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Neurogenic peptides and the cardiomyopathy of magnesium-deficiency: effects of substance P-receptor inhibition

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Abstract

Dietary deficiency of magnesium (Mg) in rodents results in cardiomyopathic lesion formation. In our rat model, these lesions develop after 3 weeks on the Mg-deficient diet; significant elevation of several cytokines, IL-1, IL-6 and TNFα also occurs. In probing the mechanisms of lesion formation, we obtained data supporting the participation of free radicals (Freedman AM *et al.*: Bioch Biophys Res Commun 1990; 170: 1102). Recently, we identified an early elevation of circulating substance P and proposed a role of neurogenic peptides during Mg-deficiency (Weglicki WB, Phillips TM: Am J Phys 1992; 262: R734). The present study was designed to evaluate the contribution of neurogenic peptides to the pathogenesis of Mg-deficiency. In the blood, substance-P and calcitonin gene related peptide (CGRP) are elevated during the first week on the diet. During the second week, circulating histamine, PGE_2 and TBAR-materials were elevated and red cell glutathione was reduced, all prior to the elevation of the inflammatory cytokines during the third week. When the rats were treated with the substance P-receptor blocker [CP-96,345], the levels of substance P and CGRP remained elevated; however, increases in histamine, PGE_2, TBAR-materials, and the decrease in red cell glutathione were inhibited; also, the development of cardiac lesions was inhibited significantly. These data support a central role for neurogenic peptides, especially substance P, in the development of cardiomyopathic lesions during Mg-deficiency. (Mol Cell Biochem **130:** 103–109, 1994)

Key words: Mg-deficiency, substance P, receptor blocker, cytokines (IL-1 TNFα), cardiomyopathy

Introduction

In our animal models, cardiomyopathic lesions develop within 2–3 weeks following onset of a Mg-deficient diet [6–8, 24]. Although the pathogenic mechanisms of lesion formation are uncertain [2], we have demonstrated that antioxidant drugs and nutrients [6–8, 24] reduced lesion size and frequency suggesting that free radicals contribute to lesion development. The basic histology of the cardiac lesions of Mg-deficiency was described 30

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years ago [12, 13] as focal necrotic inflammation surrounding small blood vessels. As lesions progress, macrophage deposition and fibroblast proliferation are seen along with debris accumulation and eventual calcification, scar formation and necrosis. We have shown that cytokines, (IL-1, IL-6, and TNFα), are increased significantly at the time of cardiomyopathic lesion formation suggesting that an inflammatory response contributes to the free radical component [26]. Recently, we identified a significant elevation of the neurokinin, substance P, during the first week on the Mg-deficient diet [25]. Along with substance P, we also found elevated levels of prostaglandin E_2 (PGE_2) during the first week and histamine during the second week. However, the levels of circulating inflammatory cytokines were not elevated during the first 2 weeks in the rat model [26]. We proposed that 'neurogenic inflammation' occurs during Mg-deficiency resulting in a cascade of events leading to inflammatory cytokine production and lesion formation [25]. The present study assesses the effect of substance P-receptor blockade on the above events and on the eventual lesion development.

Methods

Magnesium-deficiency model

Sprague-Dawley rats were placed on the Mg-deficient diet, provided with deionized drinking water and housed using a 12-hour light/dark cycle. The diet was obtained from Teklad Inc. (Madison Wisconsin); the Mg-deficient diet (Mg < 1 mmole/kg, Ca = 170 mmoles/kg) was supplemented with magnesium oxide 100 mmoles/kg for the control animals. Based on pilot studies, the substance P receptor inhibitor, CP.-96,345 (2S, 3S)-cis-2-(dipenylmethyl)-N-[(2-methoxyphenyl)-methyl])-1-azabicyclo[2.2.2]octan-3-amine) (Pfizer Inc. Central Research, Groton, CT) was formulated into sustained-release pellets (Innovative Research, Toledo, Ohio) at a dose of 3.5 mg; pellets were implanted subcutaneously at the beginning of the dietary protocol [6]. These pellets released the drug over 21 days at a daily dose of approximately 0.8 mg/Kg/day. Blood was obtained by tail vein sampling with heparinized capillary tubes throughout a three week period on the diet.

Determinations of Mg, peroxides and glutathione levels

Plasma magnesium levels were determined by atomic absorption spectroscopy. Serum lipid peroxide levels were determined by the thiobarbituric acid (TBA) procedure [17]. To prevent non-specific lipid oxidation of the serum, 0.01% butylated hydroxytoluene (BHT) was included during the 30 min heating step at 80° C. The accumulation of TBA-reactive products was measured at 532 nm and expressed as malondialdehyde (MDA) equivalents/ml serum.

Total RBC glutathione (GSH + 1/2 GSSG) was determined by the 'cyclic method' of Griffith [11] as described previously [16]. Briefly, the red cells were lysed by hypotonic treatment in the presence of 5% (w/v) 5-sulfosalicylic acid. Both total and oxidized glutathione (GSSG) were determined in the supernatants. Oxidized glutathione was determined with prior masking of GSH by 2% (v/v) 2-vinyl pyridine. Since the level of GSSG was found to constitute less than 5% of the total glutathione and was not affected by Mg-deficiency, only total glutathione was determined in most studies.

Alkaline phosphatase labeling of reporter antibodies

Pure IgG preparations of antibodies to substance P (Chemicon International, Inc., Temecula, CA), calcitonin gene-related peptide (Chemicon International), IL-1 (Cytokine Sciences, Inc., Boston, MA), IL-6 (R & D Systems, Minneapolis, MN), TNFα (Genzyme Corporation, Cambridge, MA), prostaglandin E_2 (abV ImmuneResponse Inc, Derry, NH), and histamine (Chemicon International) were conjugated to a chromatographically pure, prederivatized preparation of alkaline phosphatase (Pierce, Rockford, IL) according to the manufacturers instructions. Each alkaline phosphatase-labeled antibody preparation was passed through a prepacked desalting column (Pierce) to remove unconjugated enzyme and antibody.

Measurements of neuropeptides

Circulating concentrations of substance P and calcitonin-related peptide (CGRP) were measured by an antigen capture ELISA, using alkaline phosphatase-labeled reporter antibodies and chemiluminescence detection [25]. The wells of standard microtiter plates (Flow Laboratories, McLean, VA) were coated with a 1 μg/ml solu-

tion of streptavidin (Pierce) in 0.5 M sodium carbonate buffer, pH 9.5 for 18 hr at 4° C, washed three times in 0.01 M phosphate buffer, pH 7.2, prior to coating with antibody. Pure unlabeled preparations of anti-substance P and anti-CGRP antibodies were biotinylated by incubating with an equal volume of 50 mM N-hydroxysuccinimide-biotin for 2 hr at room temperature prior to immobilizing them onto the wells of the streptavidin-coated plates. The assay was performed by adding 25 µl of rat plasma to each well and incubating overnight at 4° C. The plates were washed 3 times in 0.01 M phosphate buffer – 0.01% Tween 20, pH 7.6 before adding 200 µl of a 1/1000 dilution of either anti-substance P or anti-CGRP enzyme-labeled antibody to each well. The plates were incubated overnight at 4° C and washed five times in phosphate-Tween buffer before adding 250 µl of a 25 mM solution of AMPPD chemiluminescent substrate (Tropix Inc., Bedford, MA) to each well. The chemiluminescent reaction product was read in a luminescence ELISA reader (Tropix Inc.) and the readings transferred directly into a computer for analysis by ANELISA-R software (Man-Tech Associates, Buffalo, NY).

Measurements of circulating cytokines

Concentrations of the IL-1, IL-6, and TNFα were measured in the plasma of Mg-deficient and Mg-sufficient animals by a biotin-avidin modification of the antigen capture ELISA assay described above. Briefly, 10 µl plasma samples were biotinylated by adding 10 µl of 50 mM N-hydroxysuccinimide-biotin and immobilized onto the wells of avidin-coated microtiter plates. Apart from this modification, the assay conditions and analyses were identical to those described for neuropeptides above. Cytokine detection was achieved by using alkaline phosphatase, labeled cytokine specific antibodies and chemiluminescence as the reporter system.

Measurement of prostaglandin E_2 and histamine

Prostaglandin E_2 (PGE_2) and histamine were measured by a standard antigen-capture ELISA [21], as described above, using PGE_2 and histamine specific antibodies for both capture and detection.

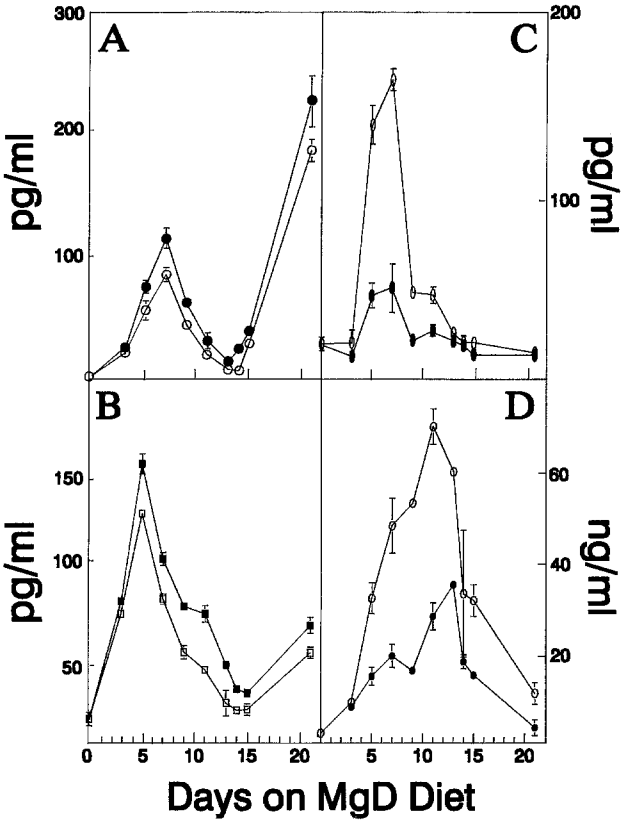

Fig. 1. The effect of the substance P receptor blocker, CP-96,345, on circulating levels of (A) substance P, (B) CGRP, (C) PGE_2, and (D) histamine. The animals were placed on the Mg-deficient diet on day zero, and implanted with sustained release pellets containing either 3.5 mg of CP-96,345 (closed symbols) or a vehicle control (open symbols). The circulating mean levels ± S.D. were followed with time on the diet.

Lesion morphometry

After three weeks on the diet, animals were anesthetized by ether induction and sacrificed by exsanguination and their myocardial tissue was harvested. The severity of myocardial injury was determined by light microscopy. The hearts were sliced into four segments, and fixed in 4% formalin. Hematoxylin/eosin (H & E) stained sections, (5 sections at different levels, not serial sections, from each of the four slices) were prepared for morphological and morphometric analyses [6]. The morphometric analysis utilized the Bioquant (R & M Biometric, Inc.), an interactive image analysis system, providing direct morphometric measurements from digitized images obtained via the (VCMTE) automated video counting and microdensitometry accessory program. The results obtained are expressed as the numerical density (# of lesions/cm^2 of tissue) and the area fraction (lesion area/tissue area).

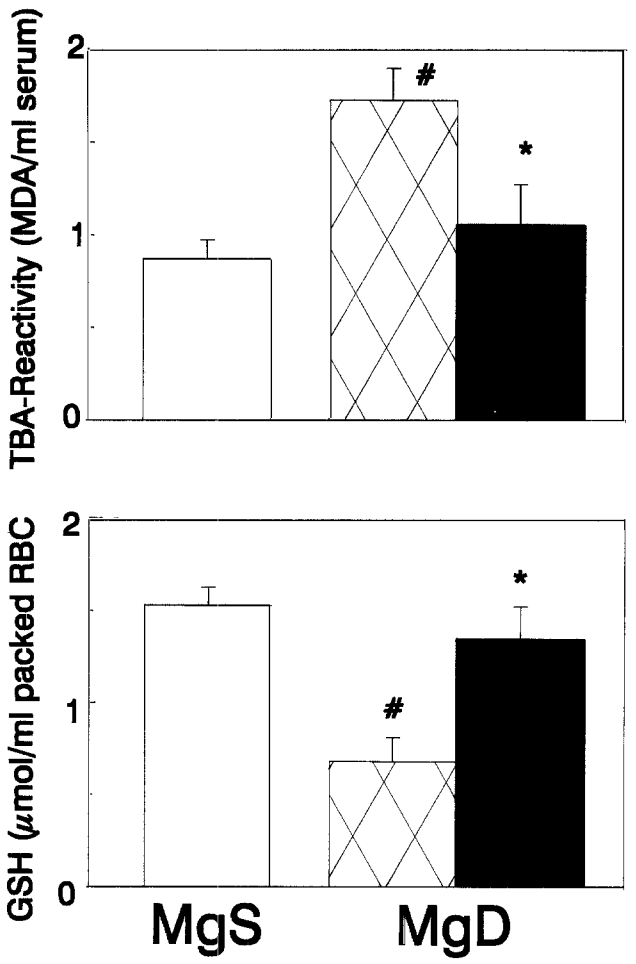

Fig. 2. The effect of the substance P receptor blocker, CP-96,345, on (A) accumulation of serum thiobarbituric acid (TBA)-reactive substances, and (B) loss of red cell glutathione during Mg-deficiency. Significant protection was provided by CP-96,345 (solid bars ± S.D.) compared to Mg-deficiency alone (hatched bars ± S.D.) # $p < 0.01$ vs MgS; * $p < 0.05$ vs MgD alone.

Statistical methods

Differences between drug-treated and un-treated Mg-deficient animals were determined using paired student T-test.

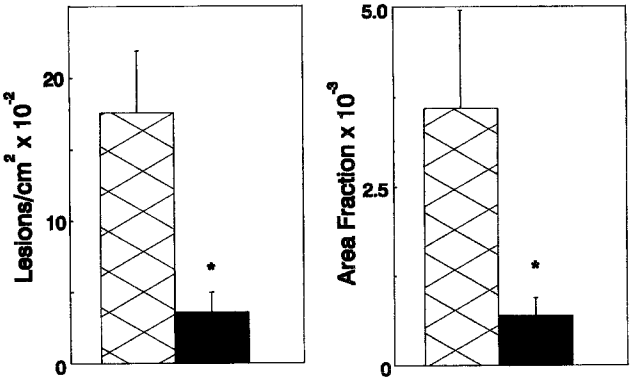

Fig. 3. The effect of the substance P receptor blocker, CP-93456, on the cardiomyopathic lesion number (left panel) and relative area size (right panel) in Mg-deficient rats at 3 weeks. Significant differences exist between Mg-deficient (hatched bar ± S.E.) and Mg-deficient plus CP-96,345 (solid bars ± S.E.) in both number of lesions/cm^2 ($p < 0.01$) and area fraction X 10^{-3} ($p < 0.05$).

Results

After one week on the Mg-deficient diet the rat plasma magnesium concentration ([Mg]— decreased from 0.74 mM ± 0.05 to 0.22 ± 0.03 mM, and dropped to 0.14 ± 0.05 mM by the third week on the diet. The plasma [Mg] of rats fed the magnesium supplemented diet was 0.79 ± 0.13 mM by the end of the third week on the diet.

Figure 1A depicts substance P elevation, with an initial peak during the first week followed by a second peak during the third week on the Mg-deficient diet. The presence of the substance P receptor inhibitor caused a slight increase in both peaks of substance P. In addition, CGRP (Fig. 1B) was elevated during the first week; it too was slightly increased by the substance P receptor inhibitor. Figure 1C depicts the elevation of PGE_2 levels concurrent with the early peak of substance P while histamine levels (Fig. 1D) were found to peak at day 11, after an initial substantial rise during the first week. Significant inhibition of PGE_2 ($p < 0.01$) on day 5 and of histamine ($p < 0.01$) on day 11 resulted from substance P receptor-inhibition (Figs 1C, 1D). The rise of substance P

Table 1. Effect of substance P inhibitor (CP-96,345) on circulating inflammatory cytokine levels in Mg-Sufficient and Mg-deficient animals. The inhibitor had no effect on these levels in Mg-sufficient rats, p values given are for Mg-deficient compared to Mg-deficient treated with the inhibitor.

	MgS		MgD		p <
	None	CP-96,345	None	CP-96,345	
IL-1	12.6 ± 2.9	10.7 ± 3.0	200.0 ± 17.5	154.3 ± 12.8	0.01
IL-6	13.3 ± 2.0	11.3 ± 0.9	187.3 ± 16.9	142.5 ± 15.3	0.01
TNFα	15.8 ± 5.6	15.2 ± 5.4	214.9 ± 36.7	132.2 ± 17.6	0.01

during the third week, paralleled the increases of IL-1, IL-6 and TNFα in untreated Mg-deficient animals. The substance P inhibitor produced a moderate, but significant ($p < 0.01$), inhibition of circulating levels of IL-1 and TNFα (Table 1). The time-course of these changes suggested that the elevation of substance P during the first week mediated subsequent metabolic responses.

As a qualitative index of *in vivo* lipid peroxidation, changes in serum peroxide accumulation were assessed. Figure 2A shows that Mg-deficiency resulted in significant elevation ($p < 0.01$) of the serum TBAR-materials suggesting increased oxidative events *in vivo* during Mg-deficiency. The results also indicate that treatment with the substance P-receptor inhibitor completely blocked the Mg-deficiency-induced rise in serum TBAR-materials.

In the hamster model [10], we found that Mg-deficiency induced a 50% loss of intracellular GSH in the red cells. In the present study, a similar degree of GSH loss was observed in the Mg-deficient rats (Fig. 2B) which was also completely prevented by treatment with the substance P-receptor inhibitor.

Significant cardiac lesions developed during Mg-deficiency and a time course study of lesion formation indicated that most of the lesions developed during the third week on the diet. Despite only moderate inhibitory effects on the circulating cytokines (Table 1), CP-96,345 significantly reduced both the number ($p < 0.05$) and size ($p < 0.01$) of the Mg-deficiency-induced lesions (Fig. 3).

Discussion

Our laboratory previously reported a marked increase in circulating substance P levels within a week of placing rats on a Mg-deficient diet [25]. From this observation, we postulated that neurogenic peptides may be playing a key role in the pathobiology of Mg-deficiency. While we have shown that a number of agents with antioxidant properties will inhibit Mg-deficient myocardial lesions [6–8, 24], CP-96,345, which only exhibits minimal antioxidant activity (< 5% that of vitamin E), was a very effective inhibitor of lesion formation (Fig. 3). In addition, vitamin E treatment had no effect on the elevated cytokine levels [22]. Thus, it is unlikely that the observed effects of CP-96,345 were due to its non-specific antioxidant activity. It has been reported that CP-96,345 may act as an antagonist of L-type calcium channels [27]. However in a pilot study, we found that nicardipine at a similar dose had no significant effects on the Mg-deficiency-induced rises in the circulating levels of SP, PGE_2 histamine, TNFα, IL-1, or IL-6, suggesting further that the observed mediated effects of CP-96,345 were due to SP-receptor blockade rather than calcium channel inhibition. Further, the binding affinities of CP-96,345 to calcium channels are significantly lower than binding to SP receptors (NK1-receptors). With the relatively low dose given to the animals (66 nmoles/kg/hr), it is estimated that the circulating levels of the agent would only be sufficient to produce selective NK1 antagonism.

A recent study reported that CP-96,345 produced a modest hypotension and a small reduction in heart rate [5]. However, these cardiovascular effects were only produced at micromolar dosages (3–10 μmoles/kg/min i.v. injection); at the low nanomolar range of inhibitor used in our study, the cardiovascular effects should be minimal. While it is not possible to eliminate the above possibility, other data strongly suggest that antihypertensive activity does not prevent lesion development. In a recently published study [7], the effects of both SH-containing and non-SH-containing ACE-inhibitors on Mg-deficiency-induced cardiomyopathy were examined: SH-containing agents were protective and the non-SH-containing agent, enalaprilat, was without effect. We concluded that the protective effects of the SH-containing ACE-inhibitors were contributed by the SH-moiety rather than by ACE-inhibition (and subsequent antihypertensive effect). In a parallel study, we reported that both D,L-propranolol and D-propranolol (the non-beta-blocking isomer) were equipotent in providing protection against lesion development [14]; we concluded that the effect was produced by an antioxidant mechanism rather than by an effect on hemodynamics.

The observed increased TBAR-materials in serum and the decreased GSH in the red cells are supportive of increased free radical lipid peroxidation during Mg-deficiency. In a previous study, it was suggested that decreased GSH of red cells was directly related to the lower serum Mg, perhaps due to diminished GSH-synthesis [4]. In the present study, feeding the animals the Mg-deficient diet resulted in a rapid decrease of serum Mg during the first week. The administration of the SP-receptor inhibitor had no effect on the serum Mg level; however, it effectively prevented the loss of GSH. These findings support the notion that the loss of GSH resulted from increased oxidation which was inhibited by SP-receptor blockade. We believe that blockade of the substance P-mediated free radical production also prevented the ac-

cumulation of lipid peroxidation products occurring during Mg-deficiency (Fig. 3).

Substance P has been reported to induce many cellular events including: inflammatory cytokine production from macrophages and endothelial cells, histamine release from mast cells, and free radical generation from macrophages and neutrophils. Presumably, most of these events are receptor-mediated. Release of neuronal substance P and CGRP from sensory neurons appears to result as a consequence of Mg-deficiency. These neuropeptides produce inflammation and release of PGE_2 and histamine. While it has been assumed that the substance P present in inflammatory exudates is neuronally derived, recent investigations suggest that there may be other cellular sources as well [1]. Our time-course studies, indicating concurrent elevations of substance P and CGRP, suggested that these peptides are released from storage sites in nerves and possibly other cells. Abdelrahman [1] has suggested that these peptides may participate in cardiovascular regulation. Lundberg *et al.* [15] stated that 'in the heart, SP- and CGRP-IR nerves were abundant in the myocardium and around coronary vessels, especially those of the right atrium'; others have detected CGRP-containing nerve fibers in cardiovascular vessels [22]. The early release of neuropeptides, along with the PGE_2 peak, suggest that molecular signaling is taking place simultaneously. The later peak of histamine suggests that mast cells and other histamine-containing cells are responding to stimulation by substance P and CGRP. Others have proposed 'a functional unit between mast cells and substance P fibers which seems to be of physiological importance in vasodilatation, plasma extravasation and the development of edema' [23]. The CP-96,345 inhibition of the histamine peak (Fig. 2D) indicates that substance P is a major stimulus of histamine response. The elevated CGRP, which has also been reported to induce histamine release might account for the incomplete inhibition of histamine release in the presence of CP-96,345 (Fig. 2D). The role of histamine in cardiac lesion development during Mg-deficiency remains to be defined.

McGillis stated that 'Production of the arachidonic acid metabolites, prostaglandin E_2, thromboxane B_2, and leukotriene C_4, characteristic of macrophage activation, is enhanced by substance P; substance P can also enhance the oxidative burst in macrophages' [19]. In addition, it has been reported that substance P activates neutrophils to produce oxy-radicals [28]. In preliminary studies, we observed that neutrophils obtained from the Mg-deficient rats were 3-fold more active than those from the Mg-sufficient rats in generating superoxide anions in the absence of stimulus. Furthermore, as part of the injurious mechanism at the endothelial cell level, substance P might induce the expression of endothelial-leukocyte adhesion molecules in the microvasculature and thus promote neutrophil-mediated cytotoxicity [18, 28]. Thus, substance P may induce 'endothelial-leukocyte interactions *in vivo*, linking factors produced by the central peripheral nervous systems with normal and pathologic trafficking of immunocompetent cells' [3].

To our knowledge, this is the first reported study of *in vivo* chronic administration of CP-96,345 in an animal model. Due to the lack of published information about the pharmacodynamics of chronic CP-96,345 treatment, indirect and subtle hemodynamic parameters cannot be excluded. Interestingly, the effects of the inhibitor on circulating inflammatory cytokines were moderate (Table 1), suggesting that the inflammatory cytokines are less dependent on substance P-mediated activity than lesion development. From the reduction in circulating lipid peroxides, preservation of RBC glutathione, and inhibition of lesions, we postulate that the cellular production of oxygen free radicals may be directly responsive to substance P-stimulation rather than resulting from the synthesis of inflammatory cytokines.

Summary

Our data suggest a rapid release of substance P and CGRP from stored cellular compartments. Overall, the results of these substance P-receptor inhibitor studies provide supporting evidence that neurogenic peptides contribute to the pathobiology of Mg-deficiency [25]. We postulate that a neurogenic peptide mechanism promotes cellular production of free radicals which may exhaust protective stores of endogenous antioxidants; subsequently, these 'antioxidant deficient' animals are more susceptible to pro-oxidant or pro-inflammatory stresses. In our studies of the hamster model of Mg-deficiency, when additional stress with catecholamines was introduced [3], more than half of the myocardial wall became necrotic. Likewise, the Mg-deficiency model may be useful in future studies involving other 'stressors': environmental, chemical, pharmacological, etc. We believe that the cardiomyopathic lesions are later markers of the pathogenic events associated with Mg-deficiency. The initial 'mechanistic' events are neurogenic and occur during the first week of rapidly evolving Mg-deficiency. Blockade by of both the early metabolic re-

sponses (PGE_2 and histamine elevations), as well as the later events (increased serum peroxides, decreased red cell glutathione, increased cardiac lesions) by CP-96,345 appears to confirm a central role for substance P in the pathobiology of cardiovascular injury in Mg-deficiency.

Acknowledgements

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