Dietary Magnesium Intake Influences Circulating Pro-Inflammatory Neuropeptide Levels and Loss of Myocardial Tolerance to Postischemic Stress

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Severe dietary Mg restriction (Mg_9, 9% of recommended daily allowance [RDA], plasma Mg = 0.25 m*M*) induces a pro-inflammatory neurogenic response in rats (substance P [SP]), and the associated increases in oxidative stress *in vivo* and cardiac susceptibility to ischemia/reperfusion (I/R) injury were previously shown to be attenuated by SP receptor blockade and antioxidant treatment. The present study assessed if less severe dietary Mg restriction modulates the extent of both the neurogenic/oxidative responses *in vivo* and I/R injury *in vitro*. Male Sprague-Dawley rats maintained on Mg_{40} (40% RDA, plasma Mg = 0.6 m*M*) or Mg_{100} (100% RDA, plasma Mg = 0.8 m*M*) diets were assessed for plasma SP levels (CHEM-ELISA) during the first 3 weeks and were compared with the Mg_9 group; red blood cell (RBC) glutathione and plasma malondialdehyde levels were compared at 3 weeks in Mg_9, Mg_{20} (plasma Mg = 0.4 m*M*), Mg_{40}, and Mg_{100} rats; and 40-min global ischemia/30-min reperfusion hearts from 7-week-old Mg_{20}, Mg_{40}, and Mg_{100} rats were compared with respect to functional recovery (cardiac work, and diastolic, systolic, and developed pressures), tissue LDH release, and free radical production (ESR spectroscopy and α-phenyl-*N*-tert butylnitrone [PBN; 3 m*M*] spin trapping). The Mg_{40} diet induced smaller elevations in plasma SP (50% lower) compared with Mg_9, but with a nearly identical time course. RBC glutathione and plasma malondialdehyde levels revealed a direct relationship between the severity of oxidative stress and hypomagnesemia. The dominant lipid free radical species detected in all I/R groups was the alkoxyl radical (PBN/alkoxyl: α_H = 1.93 G, α_N = 13.63 G); however, Mg_{40} and Mg_{20} hearts exhibited 2.7- and 3.9-fold higher alkoxyl levels, 40% and 65% greater LDH release, and lower functional recovery (Mg_{20} < Mg_{40}) compared with Mg_{100}. Our data suggest that varying dietary Mg intake directly influences the magnitude of the neurogenic/oxidative responses *in vivo* and the resultant myocardial tolerance to I/R stress. Exp Biol Med 228:665–673, 2003

Key words: dietary magnesium; substance P; glutathione; malondialdehyde; postischemic heart recovery; postischemic free radicals

Clinical evidence is strong for an association between Mg deficiency and an increased incidence of cardiovascular disease. Populations consuming less Mg from their diet or water are more prone to life-threatening arrhythmia, vasospasm, and have a higher risk of sudden death with congestive heart failure than those with higher intakes (1). Other studies also implicate a link between Mg depletion and clinical myocardial infarction (MI) (2), based largely on observations of lower myocardial and serum Mg content in acute MI patients. Animal model studies of severe Mg restriction have demonstrated progressive cardiovascular lesion formation, heightened inflammatory cell infiltration (3), decreased levels of endogenous antioxidants (glutathione, vitamin E, and ascorbate) (4, 5), and higher plasma levels of pro-oxidant metals (6) and lipid peroxidation products (7, 8). Other investigations suggest that preexisting Mg deficiency amplifies myocardial vulnerability to toxic agents (1) and imposed stresses (9). Dogs placed on a magnesium-deficient (MgD) diet developed larger infarcts than Mg-sufficient (Mg_{100} = 100% of recommended daily allowance [RDA]) animals after regional ischemia/reperfusion (I/R) (10). Moreover, Langendorff-perfused postischemic hearts from MgD rats displayed significantly lower functional recovery than the Mg_{100} group (9). In agreement with this study, we (11) showed that working hearts from rats placed on a severe MgD diet (Mg_9 = 9% RDA) displayed greater postischemic mechanical dysfunction and tissue injury, as well as heightened production of lipid peroxidation (LPO)-derived free radicals (alkoxyl, $LO^{\bullet}$) compared with the Mg_{100} group. Antioxidant treatment (vitamin E and probucol) *in vivo* during the Mg_9 dietary period significantly reduced cardiac perivascular lesion formation *in vivo* (3) and reperfusion injury *in vitro*

This work was supported by PHS Grants NIH RO1-HL62282 and RO1-HL-65178.

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Received July 25, 2002.
Accepted December 17, 2002.

1535-3702/03/2286-0665$15.00

(11), indicating that a pro-inflammatory/pro-oxidant condition developed during exposure to the Mg_9 diet.

Mg_9 rats also experience an early neurogenic inflammatory response (elevated plasma substance P [SP] levels between dietary Days 3 and 8) (12, 13), which preceded other observed inflammatory events (elevated circulating histamine and cytokines between Days 9 and 21; myocardial leukocyte infiltration and inflammatory lesions after 3 weeks) (14, 15), and myocytic necrosis (after 4 weeks) (16). The suggestion that pro-inflammatory neuropeptides may trigger a cascade of inflammatory/pro-oxidant events in this model (13) has received support from studies using specific neurokinin-1 (NK-1) receptor antagonists to induce SP receptor blockade *in vivo* (17–19). *In vivo* treatment of Mg_9 rats with SP receptor antagonists (L-703,606 or CP-96,345) (12, 17, 18) significantly reduced oxidative stress *in vivo* (preserved red blood cell [RBC] glutathione levels, decrease plasma malondialdehyde [MDA] content, decreased circulating and myocardial inflammatory cytokine levels, and reduced myocardial lesion formation), while improving postischemic tolerance of rat hearts *in vitro* (greater functional recovery, and lower tissue LDH release, protein oxidation, and lipid radical and lipid hydroperoxide production) (8, 11, 19). Interestingly, postischemic Mg_{100} rat hearts did not benefit from long-term *in vivo* treatment with L-703,606, and acute *in vitro* treatment failed to provide protection (19). Collectively, these findings suggest that excessive SP bioactivity mediated through the NK-1 receptor during severe Mg restriction must be a critical early initiator of the pro-inflammatory/pro-oxidative cascade *in vivo*, which alters myocardial susceptibility to postischemic stress *in vitro*.

In this light, it is reasonable to predict that manipulations that reduce neuronal release and/or circulating levels of SP should also provide benefits against the pathology and heightened sensitivity to postischemic stress seen with Mg deficiency. In the current study, we tested the hypothesis that varying dietary Mg intake (9%, 20%, 40%, and 100% RDA) can exert direct influences on the magnitude of circulating neuropeptide levels and oxidative stress *in vivo*, as well as the loss of myocardial tolerance to I/R stress *in vitro*. This study may have clinical ramifications because the moderate Mg-restricted diets in use (20% and 40% RDA) lead to hypomagnesemic conditions that are comparable with clinically observed levels (20).

Materials and Methods

Chemicals. All reagents and solvents were from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The TEMPO free radical standard and spin trap α-phenyl-*tert*-butylnitrone (PBN) were from Aldrich Chemicals (Milwaukee, WI), and lactate dehydrogenase (LDH) assay kits and alkaline phosphatase used for antibody labeling were purchased from Sigma. Specific antibodies directed against neuropeptides of interest were from Chemicon International (Temecula, CA), and lyophilized neuropeptides were from Bachem Bioscience (King of Prussia, PA). Heavy metal levels (Shimadzu flame emission spectrophotometer) in all solutions were below the limits of detection.

Animal Assurance. All animal experiments were guided by the principles for the care and use of laboratory animals as recommended by the U.S. Department of Health and Human Services and approved by The George Washington University Animal Care and Use Committee.

Dietary Model. Age-matched male Sprague-Dawley rats (150–175 g) were place on a low Mg diet (Mg_9 = 1.04–1.8 mmol Mg/kg feed = 5%–9% RDA), or this diet supplemented with an additional 4.0 (Mg_{20} = 20%–25% RDA), 8.0 (Mg_{40} = 40%–45% RDA), or 20.0 (Mg_{100} = 100%–105% RDA) mmol Mg oxide/kg for up to 7 weeks (11). The ranges provided for the RDA reflect the variations in background Mg in food lots. The U.S. National Research Council's RDA for normal rat maintenance and growth was estimated to be 500 ppm (21). Our Mg_{100} diet contained 509–528 ppm, including the background Mg in the feed. Diet composition (Harlan Teklad, Madison, WI) and animal housing conditions have been described (22).

Circulating Neuropeptides. Tail bleed samples (0.7 ml) were collected during the first 3 weeks of each diet and were spun in EDTA-containing (10 μl of 15%) centrifuge tubes. Plasma SP concentrations were determined by CHEM-ELISA using specific alkaline phosphatase-labeled antibodies directed against the neuropeptide (23, 24). Assays were run on fractions separated using an ISCO 3140 high-performance capillary electrophoresis system (HPCE) (17). Capture ELISA using monospecific antibodies to the neuropeptide was used to confirm results. Area integration of dietary time courses allowed determination of total plasma SP levels.

Red Blood Cell Glutathione, Plasma MDA, and Mg Levels. Total cellular glutathione (GSH + GSSG) levels were determined in packed RBC samples collected in heparinized tubes at dietary Day 21 (5). After a 50-fold dilution, hemolyzed samples were acidified with 5% 5-sulfosalicylic acid to preserve GSH. Using the "cyclic method," which combines the colorimetric reaction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) with the enzymatic specificity of GSSG reductase, the reaction was followed spectrometrically (412 nm) with (for GSSG) or without (for GSH) the presence of 2% vinyl pyridine. Using heparinized blood samples, plasma MDA levels at dietary Day 21 were measured by the thiobarbituric acid-reactive substance (TBARS) method (5). Changes in plasma Mg levels were determined by atomic absorption spectroscopy after stable hypomagnesemia had been achieve (beyond dietary Day 7) (22).

Isolated Working Rat Heart and Postischemia Model. The working heart and global I/R models were previously described (11). Cannulated hearts (nonpaced) were perfused (nonrecirculating, 37°C, pH 7.4, 95% O_2:5% CO_2) with physiologic Krebs-Henseleit buffer (KHB) supplemented with 5 m*M* glucose. After 30 min of stabili-

zation, baseline hemodynamic and biochemical measurements were taken, followed by 40-min normothermic low-flow ischemia (~0.1 ml/min coronary flow rate), and 30-min reperfusion. Parameter measurements (cardiac pressure-volume work, coronary flow rate, cardiac output, and mean aortic diastolic pressure, left ventricular peak systolic, and developed pressures via P23 Gb Statham transducers) were repeated for estimates of postischemic recovery (11). Preischemic hemodynamic parameters were not significantly different between dietary groups (Table I). LDH release into the effluent indicates tissue injury (11), and was measured spectrometrically using Sigma assay kits. Area integration of reperfusion time courses provided values for total effluent LDH activity.

Spin Trapping in Postischemic Model. PBN spin trap was used for detection and quantification of LPO-derived free radicals ($L^{\bullet}$ = alkyl; $LO^{\bullet}$ = alkoxyl) (11). The PBN solution (120 m*M* in isosmotic saline) was diluted to 3 m*M* by infusion into the aortic perfusion line of the heart. Infusion occurred during the last 5 min of control perfusion and the initial 15 min of reperfusion, when most free radical production occurs in this model (11, 25). Coronary effluent samples (5 ml) were extracted with toluene before ESR spectroscopy (11, 19).

ESR Spectroscopy. Toluene-extracted effluent was transferred into 5-mm (i.d.) quartz ESR tubes and was flushed with N_2 gas before ESR measurement (11, 19). ESR analysis was performed at 12.8°C with a Bruker ER 100 series, X-band spectrometer using previously described settings. An EPR data acquisition system (Scientific Software Services, Bloomington, IL) was used for signal verification and averaging (2×). Signal intensities were measured and PBN adduct content was determined using TEMPO nitroxide radical as an integration standard (11). Area integration of reperfusion time courses provided estimates of total free radical production.

Statistical Approaches. Analysis of variance was used to compare several means, and the Tukey test was used for all paired comparison. Significance was considered at $P < 0.05$. Least squares linear regression analysis was applied to correlate changes in magnitude of postischemic measurements.

Results

Rats placed on Mg_9, Mg_{40}, and Mg_{100} diets displayed proportional changes in their plasma Mg concentrations, indicating that varying dietary Mg content can alter the severity of hypomagnesemia in this model (Fig. 1). The Mg_{40} and Mg_9 diets led to 25.2% and 69.2% reductions in plasma Mg, respectively, compared with the Mg_{100} group. Figure 2A compares circulating SP levels and 3-week detection time courses for Mg_9, Mg_{40}, and Mg_{100} rats. Whereas the Mg_{100} rats displayed only low plasma SP levels throughout the 3-week dietary period, rats placed on increasingly restricted Mg-diets exhibited proportionately greater neuropeptide levels ($Mg_9 > Mg_{40} > Mg_{100}$). Interestingly, the biphasic detection time course in the Mg_{40} group was identical to that of the Mg_9 rats: significant elevations in SP levels began at dietary Day 3, transiently peaked on Day 7, fell by Day 11, and a secondary rise in levels emerged between Days 15 and 21 (14). Changes in total circulating SP levels from Mg_9, Mg_{40}, and Mg_{100} rats during the 21-day period clearly demonstrated that the magnitude of its rise can be modulated by the degree of dietary Mg restriction (Mg_{40} and Mg_9: 2.43- and 5.14-fold higher than Mg_{100}; Fig. 2B).

The association between dietary Mg content and the magnitude of the neurogenic response *in vivo* led us to predict that the severity of any SP-triggered inflammatory/pro-oxidant event(s) may also be modulated by the degree of dietary Mg restriction. This possibility was examined by monitoring changes in levels of *in vivo* oxidative stress markers, RBC glutathione, and plasma MDA in Mg-restricted rats. An additional dietary group (Mg_{20} yielding plasma Mg = 0.403 ± 0.034 m*M*) was introduced to more fully establish the dependency on Mg dietary content. Figure 3A demonstrates that the decline in RBC glutathione levels was directly proportional to the extent of dietary Mg restriction: levels from Mg_{40}, Mg_{20}, and Mg_9 rats fell 20.6%, 29.4%, and 50% compared with the Mg_{100} group. An inverse relationship occurred for plasma MDA formation: levels from Mg_{40}, Mg_{20}, and Mg_9 rats were elevated 20% (nonsignificant), 60%, and 148% compared with Mg_{100} rats (Fig. 3B).

Because a significant neurogenic response and systemic oxidative stress occurred even with the less severe Mg-

Table I. Preischemic Hemodynamic Properties of Perfused Hearts from Dietary Mg-Restricted and -Normal Rats

Preischemic parameter	Mg_{100}	Mg_{40}	Mg_{20}
Coronary flow rate (ml/min)	21.5 ± 1.0	22.6 ± 1.0	22.9 ± 1.6
Cardiac output (ml/min)	63.0 ± 1.9	60.7 ± 2.9	58.0 ± 4.9
Systolic pressure (mmHg)	132.9 ± 8.0	125.3 ± 2.3	123.4 ± 5.6
Aortic diastolic pressure (mmHg)	52.0 ± 1.6	51.6 ± 2.3	53.0 ± 1.8
Developed pressure (mmHg)	135.8 ± 8.0	128.1 ± 2.6	127.6 ± 6.0
Cardiac work (kg-m/g dwt/min)	0.504 ± 0.023	0.478 ± 0.19	0.466 ± 0.04

Note. After the 7-week dietary period, each excised heart was subjected to a 15-min stabilization perfusion before recording baseline hemodynamic values. Values are means ± SE of 5–8 hearts from each group.

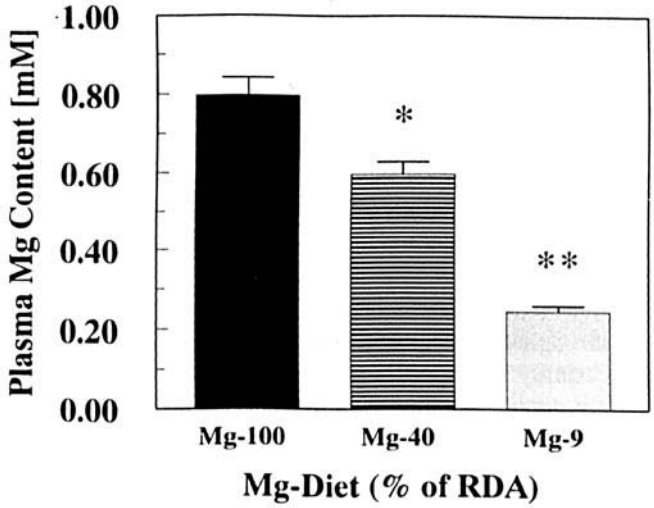

Figure 1. Effect of varying Mg dietary content on plasma Mg levels in rats. Blood samples were taken between dietary Days 7 through 10 from rats exposed to diets containing 9%, 40%, or 100% of the RDA for Mg. Plasma Mg levels were determined by atomic absorption spectroscopy. Values are means ± SE of 5–8 rats. * and ** denote significant differences at $P < 0.05$ and < 0.01 vs Mg_{100}, respectively.

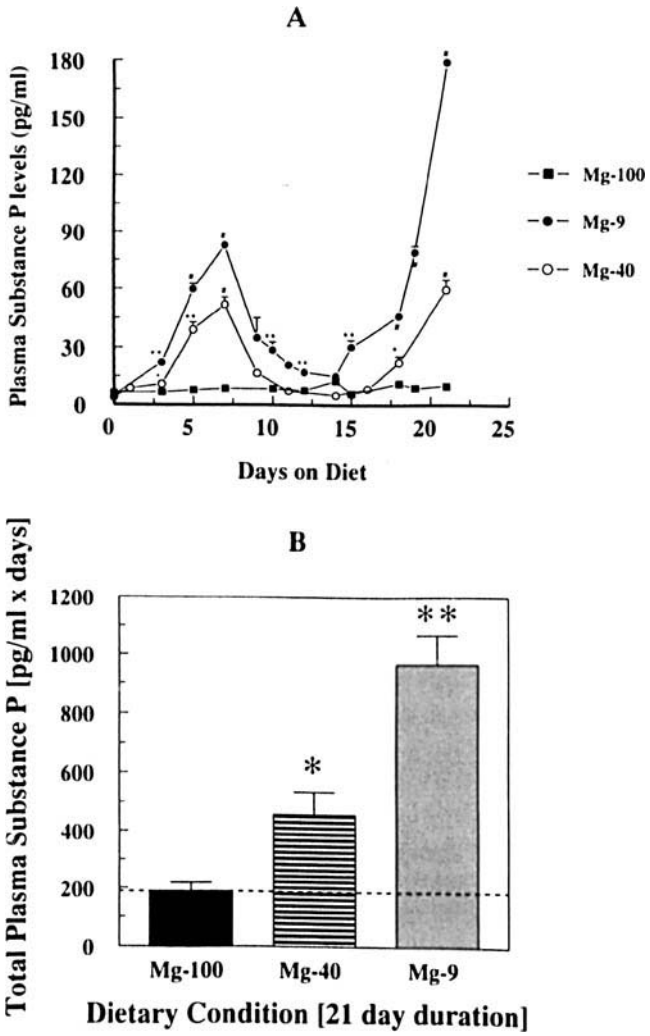

Figure 2. (A) Time course determinations of plasma SP levels after the onset of feeding an Mg_9, Mg_{40}, or Mg_{100} diet to rats. SP levels were determined by CHEM-ELISA using fractions obtained by HPCE. Values are means ± SE of 10 rats. Significant differences: $^*P < 0.05$, $^{**}P < 0.01$, and $\#P < 0.001$ vs Day 0. (B) Mg_9, Mg_{40}, and Mg_{100} diet-induced changes in total circulating levels of SP in the rat. Values are means ± SE from 4–10 rats and were determined by area integration of 21-day time courses. Significant difference: $^*P < 0.05$ and $^{**}P < 0.01$ vs Mg_{100}.

restricted diets, we determined whether hearts from these dietary groups displayed reduced tolerance to postischemic stress that was proportional to Mg intake level. Hearts from 7-week maintained Mg_{20}, Mg_{40}, and Mg_{100} rats were subjected to I/R and indices of recovery were compared. Hearts from both Mg_{20} and Mg_{40} groups were more susceptible to postischemic dysfunction then Mg_{100} hearts. Significant further reductions in cardiac output (Mg_{20} and Mg_{40}: 31.6% and 23.1% lower than Mg_{100}), peak left ventricular systolic pressure (Mg_{20} and Mg_{40}: 17% and 11.6% lower), and left ventricular developed pressure (Mg_{20} and Mg_{40}: 17.5% and 12.3% lower; Fig. 4A), together with increased mean aortic diastolic pressure (Mg_{20} and Mg_{40}: 7.6% and 6.1% higher; Fig. 4B), were largely responsible for the further decreases (Mg_{20} and Mg_{40}: 41.9% and 29.5% lower) in recovery of postischemic cardiac work observed with the moderate groups (Fig. 5). When compared with the Mg_{100} group, postischemic Mg_{40} and Mg_{20} hearts also exhibited greater tissue injury, as suggested by additional losses of tissue LDH (Fig. 6: 40.1% and 65.2%, respectively), and further increases in oxidative injury. Postischemic Mg_{40} and Mg_{20} hearts exhibited significantly heightened total production of alkoxyl radicals (Mg_{40} and Mg_{20}: 2.62- and 3.78-fold higher) compared with the Mg_{100} group (Fig. 7). Furthermore, linear regression analysis showed a strong negative correlation ($r = -0.81$) between postischemic recovery of function and total lipid radical production for individual hearts from each dietary group (Fig. 8).

Discussion

Dietary Mg Intake and Severity of *In Vivo* Pathology. We previously showed direct relationships between the occurrence of severe hypomagnesemia in the Mg_9 rat, and the resultant elevations in circulating SP levels, the development of a pro-inflammatory/pro-oxidative condition *in vivo* (13), and enhanced myocardial susceptibility to subsequent postischemic stress (11). SP receptor blockade *in vivo* (12, 17, 18) significantly reduced the pathology associated with the Mg_9 diet, suggesting that SP bioactivity through its NK-1 receptors is a critical initiator of subsequent oxidative events.

The present study complements these earlier observations by showing that altering dietary Mg content can itself directly influence the extent of neurogenic inflammation and oxidative stress *in vivo*, as well as postischemic recovery. Although moderately Mg-restricted rats typically do not develop the cardiomyopathy (16) seen with Mg_9 rats during the same time frame (4–7 weeks), they do possess many of the same pathological characteristics (inflammatory/oxidative) observed during earlier stages (within 3

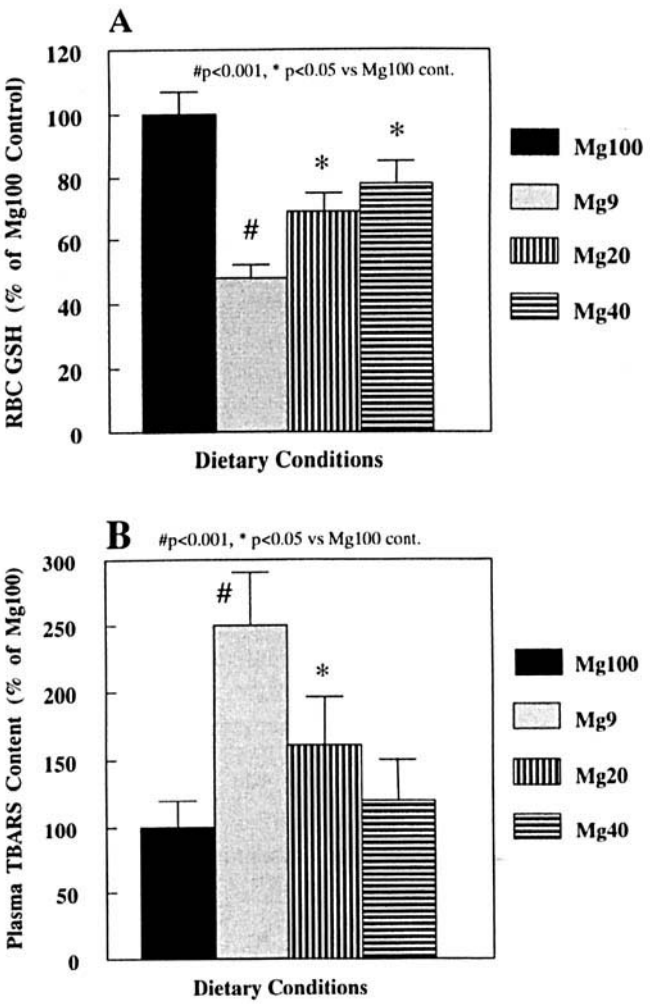

Figure 3. Effects of varying dietary Mg content during a 3-week period on RBC glutathione (A) and plasma malondialdehyde levels (B; as TBARS) in the rat. Means ± SE of 4–5 rats.

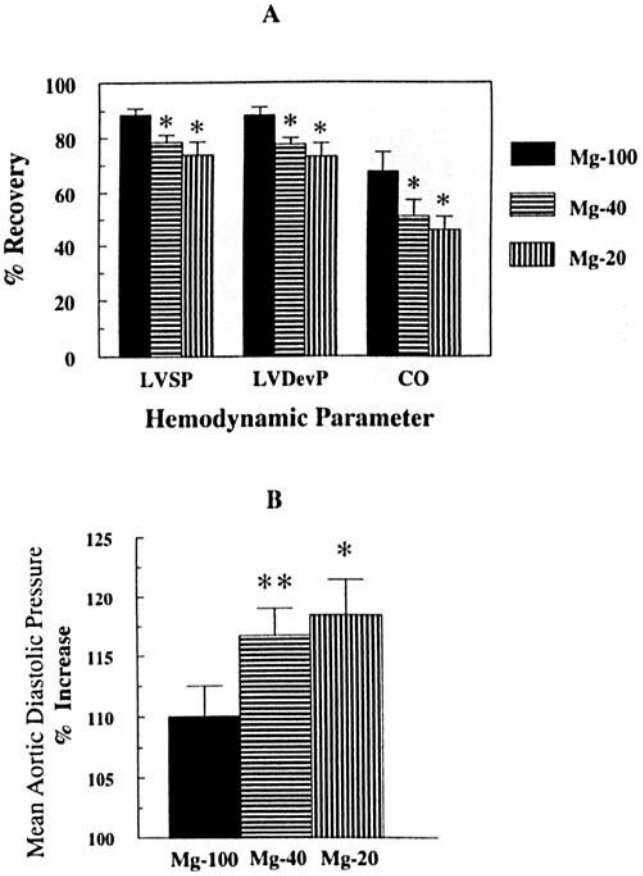

Figure 4. (A) Effect of Mg-restricted diets on recovery of postischemic hemodynamic parameters. Left ventricular systolic pressure (LVSP), developed pressure (LVDevP), and cardiac output (CO). Values are means ± SE for 5–9 rats. $^{*}P < 0.05$ vs Mg_{100}. (B) Effect of dietary Mg restriction on the percentage of increase of postischemic mean aortic diastolic pressure. Values are means ± SE from 5–9 rats. $^{*}P < 0.05$ and $^{**}P < 0.02$ vs Mg_{100}. Increasing DP, more damage.

weeks) of the severe model. Progressively lower dietary Mg intake led to graded levels of hypomagnesemia (Fig. 1). This was associated with proportionately higher circulating levels of SP (Fig. 2, A and B), and greater oxidative stress *in vivo,* as indicated by increased plasma MDA and decreased RBC glutathione levels (Fig. 3). Plasma MDA can be considered a nonspecific marker of systemic oxidative injury because it could derive from circulating polyunsaturated lipids and/or cellular and tissue membranes. RBC glutathione losses (dietary Weeks 2–3), which reflect oxidative stress in the circulation, preceded changes in myocardial antioxidant levels in Mg_9 rats (dietary Week 4) (19); this suggests that RBC glutathione is a reasonably sensitive, early index of *in vivo* oxidative stress in this model. The RBC's high susceptibility to oxidative stress may be due to: direct (and persistent) contact with MgD-activated vascular endothelium (12, 26) and circulating white blood cells (27), both of which may generated free radicals; increased RBC hemoglobin oxidation (28), which will consume glutathione; and the pro-oxidant actions of elevated extracellular iron and lipid peroxides (11).

In Mg_9 rats, the increased plasma MDA and decreased RBC glutathione levels occurred after the earliest significant rise (dietary Day 3) in circulating SP levels (29), and were significantly attenuated by treatment *in vivo* with the NK-1 receptor antagonist CP-96,345 (12). Thus, one can surmise that these oxidative events, which also characterize the moderate models to a varying degree ($Mg_9 > Mg_{20} > Mg_{40} > Mg_{100}$), are for the most part, modulated by SP.

Dietary Mg Intake and Postischemic Tolerance. Subjecting Mg_{20}, Mg_{40}, and Mg_{100} rat hearts to a standardized postischemic stress led to graded differences in functional recovery. Postischemic recoveries of cardiac output, left ventricular systolic, and developed pressures (Fig. 4A) and aortic diastolic pressure (Fig. 4B) were the most affected parameters. Likewise, the differences in recovery of cardiac work paralleled that seen for the other hemodynamic parameters (Fig. 5). Although recoveries of the above parameters were even more depressed in equally stressed postischemic hearts from 3-week Mg_9 rats (11), a direct comparison with the 7-week moderate groups was omitted because of the different dietary time frames and thus, animal age and size. Furthermore, rats maintained on the Mg_9 diet

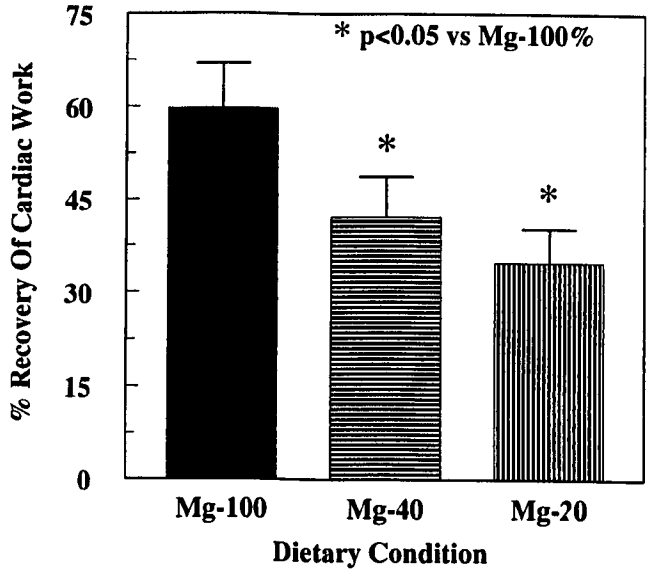

Figure 5. Effect of Mg-restricted diet on subsequent rat heart postischemic recovery of cardiac pressure-volume work. Values are means ± SE of 5–9 rat hearts. $^{*}P < 0.05$ vs Mg_{100}.

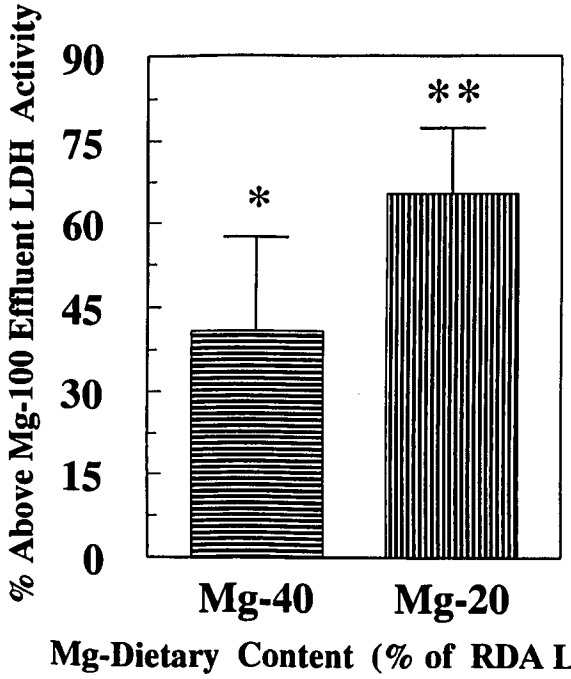

Figure 6. Effect of dietary Mg restriction on total postischemic LDH activity released into the effluent. Values are means ± SE of 4–8 rats hearts and were determined by area integration of the reperfusion time courses. $^{*}P < 0.05$ and $^{**}P < 0.02$ vs Mg_{100} (= 100%).

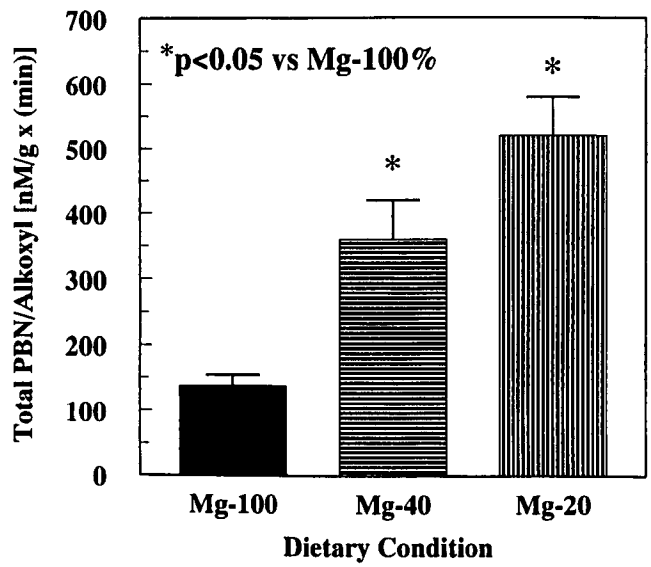

Figure 7. Effect of varying Mg dietary content on subsequent rat heart postischemic lipid radical (alkoxyl) content determined by area integration of detection time courses during reperfusion. Values represent total production and are means ± SE of 5–9 hearts. $^{*}P < 0.05$ vs Mg_{100}.

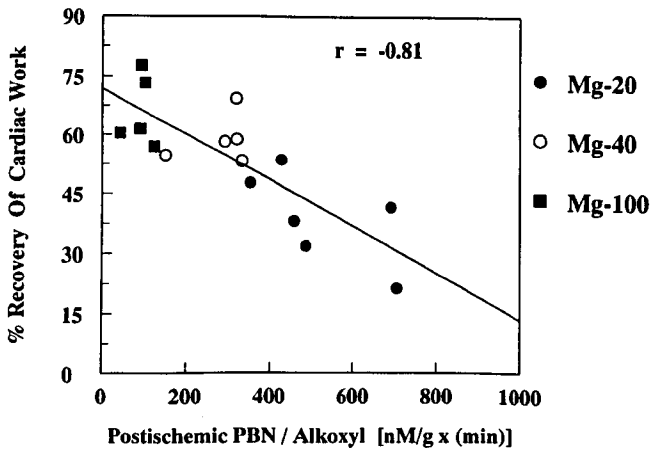

Figure 8. Linear regression analysis comparing individual rat heart postischemic recovery of cardiac work with total lipid radical production for different Mg dietary conditions. Correlation coefficient, r = −0.81.

beyond 4 weeks showed signs of developing myocardial necrosis (19) and would not provide an appropriate comparison with the moderate groups.

Explanations for the enhanced functional depression of postischemic hearts from Mg-restricted animals have been discussed (11, 19). In keeping with the inflammatory events during Mg deficiency, a plausible mechanism might involve the negative inotropic effects of inflammatory cytokines (30), which become significantly elevated in the circulation during dietary Week 3. However, hemodynamic defects were not apparent in isolated, normally perfused (KHB with normal Mg content) Mg_9 (11, 19), Mg_{20} or Mg_{40} (Table I) rat hearts before inducing I/R stress. Moreover, our use of a nonrecirculating heart perfusion model tends to argue against a possible direct involvement of circulating cytokines. In light of our findings of dose-dependent cardioprotection using NK-1 receptor blockade *in vivo* (19), we believe mechanism(s) consistent with SP bioactivity are most likely involved. Although SP may have little direct influence on functional properties of normally perfused hearts from Mg normal rats (31), it may modulate contractility by stimulating tissue resident inflammatory cells to release inflammatory cytokines. Support for this mechanism comes from reports showing that SP can promote adhesion mol-

ecule expression on vascular endothelium (32), and progressive increases in inflammatory cell numbers within the heart (33) during the Mg-restricted period (15). Because hearts from 3-week Mg_9 rats have elevated tissue SP and cytokine levels (19), functional depression might be anticipated even during normal perfusion. However, as indicated above, hemodynamic defects were absent during preischemic perfusion of these rat hearts (Table I). From the above findings, we reasoned that much of the SP content within Mg_9 hearts may not be readily available (retained within neuronal storage site) (34) to act at surface NK-1 receptors of inflammatory cells (35); that most of the cytokine content in these hearts remain confined within tissue-resident inflammatory cells and not able to exert their negative inotropic actions; and that normal perfusion does not sufficiently stimulate cytokine secretion at a level high enough to cause functional depression. Alternatively, the myocardial-resident inflammatory cells, which accumulated in response to diet-induced neurogenic event(s), may become activated when the postischemic insult was imposed (36). This would cause direct induction of oxidative stress via cytokine secretion and free radical generation (29, 37), leading to excessive consumption of endogenous antioxidants (4, 5), and enhanced free radical attack on critical macromolecules during reperfusion (11). Such a scenario provides a fundamental, though indirect, link to SP as an early mediator of events *in vivo* leading to the enhanced injury exhibited by postischemic Mg_9 rat hearts (19).

An analogous SP-triggered mechanism may account for the graded severity of postischemic injury observed in hearts from moderately Mg-restricted animals. In this instance, changes in circulating SP levels (Fig. 2) caused by moderate reductions in dietary Mg content would render proportional changes in the bioavailability/bioactivity of this neuropeptide, as well as in the pro-oxidative events that followed. As an predicted outcome, the severity of postischemic injury to Mg_{20} and Mg_{40} rat hearts did parallel the extent of diet-induced hypomagnesemia. In addition to graded changes in hemodynamic recovery, postischemic Mg_{20} and Mg_{40} hearts ($Mg_{20} > Mg_{40} > Mg_{100}$) experienced heightened tissue injury (Fig. 6: cardiac LDH release), which was related to the extent of Mg intake and diet-induced hypomagnesemia. Detection of excessive LDH activity in venous effluent suggests that plasma membrane structural and functional integrity was compromised during reperfusion (19), and that the severity of biomembrane injury can be influenced, at least indirectly, by prior dietary Mg intake levels. Loss of membrane integrity during reperfusion may partially be a consequence of enhanced primary free radical production (superoxide anion and hydroxyl radical) (25) that can initiate the LPO pathway. This view is supported by our findings that tissue antioxidant consumption (vitamin E and glutathione: 35% and 37% more, respectively) was far greater in postischemic Mg_9 rat hearts compared with Mg_{100} despite the presence of similar preischemic levels (19); and that the enhanced production of LPO-derived alkoxyl radicals (Fig. 7) paralleled the severity of diet-induced hypomagnesemia and directly correlated with loss of functional recovery (Fig. 8).

Mg deficiency has been linked to vasospasm *in vivo* (38), and it is possible that the more severe postischemic dysfunction exhibited by hearts from MgD rats is linked to Mg depletion in cardiovascular tissue before I/R exposure. However, the most affected tissues with respect to changes in Mg status during Mg deficiency appear to be bone and skeletal muscle (39), whereas the heart was able to conserve its Mg^{2+} for an extend time, in spite of substantial hypomagnesemia. Others reported that rats receiving a severely Mg-restricted diet for 4 (9) or 6 (40) weeks, experienced no significant loss of cardiac Mg^{2+}; moreover, after 11 weeks on a moderate Mg_{20} diet, only 7% and 10% losses of cardiac and aortic Mg^{2+} content, respectively, were observed (4). Thus, the *in vivo* cardiac predisposition to I/R stress that develops during Mg deficiency may not be directly associated with changes in cardiac tissue Mg^{2+} levels.

Neuropeptide Release. The reason(s) for the rise in circulating SP levels remains controversial because not all investigators have detected this event during dietary Mg restriction. Malpuech-Brugere *et al.* (41) demonstrated a significant elevation of circulating interleukin-6 levels in rats by Day 4 of a severely Mg-restricted diet, but this was not associated with elevations in circulating SP levels. Although contradictory to our previous (17, 19) and current findings (Fig. 2), this disparity may partly be explained by differences in experimental model and assay procedures. The study by Malpuech-Brugere *et al.* (41) involved a different rat strain (Wistar versus our Sprague-Dawley) and smaller/younger animals (60 g versus our 175 g); used a different SP assay system (RIA without internal standards versus our HPCE/ELISA); and examined a blood sampling time (dietary Day 4) that was earlier than the peak detection time (dietary Day 7) reported in our investigation (12).

Release of neuromediators (42–44) relies on mechanism(s) involving excessive calcium influx into neuronal tissue via voltage-dependent L- or N-type calcium channels or the ligand-gated NMDA (*N*-methyl-D-aspartate) receptor/calcium channel complex. Although it is unclear whether SP is released directly from CNS (brain and spinal cord) or peripheral neuronal tissue, the relatively short half-life (minutes) of released SP and potential difficulties regarding its penetration of the blood-brain barrier seem to favor the peripheral neurons as the principle source. SP has been localized in peripheral neurons (34, 45), along with the NMDA receptor/channel complex (46, 47). We believe that the initial transient rise in SP levels (Fig. 2, Days 3–8) may be predominantly modulated by the neuronal Mg-gated NMDA receptor/channel complex because of its known hypersensitivity to declining extracellular Mg levels. The hypersensitivity of this complex to Mg appears to depend on the subunit composition of the heteromeric channel. In the mouse model (48), active complexes were only observed when the ζ 1 channel subunit was expressed with one of

four different ∈ subunits, and these complexes displayed variable sensitivity to Mg blockade. Similar functional variations were detected with the heteromeric NMDA receptor channels in the rat (designated NMDAR1 + NMDAR-2A, -2B, -2C, or -2D) (49). These observations imply that NMDA receptor mediated-neuromodulator release is finely regulated in its response to small changes in extracellular Mg levels. Curiously, this (Mg) control may not exist with respect to the later second peak (dietary Days 17–21) of circulating SP. The neuronal release mechanism that contributes to the first SP peak may not be involved with the second release because severe dietary Mg restriction (Mg_9) in the rat resulted in a maximal decline of plasma Mg levels (0.78 to 0.25 m*M*) within 7 days (22), yet the second release of SP does not appear until dietary Days 17 to 21 (Fig. 2); and SP release during dietary Days 17 to 21 was not accompanied by corelease of calcitonin gene-related peptide (CGRP), which would have been expected had the same neuronal release mechanism elicited during the first week of the diet been involved (12). These observations also permit speculation that the second SP peak may originate from non-neuronal sources. Investigations using RT-PCR analyses of inflammatory cells (mast cells, macrophages, neutrophil, and monocytes) and endothelial cells have demonstrated that these non-neuronal cells possess mRNA for both SP and NK-1 receptor expression (*de novo* synthesis) (50–53). The possibility of a non-neuronal origin of the second SP peak requires further consideration. Likewise, we cannot fully exclude the contribution made by peripheral blood mononuclear cells toward the first SP peak; however, their lack of the Mg-gated NMDA receptor, as well as findings that intracellular Mg did not decline within these cells from hypomagnesemic patients (54, 55), tends to argue against this possibility.

In summary, we have shown that manipulating dietary Mg intake levels in the rat directly influenced oxidative stress *in vivo*, and the associated loss of myocardial tolerance to postischemic stress *in vitro*. The severity of this later pathology may be related to the following sequence of events: hypersensitivity of neuronal NMDA receptor/channel to declining extracellular Mg levels permits graded reduction of Mg inhibition at the calcium channel, allowing calcium influx and release of SP; the resultant changes in circulating SP levels and its bioactivity at the NK-1 receptor initiates a cascade of inflammatory/oxidative events *in vivo*; and imposed postischemic stress *in vitro* activates myocardial-resident inflammatory cells that accumulated during the dietary period, inducing further oxidative stress (cytokine secretion, free radical production, and antioxidant consumption), which places the heart at greater risk of postischemic injury. Because the moderately Mg-restricted diets (20% and 40% RDA) in use can lead to levels of hypomagnesemia that are comparable (20) with that seen in clinical situations (cardiovascular disease, diabetes, alcoholism, gestational hypomagnesemia, AIDS/HIV, and diuretic use), Mg replacement therapy may be an appropriate remedy to lessen potential ill effects caused by this metal deficiency.

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