

knowledge, this is the first time anyone has shown an upregulation of SPT 1 and SPT 2 by MgD of any tissue or cell type in any species. The de novo pathway of sphingolipid synthesis via the activation of these enzymes has gained considerable attention over the past decade (16, 17, 23, 31, 34, 41). SPT catalyzes the first step in the biosynthesis of sphingolipids (leading to formation of ceramides) and has been shown to be activated by UV radiation, endotoxins, cytokines, retinoic acid, balloon-injured carotid arteries, phorbol ester, daunorubicin, and etoposide-induced apoptosis among other agents (16, 34, 40, 41). Ceramide synthesis has been shown to be the active messenger in most of these events and agencies. Our report suggests that MgD should be added to the list of stimuli known to activate the SPT pathway, at least in rats.

Our new findings thus extend our previous reports that low levels of Mg^{2+} in primary cultured peripheral and cerebral vascular smooth muscle cells result in elevated levels of ceramides and the activation of SMase, concomitant with reductions in membrane SM content (38). Although the present study did not measure the cellular levels of ceramide, we show that dietary deficiency of Mg produces an activation of SPT 1, SPT 2, and Cyto *c*, most likely resulting in increased plasma and cellular levels of ceramide.

As is now known and accepted, ceramides play leading roles in apoptotic events in a number of cells and tissues following treatment with a variety of agents (and agencies), including ionizing radiation, UV light, interleukin-1, γ-interferon, TNF-α, chemotherapeutic agents, endotoxins, and oxidative stresses (43, 52, 55, 62). We have found that several different ceramides (e.g., C_2, C_6, and C_{16} ceramides) can acutely induce apoptosis in primary 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 transferase-mediated dUTP nick end labeling, acridine orange, propidium iodide, annexin V, and caspase-3) (6). Low Mg^{2+} environments exacerbated both the rapidity and degree of programmed cell death in these cells (6, 30). The addition of myriocin (a specific inhibitor of SPT-1 and SPT-2 biosynthesis) inhibited the apoptotic events by about 50–60%, suggesting that the biosynthesis of ceramides via the de novo pathway was, most likely, accounting for a large part of the MgD-induced apoptotic events in the vascular cells, thus bolstering our hypothesis. We hypothesize that the remainder of the MgD-induced apoptosis in the vascular cells and cardiac cells is, most likely, attributed to a synthesis and release of ceramides via activation of SMase. The fact that we found that 21 days of MgD (similar to the present protocol) in rats resulted in DNA fragmentation and the activation of caspase-3 in ventricular, atrial, and vascular smooth muscle cells (6) lends further support to our contentions.

Programmed cell death is a physiological, fundamental cellular response that plays critical roles in the development and regulation of tissue homeostasis by the elimination of unwanted cells (for recent reviews, see Refs. 24 and 28). In mammals, apoptosis is mediated by an extrinsic and intrinsic

Table 5. *Serum sphingomyelin in normal and MgD rats with and without Mg added to the drinking water*

Group	Sphingomyelin
Controls	81.2 ± 6.2
MgD	60.4 ± 3.2*
MgD + 15	70.2 ± 3.8*
MgD + 40	72.8 ± 4.2*
MgD + 100	79.4 ± 8.4

Values (in mg/dl) are means ± SE; n = 10–12 animals/group. *$P < 0.01$, significantly different from controls and all other groups (ANOVA).

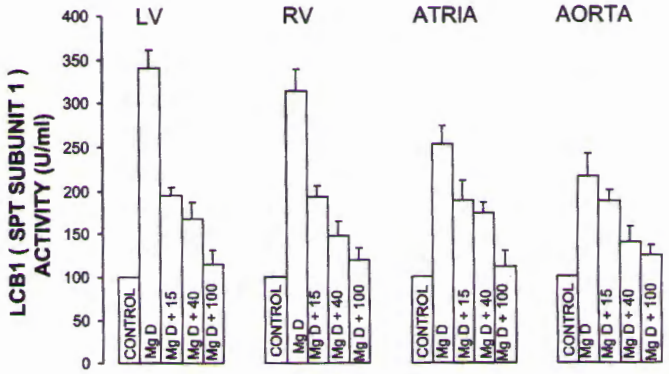

Fig. 1. Serine palmitoyl transferase (SPT)-1 levels in left ventricular (LV) muscle, right ventricular (RV) muscle, atria, and abdominal aortic smooth muscle in normal and Mg-deficient (MgD) rats with and without Mg added to the drinking water. LCB1, long chain base subunit 1. N = 10–12 animals per group. Mean values for MgD animals are significantly different from all other groups (ANOVA, $P < 0.01$).

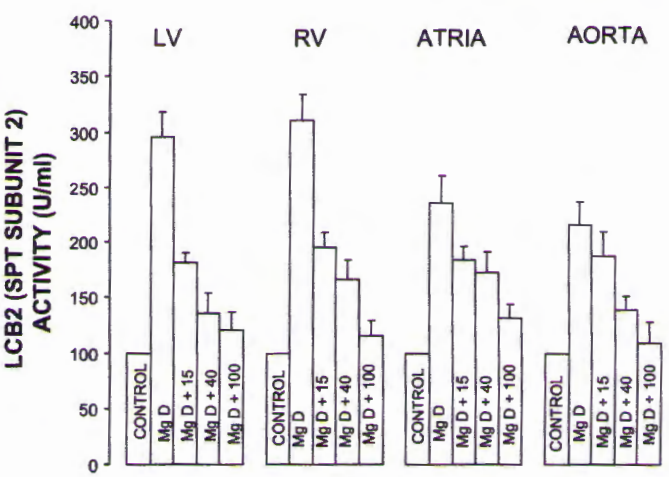

Fig. 2. SPT-2 levels in LV muscle, RV muscle, atria, and abdominal aortic smooth muscle in normal and MgD rats with and without Mg added to the drinking water. N = 10–12 animals per group. Mean values for MgD are significantly different from all other groups (ANOVA, $P < 0.01$).

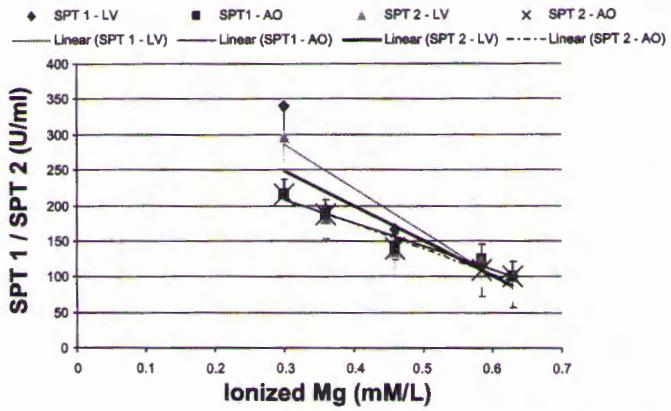

Fig. 3. Linear correlations between SPT 1 (and SPT 2) in LV and aortic smooth muscle and serum ionized magnesium levels in normal and MgD rats with and without Mg added to the drinking water. N = 10–12 animals per group. AO, aortic. Bars are SEs. SPT 1-LV = dotted line with ♦ (linear regression equation: $y = -0.0013x + 0.713$; $r = 0.82$), SPT 1-AO = dashed line with ■ ($y = -0.0029x + 0.913$; $r = 0.95$), SPT 2-LV = solid line with ▲ ($y = 0.016x + 0.733$; $r = 0.79$), and SPT 2-AO = short/long dashed line [$x(y = -0.00284 + 0.885)$; $r = 0.97$].

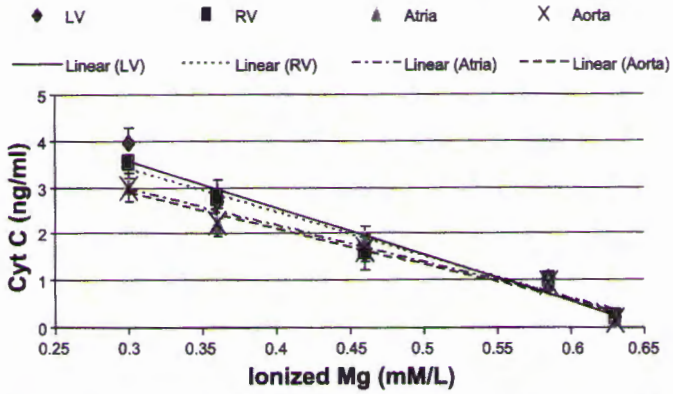

Fig. 5. Linear correlations between cytochrome c (Cyt c) in LV muscle, RV muscle, atrial muscle, and aortic smooth muscle and serum ionized magnesium levels in normal and MgD rats with and without Mg added to the drinking water. N = 10–12 animals per group. Bars are SEs. LV = ♦ ($y = -9.98 + 6.552$; $r = 0.94$); RV = ■ ($y = -9.356 + 6.194$; $r = 0.98$); atria = ▲ ($y = -7.774 + 5.271$; $r = 0.97$); aorta = $x(y = -7.661 + 5.162$; $r = 0.97$).

pathway and is almost always caspase dependent. Recently, we provided convincing evidence that short-term MgD produces apoptosis, at least in ventricular, atrial, and vascular muscle cells via the extrinsic pathway (6, 30). However, the present study that demonstrates profound release of free Cyto c (presumably from mitochondria) suggests rather strongly that MgD also produces apoptosis in these cardiovascular cells via the intrinsic pathway of programmed cell death, particularly as a release of free Cyto c is a sine qua non for identifying (and implicating) this pathway (24, 28). Our new findings suggest that the activation of this intrinsic pathway is most likely brought about as a consequence of a generation of ceramides because of the activation of SPT 1 and SPT 2 by low plasma and cellular ionized Mg. This suggestion is strengthened by our experiments on primary cultured cerebral and vascular smooth muscle cells, which show that low levels of Mg^{2+} activate the protein apoptotic protease factor-1, which must be activated by a binding to free Cyto c (28). Such binding between these molecules must take place in order for apopototic events to occur via the intrinsic pathway (28). It is thus clear from the present work that it is the lowering of the ionized Mg per se that is critical to the activation of the cascade in the cardiovascular tissues.

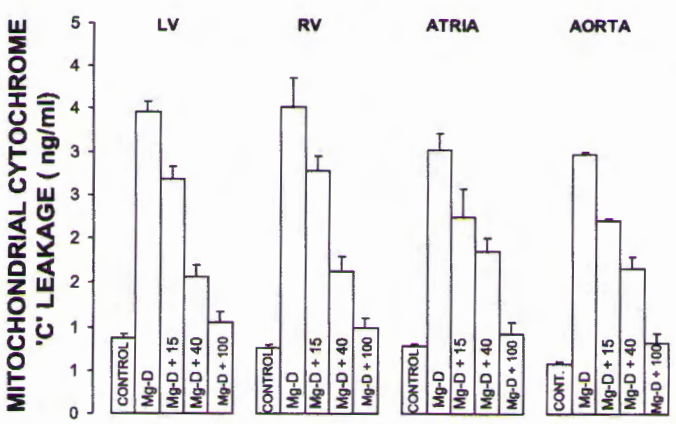

Fig. 4. Cytochrome c levels in LV muscle, RV muscle, and abdominal smooth muscle in normal and MgD rats with and without Mg added to the drinking water. Designations and numbers of animals are identical to those in Figs. 1 and 2. Mean values for MgD animals are significantly different from all other groups (ANOVA, $P < 0.01$).

Ever since the first demonstration that the measurement of ionized Mg in whole blood, plasma, or serum with specially designed Mg^{2+} ion-selective electrodes can show subtle, significant alterations from normality in different types of disease states in humans, despite no significant changes in either total serum or plasma Mg (7), a considerable body of evidence has been brought forth to indicate that several types of cardiovascular diseases are associated with significant falls in ionized Mg in blood, plasma, and sera, which are often prognostic and diagnostic of the disease state in humans (1, 7, 9, 12, 19, 20, 36, 39, 46, 48, 49, 53, 59, 64). This present report indicating strong correlations of serum levels of ionized Mg to SPT 1, SPT 2, and Cyto c in several types of cardiovascular tissues, but not to serum total Mg, strengthens the hypothesis that the free level of this divalent is crucial to the regulation of the cardiovascular system (1, 7).

Over the past three decades, experimental and epidemiological studies have been published that suggests a striking linkage between dietary deficiency of Mg and diverse types of cardiovascular problems, e.g., high blood pressure, coronary artery disease, ischemic heart disease, congestive heart failure, atherogenesis, sudden cardiac death, vasospasm, irregular heart rhythms, peripheral arterial diseases, diabetic vascular-related diseases, myocardial infarction, and strokes (1–3, 7, 9, 11, 13–15, 56, 57, 61). More than five decades ago, Kobayashi (26) showed in an epidemiological study that when the hardness of drinking water was elevated, the rate of death from cardiovascular diseases decreased. This concept has gained considerable credibility over the past 50 years from a large number of studies from different parts of the globe (for reviews, see Refs. 13–15 and 47); the death rates are lower in hard water areas than in soft water areas. Despite the fact that the hardness of waters is due to the concentrations of calcium 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 (15, 29, 32, 47). More than 20 years ago, it was suggested that as little as 6–30 mg/l of Mg in drinking water

should be cardioprotective (29, 32). Recently, using the same model of dietary deficiency of Mg as in the current studies (21 days of MgD), we showed for the first time in well-controlled experiments that as little as 15 mg/l in drinking water either prevented or ameliorated formation of reactive oxygen species, DNA fragmentation, caspase-3 activation, activation of apoptosis, hydrolysis of both SM (and thus potentially ceramides), and elevation of ionized calcium levels (6). Although the present studies could be used to reinforce the hypothetical concepts of previous investigators (29, 32), our experiments demonstrate that something in excess of 40 mg/l, but <100 mg/l, of Mg in the drinking water must be imbibed to prevent the activation of SPT 1 and SPT 2, as well as the mitochondrial release of Cyto *c*, at least in rats. From the present data and that published previously (6), it could be speculated that between 15 and 50 $mg \cdot l^{-1} \cdot day^{-1}$ of water-borne, bioavailable Mg should be both cardio- and vasculoprotective. Such an insight, to our knowledge, has not been possible to make from any previous study.

We believe, at the very least, that this study when taken together with our previous one (6) would seem to support the hypothesis suggested more than 20 years ago (29): 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^{2+} intakes varying from <5 to >100 mg/l, may, as we suggested recently (6), represent an excellent way to overcome and control the marginal intakes of Mg obtained with most Western diets (13, 29, 33). In addition, in view of the present results and those shown previously (6), it is probably propitious to suggest that all desalinated purified recovered and recycled waters, harvested rainwaters, tap waters, and all bottled waters given to humans should be supplemented with bioavailable Mg^{2+} to ameliorate the induction of cardiovascular risk factors and cardiovascular disease processes worldwide.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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