Short-term magnesium deficiency upregulates sphingomyelin synthase and p53 in cardiovascular tissues and cells: relevance to the de novo synthesis of ceramide

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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, Brooklyn, New York; [5]Bio-Defense Systems, Incorporated, Rockville Centre, New York; and [6]Instituto Bien de Salud, Lima, Peru

Altura BM, Shah NC, Li Z, Jiang X, Zhang A, Li W, Zheng T, Perez-Albela JL, Altura BT. Short-term magnesium deficiency upregulates sphingomyelin synthase and p53 in cardiovascular tissues and cells: relevance to the de novo synthesis of ceramide. *Am J Physiol Heart Circ Physiol* 299: H2046–H2055, 2010. First published October 8, 2010; doi:10.1152/ajpheart.00671.2010.— The present study tested the hypotheses that *1*) short-term dietary deficiency of magnesium (21 days) in rats would result in the upregulation of sphingomyelin synthase (SMS) and p53 in cardiac and vascular (aortic) smooth muscles, *2*) low levels of Mg^{2+} added to drinking water would either prevent or greatly reduce the upregulation of both SMS and p53, *3*) exposure of primary cultured vascular smooth muscle cells (VSMCs) to low extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) would lead to the de novo synthesis of ceramide, *4*) inhibition of either SMS or p53 in primary culture VSMCs exposed to low $[Mg^{2+}]_o$ would lead to reductions in the levels of de novo ceramide synthesis, and *5*) inhibition of sphingomyelin palmitoyl-CoA transferase (SPT) or ceramide synthase (CS) in primary cultured VSMCs exposed to low $[Mg^{2+}]_o$ would lead to a reduction in the levels of de novo ceramide synthesis. The data indicated that short-term magnesium deficiency (10% normal dietary intake) resulted in the upregulation of SMS and p53 in both ventricular and aortic smooth muscles; even very low levels of water-borne Mg^{2+} (e.g., 15 $mg \cdot l^{-1} \cdot day^{-1}$) either prevented or ameliorated the upregulation in SMS and p53. Our experiments also showed that VSMCs exposed to low $[Mg^{2+}]_o$ resulted in the de novo synthesis of ceramide; the lower the $[Mg^{2+}]_o$, the greater the synthesis of ceramide. In addition, the data indicated that inhibition of either SMS, p53, SPT, or CS in VSMCs exposed to low $[Mg^{2+}]_o$ resulted in marked reductions in the de novo synthesis of ceramide.

ceramide synthase; de novo ceramide; water-borne magnesium; cardiac muscle; vascular muscle

SEVERAL DIFFERENT CONTROL MECHANISMS, including physiological, nutritional, and biochemical factors, are responsible for normal functions of the cardiovascular system. These homeostatic factors maintain the patency of blood vessels, cardiac output, and the fluidity of blood. Pathological changes in peripheral blood vessels and the chambers of the heart can result in alterations of vascular wall geometry, disturbances in oxygenation, and nutritional status of the blood vessels and myocardium as well as the tissues they perfuse. Although numerous theories and hypotheses have been generated to account for the hypertrophy of resistance vessels and alterations in cardiac output in the etiology of essential hypertension, there is no agreement as to the precise mechanisms (for reviews, see Refs. 30, 35, and 49).

Disturbances in diet are known to promote lipid deposition and accelerate the growth and transformation of smooth muscle cells (SMCs) in the vascular walls (35). Over the past four decades, an accumulation of epidemiological and experimental data have indicated that a reduction in the dietary intake of magnesium (Mg), as well as low Mg content in drinking water, are risk factors for the development of hypertension, atherogenesis, vasospasm, sudden cardiac death, and stroke by ill-defined mechanisms (e.g., see Refs. 2–5, 10, 11, 13, 18, 25, 26, 32, 37, 40, 57, and 59). Hypermagnesemic diets have been shown to ameliorate hypertension and atherogenesis (2–5, 13, 40, 57, 59). At present, the average dietary intake of Mg has declined from ~450–485 mg/day in 1900 to ~185–235 mg/day for large segments of the North American population (2, 3, 26). 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 in subjects living in hard-water areas (21, 25, 26, 42, 65).

Using sensitive, specific Mg^{2+}-selective electrodes, it has been shown that patients with hypertension, ischemic heart disease, stroke, and atherosclerosis exhibit a significant depletion of serum/plasma ionized, but not total, Mg (2, 3, 12, 14, 27, 59). Dietary deficiency of Mg in rats and rabbits has been shown to cause vascular remodeling concomitant with hypertension and atherosclerosis (i.e., arteriolar wall hypertrophy and alterations in the matrixes) of unknown origin (4, 5, 13, 36).

A little over a decade ago, using cerebral and peripheral vascular SMCs (VSMCs) in culture, it was shown that a variation in free Mg^{2+} causes sustained changes in membrane phospholipids and second messengers as well as the activation of intracellular signal transcription molecules (i.e., NF-κB, the proto-oncogenes c-*fos* and c-*jun*, MAPK, MAPKK, PKC isozymes, and tyrosine kinase) (3, 7, 9, 47, 48). Such paradigms, using variations in Mg^{2+}, also cause membrane oxidation, truncation of membrane fatty acids, and the activation of apoptotic pathways (i.e., caspase-3, apoptotic protease activation factor-1, and release of mitochondrial cytochrome *c*) concomitant with the significant activation of sphingomyelinase (SMase) and alterations in membrane sphingomyelin (SM), leading to the release of ceramides in these cultured VSMCs (Refs. 3, 38, 47, and 48 and unpublished observa-

tions). Very recently, using a short-term (21 days) rat model of dietary deficiency of Mg, we noted decreased levels of serum SM, lipid peroxidation, apoptosis, and fragmentation of DNA in the left ventricular and right ventricular muscles as well as in atrial and vascular smooth muscles (10). These alterations (including the change in serum SM) in these tissues were highly correlated ($P < 0.01$) with the levels of serum ionized Mg levels (10, 11).

The de novo synthesis of SM is brought about via the action of serine palmitoyl-CoA transferase (SPT), 3-ketosphinganine reductase, ceramide synthase (CS), dihydroceramide desaturase, and SM synthase (SMS) (68). SMS requires phosphatidylcholine (PC) and ceramide as substrates to manufacture SM and diacylglycerol (DAG; see Ref. 68). This reaction directly affects SM, PC, and ceramide as well as DAG levels. We (48) have previously noted, using primary cerebral and peripheral vascular muscle cells in culture, that a variation in extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) influences the cellular levels of SM, PC, DAG, and ceramide. Ceramide, either released as a consequence of SMase acting on SM or activation of SPT, CS, or activation of SMS, 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, senescence, and programmed cell death (8, 16, 17, 19, 28, 29, 34, 51, 67, 72, 73). Although the activation of SMase, SPT-1, and SPT-2 (the rate-limiting enzymes for the biosynthesis of ceramide) by low $[Mg^{2+}]_o$ results in (and ensures) ceramide production in cardiovascular tissues (10, 11), the activation of CS and/or SMS by low $[Mg^{2+}]_o$ result in additional levels of ceramide. Since SMS activity exhibits links to cell membrane structures and many cell functions (23, 39, 42, 62, 68), it could have far-reaching effects on the cardiovascular system. We hypothesized that short-term Mg deficiency in 1) intact rats would upregulate SMS activities in cardiac and vascular smooth muscles and 2) VSMCs incubated with an inhibitor of SMS (i.e., D-609) (44) would demonstrate reduced cellular levels of ceramide. As other biochemical pathways mentioned (i.e., SPT and/or CS) could also contribute to the de novo synthesis of ceramide in response to low levels of $[Mg^{2+}]_o$, an additional aim of this study was to investigate these pathways as well in VSMCs.

The p53 tumor suppressor protein is a transcription factor that can be activated by numerous agents, including DNA damage, ionizing radiation, ultraviolet irradiation, ribonucleoside triphosphate depletion, metabolic stress, and aging as well as myocardial infarction, reperfusion injury, ischemia, atherogenesis, and stroke (for recent reviews, see Refs. 15, 43, 46, and 69). This protein is known to have key roles in the regulation of cell growth (for a recent review, see Ref. 69) and apoptosis (43, 45). It is known that exposure of tissues and cells to radiation or chemotherapeutic agents produces programmed cell death by a mechanism that leads to damage of DNA (genotoxic stress) and that p53 accumulates in cells when DNA is damaged (43, 46). Atherosclerotic plaques demonstrate DNA damage, activation of DNA repair pathways, increased expression of p53, and apoptosis (46). Recently, we (10, 11) have demonstrated that short-term Mg deficiency in intact rats leads to fragmentation of DNA and programmed cell death in ventricular, atrial, and vascular smooth muscles. We have also reported that rabbits exposed to dietary Mg defi-

ciency (for 2–8 wk) demonstrate rapid atherogenesis (13) and increased levels of p53 (histochemically) in the thickened plaques (B. M. Altura, B. T. Altura, and J. G. Stempak, unpublished observations). Although the precise mechanism(s) by which p53 can trigger apoptosis in cardiovascular tissues and cells is not known, recent studies (22, 60) in certain cell types have suggested that the upregulation of p53 is associated with the de novo synthesis of ceramide. We hypothesized that inhibition of p53 activation in Mg-deficient (MgD) vascular muscle cells would lead to a reduction in the de novo synthesis of ceramide.

Interestingly, in preliminary unpublished observations, we (B. M. Altura and B. T. Altura) have shown that the increases in blood pressure observed in rats placed on MgD diets are lowered when the animals are pretreated with known inhibitors of p53 (e.g., pifithrin). We (7, 10) have previously noted that incubation of primary vascular muscle cells with lower than normal levels of $[Mg^{2+}]_o$ resulted in the rapid activation of several DNA-binding subunits of NF-κB. Since, in view of these recent findings, SMS may be a modulator of p53 activation and NF-κB in pathogenic states, such as hypertension and atherogenesis, we hypothesized that short-term Mg deficiency would result in the upregulation of p53 in cardiac and vascular smooth muscles. The pathophysiological similarities between ceramide and p53 (i.e., both induce cell cycle arrest, guide cells to apoptosis, and are associated with DNA damage) (22) led us to examine this potential relationship in cardiovascular tissues and cells.

We designed experiments to determine whether 1) short-term Mg deficiency in rats would lead to the upregulation of SMS and p53 in cardiac and vascular smooth muscles, 2) inhibition of SMS in vascular muscle cells exposed to low levels of $[Mg^{2+}]_o$ would lead to a reduction in the levels of de novo ceramide synthesis, 3) inhibition of SPT and/or CS would lead to a reduction in the levels of de novo ceramide synthesis in VSMCs exposed to low $[Mg^{2+}]_o$, 4) an inhibitor of p53 upregulation induced by low $[Mg^{2+}]_o$ would lead to cellular reductions in the de novo synthesis of ceramide, and 5) imbibing low levels of a water-soluble Mg salt in drinking water would inhibit or reverse the predicted effects of dietary deficiency of Mg in cardiovascular tissues of rats.

MATERIALS AND METHODS

Animal diets, sera, and organ tissue collections. Mature male and female Wistar rats (200 ± 65 g) were used for all experiments. All experiments were approved by the Animal Use and Care Committee of the State University of New York Downstate Medical Center. Equal numbers of paired male and female animals were used for all nutrition experiments. Control (600 ppm Mg) and 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). All animals were given their respective diets for 21 days as previously described (10, 11). MgD animals were allowed to drink triply distilled water ($Mg^{2+} < 10^{-6}$ M) containing one of four different levels of Mg aspartate-HCl (0, 15, 40, or 100 mg/l Mg, Verla Pharm, Tutzing, Germany). All control animals received a normal Mg-containing diet (either 600 or 1,000 ppm) as well as triply distilled water to drink. On the 22nd day, sera and tissues (the left and right ventricles, atria, abdominal aorta between the superior mesenteric arteries, and renal arteries, cleaned of all connective tissues) were collected quickly after anesthesia (45 mg/kg im pentobarbital sodium). 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, similar to previously described methods (10, 11). Serum samples were analyzed within 2 h after collection, as previously described (10, 11). Total Mg levels were measured by standard techniques in our laboratory (Kodak DT-60 Analyzer, Ektachem Colorimetric Instruments, Rochester, NY). The method favorably compares with atomic absorption techniques for total Mg (12). A Mg^{2+}-selective electrode with a novel neutral carrier-based membrane (NOVA 8 Analyzer, NOVA Biomedical Instruments, Waltham, MA) was used to measure the free divalent cation in the sera (12). The ion-selective electrode was used in accordance with established procedures developed in our laboratory, having an accuracy and precision of 3% (12).

Biochemical tissue measurements of SMS and p53. For the SMS assays, tissues (0.1 g) were homogenized in a buffer containing 50 mM Tris·HCl (pH 7.5), 1.0 mM EDTA, 5% sucrose, and protease inhibitors (23). Homogenates were centrifuged at 5,000 rpm for 10 min, and the supernatant was used for the SMS activity assays (23). The reaction system contained 50 mM Tris·HCl (pH 7.4), 25 mM KCl, C6-NBD-ceramide (3 μg/ml), and PC (0.1 mg/ml). The mixture was incubated at 37°C for 2 h. Lipids were extracted in chloroform-methanol (2:1), dried under N_2 gas, and separated by TLC using chloroform-methanol-20% NH_4OH [14:6:1 (vol/vol/vol)] (23). Plates were scanned with a PhosphorImager (Molecular Dynamics, Sunnydale, CA), and the intensity of each band was measured using Image-Pro Plus version software (Media Cybernetics) (23).

For the p53 assay, we used a p53 EIA Assay Kit from Assay Designs (Ann Arbor, MI). The Assay Designs p53 TiterZyme Enzyme Immunometric Assay Kit is a complete kit for the quantitative determination of wild-type and mutant p53 in human, mouse, and rat samples. The kit uses a monoclonal antibody to p53, which is immobilized on a microliter plate so as to bind p53 to either samples or standards. Briefly, tissues were homogenized in 1–5 volumes of RIPA buffer (20 mM Tris·HCl, 0.5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxylate, 0.05% SDS, 1.0 mM PMSF, 1 μg/ml aprotinin, and 2 μg/ml leupeptin) on ice. Homogenates were then centrifuged at 10,000 g for 10 min. Supernatants were then collected and diluted in the assay buffer (1:5). Lyophilized authentic p53 standards were used. A polyclonal antibody to p53 labeled with horseradish peroxidase was added to the samples and standards. This polyclonal antibody binds to the p53 protein captured on the microliter plates. Standards and samples (100 μl) were placed in contact with 100 μl of the antibody working dilution (1:15) into the appropriate wells and then washed five times with 400 μl of wash solution. TMB substrate solution (3,3′,5,5′-tetramethylbenzidine and H_2O_2; 200 μl) was added to each well, and samples were incubated at room temperature on a plate shaker for 30 min at ~500 rpm. After this, 50 μl of stop solution (1 N sulfuric acid) was added to each well. Optical density at 450 nm (with correction between 570 and 590 nm) was then read with the appropriate blanks subtracted from each reading. The corrected sample optical density readings were next plotted against the standard curve for p53 (in pg/ml).

Isolation of vascular muscle and primary culture of aortic and cerebral VSMCs. Male mongrel (15 ± 3 kg) dogs ($n = 10–12$ dogs/group) were anesthetized with pentobarbital sodium (40 mg /kg iv) and killed by bleeding from the common carotid arteries. After a craniotomy, the brains were rapidly removed and placed in normal Krebs-Ringer bicarbonate (NKRB) solution at room temperature, and the middle cerebral and basilar cerebral arteries were excised and cleaned of arachnoid membranes and blood elements, as previously described (48, 73). Vessels were cut into segments of ~3–4 mm in length (48). Rat aortic and canine cerebral VSMCs were isolated according to established methods (73) in our laboratory ($n = 10–12$

animals/group) and cultured in DMEM containing 1.2 mmol/l $[Mg^{2+}]_o$, FCS, and antibiotics at 37°C in a humidified atmosphere composed of 95% air-5% CO_2 (73). After confluence had been reached, VSMCs were placed in media containing either 0.30, 0.6, or 1.2 mmol/l $[Mg^{2+}]_o$ for varying periods of time (120 min or 18–20 h). It should be stressed that these experiments using cell cultures and those below on primary VSMCs in culture were never part of the whole animal nutritional experiments (described above); these experiments and others were separate from the nutritional experiments.

Influence of $[Mg^{2+}]_o$ on ceramide levels in primary cultures of VSMCs. Cells were exposed for either 120 min or 18 h in NKRB solutions containing different concentrations of $[Mg^{2+}]_o$ (either 1.2 or 0.3 mM). We then extracted the lipids in the cells by first treating them with 0.1 M KOH in chloroform-methanol [1:2 (vol/vol)] at 37°C for 1 h. The ceramide was next converted into ceramide-1-[^{32}P]phosphate by *Esherichia coli* DAG kinase in the presence of [γ-^{32}P]ATP (50), and the lipids were then separated on high-performance TLC plates in a solvent system consisting of a chloroform-acetate-methanol-acetic acid-water [50:20:15:10:5 (vol/vol/vol/vol/vol)] mixture. After autoradiography, spots corresponding to ceramide-1-phosphate were carefully scraped into vials, and the radioactivity was then counted in a scintillation counter (LS-6500, Beckman). Quantitation of ceramide levels was based on standard curves of known amounts of authentic ceramide. The results were expressed as picomoles per 10^8 cells.

Influence of an inhibitor of SMS on the de novo synthesis of ceramide in VSMCs exposed to low $[Mg^{2+}]_o$. Before the cells were radiolabeled (as a measure of de novo ceramide synthesis), VSMCs were treated with either 50 or 350 μM of the potassium salt of D-609 (BioMol Research Laboratories, Plymouth Meeting, PA) for 3 h in NKRB solution [composed of (in mmol/l) 118 NaCl, 4.7 KCl, 1.2 $KHPO_4$, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 10 glucose, and 25 $NaHCO_3$] (1, 73). Precise concentrations of free Mg^{2+} in our cultures and modified NKRB solution were determined with our specific ion-selective electrode for Mg^{2+}. After the treatment of VSMCs with the above inhibitors, cells were labeled with [^{3}H]palmitic acid (4–20 μCi/ml) at 37°C for either 3 or 18 h, rinsed with fresh NKRB solution, and transferred to NKRB solutions (with the inhibitor D-609) containing either 0.30, 0.60, or 1.2 mmol/l $[Mg^{2+}]_o$, similar to previously described methods (48).

Influence of an inhibitor of SPT and an inhibitor of CS on the de novo synthesis of ceramide in VSMCs exposed to low $[Mg^{2+}]_o$. Before cells were radiolabeled (47), VSMCs were treated with either 75 μM fumonisin B1 (an inhibitor of CS; Sigma-Aldrich, St. Louis, MO) or 75 nM ISP-1(myriocin, an inhibitor of SPT, Sigma-Aldrich) for 3 h in NKRB solution. After the treatment of VSMCs with these inhibitors, VSMCs were labeled with [^{3}H]palmitic acid (4–20 μCi/ml) at 37°C for either 3 or 18 h, rinsed with fresh NKRB solution, and transferred to NKRB solutions (with either fumonisin B1 or ISP-1) containing either 0.3, 0.6, or 1.2 mmol/l $[Mg^{2+}]_o$.

Influence of an inhibitor of p53 on the de novo synthesis of ceramide in VSMCs exposed to low $[Mg^{2+}]_o$. Before cells were radiolabeled (48), VSMCs were treated with either 10 or 50 μM pifithrin-α (Sigma-Aldrich) for 3 h in NKRB solution. After the treatment of VSMCs with the inhibitor (in NKRB solution), cells were labeled with [^{3}H]palmitic acid (4–20 μCi/ml) at 37°C for 3 h and rinsed with fresh NKRB solutions (with 10 or 50 μM pifithrin) containing either 0.3, 0.6, or 1.2 mmol/l $[Mg^{2+}]_o$, similar to previously described methods (48).

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, linear correlation coefficients were calculated by the method of least squares. P values of <0.05 were considered significant.
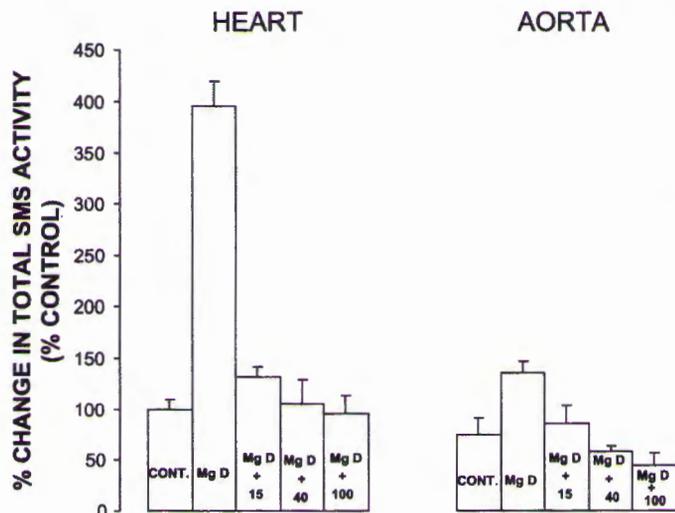
HEART   AORTA

Fig. 1. Sphingomyelin (SM) synthase (SMS) levels in the whole heart and aortic smooth muscles in normal and Mg-deficient (MgD) rats with and without Mg^{2+} added to their drinking water. Concentrations of Mg^{2+} per liter added to drinking water were as follows: 15 mg/l (MgD +15), 40 mg/l (MgD + 40), and 100 mg/l (MgD + 100). All values are means ± SE; $n = 10$–14 animals/group. MgD mean values were highly significantly different from controls (Cont; $P < 0.001$); MgD + 15, MgD + 40, and MgD + 100 values were all significantly different from MgD values ($P < 0.001$ by ANOVA).

RESULTS

Influence of diet on water consumption, food intake, and overall physiological condition. As recently demonstrated using an identical dietary regimen of Mg in controls and MgD animals (10, 11), 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 $mg·l^{-1}·day^{-1}$ Mg^{2+}, MgD + 40 $mg·l^{-1}·day^{-1}$ Mg^{2+}, or 100 $mg·l^{-1}·day^{-1}$ Mg^{2+} in drinking water] (10, 11). All of the MgD subgroups ($n = 10$–14 animals/group), irrespective of the amount of Mg in the diets or drinking water, showed no loss of 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 Mg diet ($n = 10$–14 animals/group) resulted in a total serum Mg level of ~1.00 ± 0.006 mM, whereas animals receiving the MgD diet demonstrated a total serum Mg level of ~0.4 ± 0.03 mM ($P < 0.05$).The serum level of ionized Mg in both the normal, control groups had a mean value of 0.6 ± 0.006 mM, whereas in the MgD group, the serum ionized level was reduced (by 50%) to 0.3 ± 0.01 mM ($P < 0.05$).

Feeding MgD animals various levels of Mg in their drinking water (as previously noted in Refs. 10 and 11) resulted in concentration-dependent rises in both the total and ionized serum levels of Mg. Feeding the animals 15 and 40 $mg·l^{-1}·day^{-1}$ Mg^{2+} in drinking water raised total serum Mg levels to 70% and 85%, respectively, of normal ($n = 10$–14 animals/group, $P < 0.05$), whereas feeding the animals 100 $mg·l^{-1}·day^{-1}$ Mg^{2+} in drinking water elevated serum total Mg levels to normal, i.e., 1.0 mM. With respect to the serum ionized level, feeding the MgD animals 100 mg/l Mg^{2+} in their drinking water restored the level to normal, whereas feeding

the MgD animals 15 and 40 mg/l Mg^{2+} in drinking water elevated serum ionized levels to 60% and 68% of normal.

Influence of dietary Mg intake on SMS levels in cardiac and vascular smooth muscles. Figure 1 shows that feeding rats a MgD diet for 21 days resulted in an ~400% rise in SMS enzymatic activity in ventricular muscles and an ~50% elevation in SMS activity in aortic vascular muscle cells ($P < 0.01$). Interestingly, feeding the MgD animals as little as 15 mg/l Mg^{2+} in drinking water completely prevented the rises in SMS activities in cardiovascular tissues ($P < 0.001$).

Influence of dietary Mg intake on p53 levels in cardiac and vascular smooth muscle. Figure 2 shows that feeding rats a MgD diet for 21 days resulted in ~900% rises in p53 in ventricular and vascular muscle cells and a rise of ~800% in atrial muscle cells ($P < 0.0001$). Feeding these MgD animals various levels of Mg^{2+} in drinking water, unlike that seen with SMS, demonstrated a range of sensitivities in the tissues. For example, although vascular and atrial muscle cells showed that as little as 15 mg/l Mg^{2+} in drinking water could significantly inhibit (by ~25%) the rise in p53 levels in these tissues ($P < 0.01$), much more of a Mg^{2+} intake (in drinking water) is needed (e.g., 40 or 100 mg/l Mg^{2+}) to significantly inhibit the rises observed in right and left ventricular muscles.

Influence of low $[Mg^{2+}]_o$ on ceramide levels in primary cerebral and aortic SMCs. Figure 3 shows that exposure of primary canine cerebral and rat aortic SMCs to low $[Mg^{2+}]_o$ resulted in an ~75% rise of ceramide levels after 120 min and over a 200% elevation in ceramide levels after 18 h.

Influence of low $[Mg^{2+}]_o$ and D-609 on the de novo synthesis of ceramide in cerebral and aortic smooth muscle. The results shown in Fig. 4 demonstrate that treatment of either cerebral arterial smooth muscle or aortic smooth muscle with low $[Mg^{2+}]_o$ produced both concentration- and time-dependent increases in the de novo synthesis of ceramide; the lower the concentration of $[Mg^{2+}]_o$, the greater the increase in the de novo synthesis of ceramide. In addition, our results demonstrate that the longer the exposure to the low $[Mg^{2+}]_o$, the greater the increase in the de novo synthesis of ceramide.

Treatment of vascular smooth muscles (in low $[Mg^{2+}]_o$) with D-609 resulted in both concentration- and time-

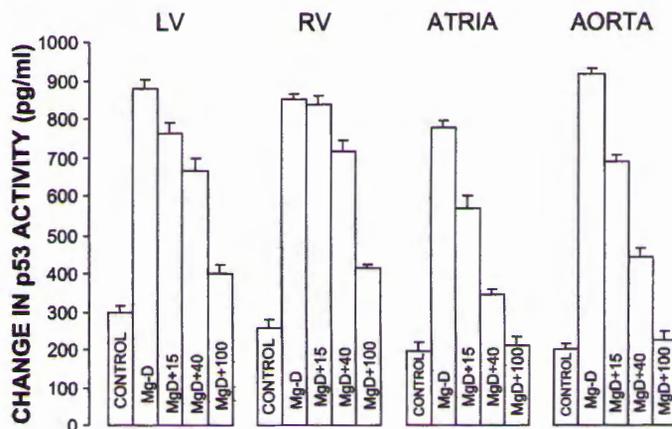LV   RV   ATRIA   AORTA

Fig. 2. Unit change in measured p53 levels in left ventricular (LV), right ventricular (RV), atrial, and aortic smooth muscles in normal and MgD rats with and without Mg^{2+} added to their drinking water. Mean values for MgD animals were highly significantly different from controls ($P < 0.001$). All subgroup MgD + 100 values, except for the atria, were significantly different from controls ($P < 0.05$).
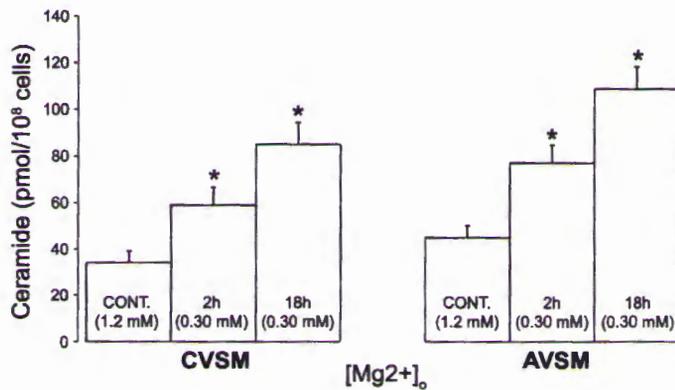
Fig. 3. Influence of extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) on ceramide levels in primary cultures of cerebral (CVSM) and aortic vascular smooth muscle (AVSM) cells. Values are means ± SE; n = 10–12 animals/group and at least 300–450 cells/group. *Values were significantly different from controls ($P < 0.01$). Mean values for CVSM and AVSM at 18 h were significantly different from mean values at 2 h ($P < 0.01$).

dependent inhibition of the rise in the de novo synthesis of ceramide (Fig. 4).

Influence of inhibitors of SPT and CS on the de novo synthesis of ceramide in cerebral and aortic smooth muscles exposed to low $[Mg^{2+}]_o$. The data shown in Fig. 5 demonstrate that treatment of VSMCs, exposed to low $[Mg^{2+}]_o$, with either fumonisin B1 or ISP-1 resulted in inhibition of the elevations in ceramide induced by the reduced levels of $[Mg^{2+}]_o$. Relatively, it appears that ISP-1 induces a much greater inhibition than fumonisin B1. However, although not shown, not even higher concentrations of ISP-1 (up to 250 nM) were able to produce complete inhibition of the de novo synthesis of ceramide induced by exposure to low $[Mg^{2+}]_o$.

Influence of pifithrin on the de novo synthesis of ceramide in cerebral and aortic smooth muscles exposed to low $[Mg^{2+}]_o$. The results shown in Fig. 6 demonstrate that treatment of either cerebral arterial smooth muscle or aortic smooth muscle (in low $[Mg^{2+}]_o$) with pifithrin produced both concentration-de-

pendent and time-dependent inhibition of the de novo synthesis of ceramide; the higher the concentration of pifithrin, the greater the inhibition of the de novo synthesis of ceramide induced by low $[Mg^{2+}]_o$. In addition, like that observed with D-609, the longer the exposure to pifithrin, the greater the inhibition of the de novo synthesis of ceramide.
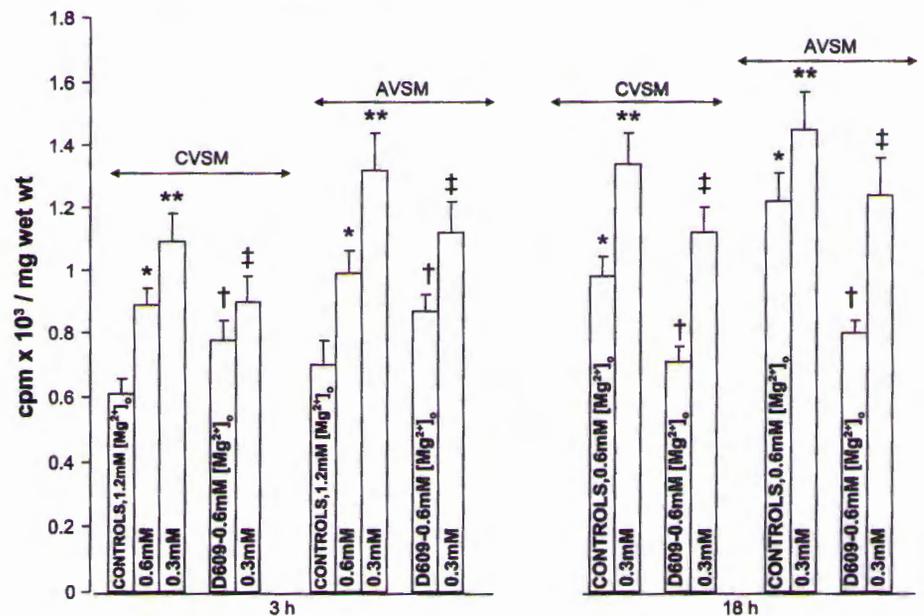
Interestingly, a comparison of the concentration and time dependency of both D-609 and pifithrin in causing inhibition of the de novo synthesis of ceramide seemed to indicate a parallel.

DISCUSSION

The results reported here are the first demonstration that short-term dietary deficiency of Mg in an intact mammal results in the activation of SMS and p53 in diverse cardiovascular tissues and cells. To our knowledge, this is the first time anyone has shown an upregulation of SMS and p53 by Mg deficiency of any cell type in any species. The de novo pathway of sphingolipid synthesis has gained considerable attention over the past decade (23, 29, 39, 52, 53). SMS transfers the phosphocholine group from PC to ceramide, thus generating SM and DAG (29). SMS, therefore, can directly regulate, in opposite directions, ceramide and DAG levels within cells.

Our new experiments confirm and add further support to the concept that lowered levels of Mg can lead to the formation of ceramide in cardiovascular tissues and cells (48). We also demonstrate, for the first time, that low $[Mg^{2+}]_o$ leads to the de novo synthesis of ceramide in cerebral and peripheral VSMCs and that this de novo ceramide synthesis is due to the activation of at least three enzymes in the sphingolipid pathway, namely, SMS, SPT, and CS. SPT catalyzes the first step in the biosynthesis of sphingolipids, namely, the condensation of L-serine with palmitoyl-CoA, giving rise to 3-ketosphinganine (leading to the formation of ceramides) (34, 51, 68); we (11) have recently demonstrated that short-term Mg deficiency (in the same model used here) upregulates SPT in the cardiovascular tissues examined in the present study. 3-Keto-sphinganine is reduced to form sphinganine, which is then acylated by CS to

Fig. 4. Influence of D-609 on [³H]palmitic acid incorporation into CVSM and AVSM as a function of $[Mg^{2+}]_o$ during either a 3- or 18-h incubation period. [³H]palmitic acid incorporation was used as a measure of the de novo synthesis of ceramide. Values are means ± SE; n = 10–12 animals/group. *Experimental mean values are significantly different from their respective control values in the paired Mg^{2+} concentrations ($P < 0.05$). **Mean values are significantly different from their respective control values and mean values in 0.6 mM $[Mg^{2+}]_o$ ($P < 0.05$ by ANOVA). †Mean values in D-609 are different from their respective paired control values in identical concentrations of $[Mg^{2+}]_o$ ($P < 0.05$). ‡Mean values are different from their respective paired values in identical concentrations of $[Mg^{2+}]_o$ ($P < 0.05$). Mean values at 18 h are significantly different from their respective paired values at 3 h ($P < 0.01$).
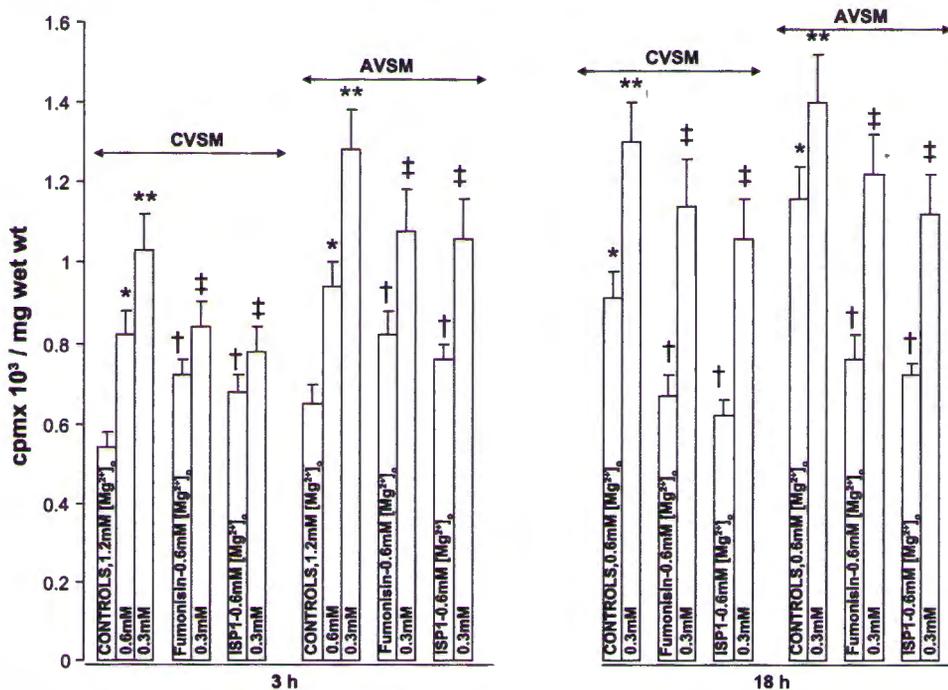
Fig. 5. Influence of fumonisin B1 and myriocin (ISP-1) on [³H]palmitic acid incorporation into CVSM and AVSM as a function of $[Mg^{2+}]_o$ during either a 3- or 18-h incubation period. Values are means ± SE; $n = 10$–12 animals/group. *Experimental mean values are significantly different from their respective paired mean control values ($P < 0.01$). **Mean values are significantly different from their respective control values as well as mean values in 0.6 mM $[Mg^{2+}]_o$ ($P < 0.01$ by ANOVA). †Mean values in either fumonisin B1 or myriocin are significantly different from their paired control values as well as their paired values in identical concentrations of $[Mg^{2+}]_o$ in the absence of fumonisin B1 or myriocin ($P < 0.05$ by ANOVA). ‡Mean values that are significantly different from controls and their respective paired mean values in the absence of the inhibitor and mean values denoted by single dagger ($P < 0.05$ by ANOVA). Mean experimental values at 18 h are significantly different from their respective paired mean values at 3 h ($P < 0.01$). cpm, Counts per minute.

produce dihydroceramide and desaturated to generate ceramide (53). We show in the present study that inhibition of SMS, SPT, or CS by specific inhibitors results in a marked reduction in the de novo synthesis of ceramide (as measured by the uptake of [³H]palmitic acid) in cerebral and peripheral VSMCs.

It has been widely demonstrated that ceramides can be produced in many types of cells and tissues when they are exposed to ultraviolet radiation, ionizing radiation, endotoxins, cytokines, retinoic acid, balloon injury of carotid arteries, phorbol esters, serum deprivation, and daunorubicin as well as in etoposide-induced apoptosis, among other agents (16, 19,

28, 29, 34, 51, 54, 56, 67, 73). Many of these agents activate SPT, CS, SMS, and SMase to produce ceramides in many cell types (19, 28, 34, 39, 52, 53). Ceramide synthesis and release appears to be the active messenger in most of these events and agencies. The present study suggests that Mg deficiency should be added to the list of stimuli known to activate SMS, SPT, and CS pathways, at least in rats. One of the major pathways leading to ceramide generation is via the hydrolysis of SM through the activation of SMases (16, 31). Previously, we have shown that the production of low-$[Mg^{2+}]_o$ environments, either in vivo (e.g., identical model of Mg deficiency used here) (10, 11) or in primary cultured vascular cells (48), results in the
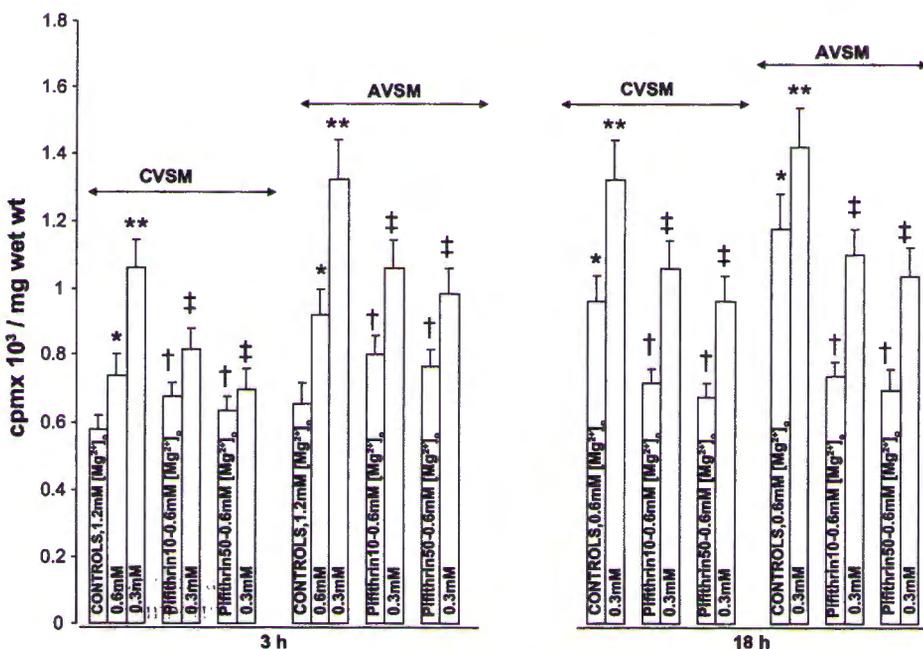

Fig. 6. Influence of pifithrin on [³H]palmitic acid incorporation into CVSM and AVSM as a function of $[Mg^{2+}]_o$ during either a 3- or 18-h period. Values are means ± SE; $n = 10$ animals/group. *Mean values are significantly different from their respective control mean values ($P < 0.01$). **Mean values which are significantly different from their respective control values and the mean values in 0.6 mM Mg ($P < 0.05$ by ANOVA). †Mean values for pifithrin (10 or 50 μM) in 0.6 mM $[Mg^{2+}]_o$ are significantly different from their respective paired values in the absence of pifithrin ($P < 0.05$). ‡Mean values for pifithrin (10 or 50 μM) in 0.3 mM $[Mg^{2+}]_o$ are significantly different from their respective paired values in the absence of pifithrin ($P > 0.05$). Mean paired values at 18 h are significantly different from their respective mean values at 3 h.

activation of SMase and SPT (SPT-1 and SPT-2) and the production of ceramide. Thus, collectively, the present work, when viewed in light of the latter experiments, indicates that ceramide is most likely generated in cardiovascular tissues and cells in low $[Mg^{2+}]_o$ by four major enzymes in the sphingolipid pathway.

The data presented here, as exemplified by both measurement of basal levels of ceramide in VSMCs and assay of de novo production of ceramide in these cells (as measured by the uptake of [^{3}H]palmitic acid), clearly demonstrate that exposure to lowered levels of $[Mg^{2+}]_o$ (i.e., 0.30 and 0.60 mM) results in sizable quantities of this putative messenger, which, in large measure, could be responsible for the activation of apoptotic events in vascular and cardiac muscles observed in MgD animals (10, 11, 64) and in primary cultured VSMCs (38). It is now widely accepted that ceramides play leading roles in apoptotic events in a number of cells and tissues after treatment with a variety of agents (and agencies), including ionizing radiation, ultraviolet light, IL-1, INF-γ, TNF-α, chemotherapeutic agents, endotoxins, and oxidative stresses (16, 19, 28, 31, 53, 56, 62). We have found that several different ceramides (e.g., C_2-ceramide, C_6-ceramide, and C_{16}-ceramide) can acutely induce apoptosis in primary canine cerebral vascular and rat peripheral (i.e., mesenteric arterial and aorta) VSMCs, as verified by several types of assays (i.e., TUNEL, acridine orange, propidium iodide, annexin V, and caspase-3) (Ref. 10 and unpublished observations). Low-Mg^{2+} environments exacerbated both the rapidity and degree of programmed cell death in these cells (Refs. 10, 11, and 38 and unpublished observations). The addition of either myriocin (a specific inhibitor of SPT-1 and SPT-2 biosynthesis), fumonisin B1 (a specific inhibitor of CS), or D-609 (an inhibitor of SMS) attenuated, to different degrees (50–80%), apoptotic events in these cells induced by low levels of $[Mg^{2+}]_o$, suggesting that the biosynthesis of ceramides via the de novo pathway (as demonstrated by the uptake of [^{3}H]palmitic acid) was, most likely, accounting for a large part of the MgD-induced apoptotic events in the vascular cells (unpublished observations), thus bolstering our hypothesis. We hypothesize that the remainder of the Mg deficiency-induced apoptosis in the vascular cells (and probably the cardiac cells) can be, most likely, attributed to the synthesis and release of ceramides via the activation of SMase. The fact that 21 days of short-term Mg deficiency (similar to the present protocol), in rats, resulted in DNA fragmentation, the release of cytochrome c, and the activation of caspase-3 in ventricular, atrial, and vascular SMCs (Refs. 10 and 11 and unpublished observations) lends further support to our hypothesis. The present study, when viewed in light of in vitro work on perfused rat hearts obtained from MgD animals (71) and in vitro studies from our laboratory on perfused working rat hearts (6, 72), demonstrate that even short-term Mg deficiency results in reductions in a variety of hemodynamic cardiac functions. These previous studies on perfused rat hearts clearly show 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 the cellular loss of intracellular Mg^{2+} concentration, activation of SMS, SMase, and p53, increased cellular and plasma levels of ceramide, and programmed cell death.

It is now accepted that apoptotic events play major roles in the development of atherogenesis and hypertension (15, 35). Although it has been suggested that sphingolipids might play important roles in the pathophysiology of these cardiovascular pathogenic events, by unknown effects on vascular smooth muscles (2, 10, 17), up until our studies began >15 yr ago (2, 47, 48), the importance of Mg deficiency and its relation to sphingolipid metabolism were not known.

One of ceramide's major pathophysiological actions is its ability to induce cell differentiation and transformation (28, 29, 50, 62, 67). Abnormal cell differentiation, transformation, and growth are key events in the development of both atherogenesis (15, 35) and hypertension (30, 35, 49). Hyperplasia and cardiovascular hypertrophy are common events in hypertension and key elements of target organ damage. However, the precise mechanisms regulating alterations in tissue mass are not completely understood. Approximately 15 yr ago, apoptosis was identified as a mechanism of cardiomyocyte damage in heart failure (64). The tumor suppressor protein p53 is known to play key roles in cell transformation, growth, and apoptotic events (43, 45, 46). Both ceramide and p53 can induce cell cycle arrest (and senescence), induce programmed cell death, and are associated with DNA damage (genotoxic events) (22, 43, 45, 46, 69). It has been previously demonstrated that Mg deficiency can produce all three of these patophysiological events in several cell types, including cardiac and vascular SMCs (Refs. 3, 7, 9, 10, 11, 38, 55, 63, and 71 and unpublished observations). Approximately 10 yr ago, Dbalbo et al. (22) demonstrated that when Molt-4 leukemic cells are exposed to low concentrations of either actinomycin D or γ-irradiation, p53-dependent apoptosis was induced, which was rapidly followed by an increase in ceramide levels, suggesting that p53 may regulate ceramide levels in certain cells and tissues. Recently, these investigators confirmed their earlier findings and found that the SPT inhibitor ISP-1 (used here) and the CS inhibitor fumonisin B1 (also used here) led to a marked attenuation in de novo ceramide generation in response to p53 stimulation (52). These investigators suggested that p53 "specifically drives de novo ceramide synthesis by activation of a CS" (52). Our present study suggests that MgD environments drive ceramide synthesis, at least in VSMCs, via the activation of three enzymes in the sphingolipid pathway: SPT, SMS, and CS. In addition, the present study clearly shows that, in at least two different types of VSMCS, inhibition of upregulation of p53 (as induced by Mg deficiency) results in marked reductions in the de novo synthesis of ceramide. It should be pointed out that atherosclerotic plaques in vascular walls in hypertension have been shown to demonstrate considerable DNA damage, activation of DNA repair pathways, increased expression of p53, apoptosis (14, 34, 45), and increased levels of ceramide (17). Experimentally, Mg deficiency can result in accelerated atherogenesis in rabbits (13), which is associated with increased levels of p53 in the thickened atherosclerotic plaques (J. G. Stempak, B. M. Altura, and B. T. Altura, unpublished observations). In preliminary studies, we found that inhibitors of ceramide generation (i.e., fumonisin B1 and ISP-1) as well as pifithrin lowered arterial blood pressures toward normal levels in Mg deficiency-induced hypertension in living rats (unpublished observations). We thus hypothesize that Mg deficiency probably plays key roles in the generation of hypertension via the upregulation of p53, SMS, SPT, and CS,

particularly as the majority of individuals who consume Western types of diets have 30–65% short falls in daily dietary Mg intake (2, 26, 57, 58).

Although it has been repeatedly shown that prolonged administration of Mg^{2+} (oral and intravenous) can lower blood pressure in both experimental and clinical forms of hypertension (2–5, 18, 40, 57, 58), the precise mechanism(s) is not known. It has been suggested, often, that Mg^{2+} lowers blood pressure by promoting vasodilation and decreasing work load on the myocardium via direct actions on Ca^{2+} channels (and cellular redistribution) in vascular and cardiac muscle cells (1–6, 18, 57, 59). In view of our present findings and those previously published (10, 11, 47, 48), we believe that Mg's effects on ceramide and sphingolipid metabolism must now be taken into consideration in helping to explain the blood pressure-lowering actions of this divalent cation.

Over the past 40 yr, epidemiological and experimental evidence has been published that suggests a striking linkage between dietary deficiency of Mg and diverse types of cardiovascular maladies, e.g., hypertension, atherogenesis, coronary artery disease, congestive heart failure, sudden cardiac death, vasospasm, irregular heart rhythms, peripheral arterial diseases, diabetic vascular-related diseases, myocardial infarction, free radical generation, dyslipidemias, and strokes (2–6, 10, 11, 13, 14, 21, 24, 25, 40, 55, 57, 59, 65, 71, 73). More than 50 yr ago, Kobayashi (33) 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 five decades from a large number of studies from different areas of our planet (24, 25, 37, 41, 42); the death rates by sudden cardiac death are lower in hard water areas than in soft water areas. Despite the fact that the hardness of water is due to the concentration of Ca^{2+} and/or Mg, the overwhelming evidence, to date, supports the idea that it is the Mg content that is responsible for most of the protective effects of hard water (24, 41, 58). More than two decades ago, it was suggested that as little as 15–30 $mg \cdot l^{-1} \cdot day^{-1}$ Mg^{2+} in drinking water should be cardioprotective (37, 41). Recently, using the same model of dietary deficiency of Mg as in the present study (21 days of MgD), we showed, for the first time, in well-controlled experiments that as little as 15 $mg \cdot l^{-1} \cdot day^{-1}$ Mg^{2+}, in drinking water, either prevented or ameliorated the formation of ROS, DNA fragmentation, caspase-3 activation, mitochondrial release of cytochrome c, activation of apoptosis, hydrolysis of SM, upregulation of SPT-1 and SPT-2, and elevation of ionized Ca^{2+} (10 ,11). Although the present work indicates that as little as 15 $mg \cdot l^{-1} \cdot day^{-1}$ Mg^{2+} in drinking water prevents the upregulation of SMS in cardiac and vascular muscles, something in excess of 40, but <100, $mg \cdot l^{-1} \cdot day^{-1}$ Mg^{2+} in water must be imbibed to prevent the upregulation of p53 in these tissues, at least in rats. From the present data as well as those previously published (10, 11), it could be speculated that between 15 and 50 $mg \cdot l^{-1} \cdot day^{-1}$ of water-borne Mg^{2+} should be both cardioprotective and vascular protective. Such an insight, to our knowledge, has not been possible to advance until our experiments.

While the activation of SMS and upregulation of p53 most likely play important roles in the biological synthesis of ceramide in Mg deficiency, it must be pointed out that until further

studies are done, this could be one of many ways in which Mg deficiency is a cardiovascular risk factor.

We believe, at the very least, that this study, when taken together with our previous studies (10, 11), strengthens support for the hypothesis suggested more than two decades ago (37, 41), that water intake (e.g., from tap waters, well waters, bottled waters, and beverages using tap/well waters) in humans varying between 1 and 2 l/day, with Mg^{2+} intakes varying from <5 to >100 mg/l, may, as we have recently suggested (10, 11), represent an excellent way to overcome and control the marginal intakes of Mg obtained with most Western diets. In addition, in view of our previous findings and those presented here, it is probably propitious to suggest that all desalinated-purified recovered/recycled waters, harvested rainwaters, well waters, tap waters, and all bottled waters given to humans should be supplemented with bioavailable Mg^{2+} to ameliorate/prevent the induction of cardiovascular risk factors and disease processes worldwide.

DISCLOSURES

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

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