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Magnesium Deficiency-related Changes in Lipid Peroxidation and Collagen Metabolism *In Vivo* in Rat Heart

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Magnesium deficiency is known to produce a cardiomyopathy, characterised by myocardial necrosis and fibrosis. As part of the ongoing investigations in this laboratory to establish the biochemical correlates of these histological changes, the present study probed the extent of lipid peroxidation and alterations in collagen metabolism in the heart in rats fed a magnesium-deficient diet for 28, 60 or 80 days. While lipid peroxidation was measured by the thiobarbituric acid reaction, collagen turnover rates and fibroblast proliferation were assessed using [^{3}H]-proline and [^{3}H]-thymidine, respectively. Tissue levels of magnesium and calcium were determined by atomic absorption spectrophotometry. A 39% increase in the cardiac tissue level of thiobarbituric acid reactive substances was observed on day 60 of deficiency ($p < 0.001$). A marked drop in collagen deposition rate (59%, $p < 0.001$%) on day 28 but a significant rise in fractional synthesis rate (12%, $p < 0.001$) and collagen deposition rate (24%, $p < 0.001$) on day 60 were observed. A fibroproliferative response in the heart was evident on day 80 but not at earlier time-points. Thus, the present study provides evidence of increased lipid peroxidation and net deposition of collagen in the myocardium in response to dietary deficiency of magnesium. These changes were, however, not directly related to alterations in the tissue levels of Mg. It is suggested that the increase in cardiac collagen synthesis and fibroplasia associated with Mg deficiency may represent reparative fibrogenesis, upon oxidative damage to the cardiac muscle, and is mediated by a mechanism independent of changes in cardiac tissue levels of Mg. © **1997 Elsevier Science Ltd. All rights reserved**

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INTRODUCTION

The interstitial collagens, as major constituents of the cardiac extracellular matrix, play a crucial role in maintaining the structural and functional integrity of the heart (Weber, 1989, 1995). Until recently, it was believed that these structural proteins turn over too slowly to be of any pathophysiologic consequence. However, it is now evident that collagen turnover in the heart is dynamic and is under stringent regulation (Laurent, 1987; Bishop *et al.*, 1994; Weber *et al.*, 1995). In certain pathological states, accumulation of connective tissue components, notably the collagens, leads to fibrosis and eventual cardiac failure (Eghbali and Weber, 1990). As collagen levels in any tissue are determined by the balance between rates of synthesis and degradation, investigations on alterations in collagen turnover would contribute considerably to a better understanding of cardiac fibrogenesis in response to various pathophysiological stimuli.

It has been known for a long time that Mg deficiency produces a cardiomyopathy, characterised by focal myocardial necrosis and fibrosis (Bloom, 1988; Kumar *et al.*, in press). Based on several lines of investigation, it has been proposed that systemic oxidative injury, involving inflammatory cytokines and the neuropep

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tide Substance P, may explain the pathobiology of cardiac muscle damage in Mg deficiency (Weglicki *et al.*, 1992, 1994). However, the molecular basis of cardiac fibrogenesis associated with Mg deficiency remains unclear in the absence of investigations on related changes in collagen metabolism.

Over the last few years, this laboratory has been interested in the biochemical correlates of the cardiomyopathy of Mg deficiency. As part of the ongoing investigations, the present study evaluated the extent of lipid peroxidation in the heart to verify the postulation of increased oxidative damage to the cardiac muscle in Mg deficiency. Further, the effects of Mg deficiency *in vivo* on fractional rates of collagen synthesis and rates of collagen degradation and deposition in rat heart were assessed, employing flooding dose methods, to establish the biochemical basis of cardiac fibrosis in Mg deficiency. This communication presents evidence of increased lipid peroxidation and considerable alterations in rates of collagen turnover and cardiac fibroblast proliferation *in vivo* in a rodent model of acute Mg deficiency. Interestingly, although serum Mg was significantly reduced (hypomagnesemia), cardiac tissue levels of Mg remained unchanged throughout the study period, implying that biochemical changes in the heart associated with Mg deficiency are not directly related to alterations in tissue levels of Mg. It appears that dietary deficiency of Mg may promote reparative cardiac fibrogenesis, upon oxidative damage to the cardiac muscle, through a mechanism that does not involve a lowering of myocardial Mg. The possible implications of these observations are discussed.

MATERIALS AND METHODS

Animal feed was procured from Zeigler Bros. Inc., P. A., U.S.A. Feed composition, based on the nutrient requirements for rats proposed by the American Institute of Nutrition is given in Table 1 (Anonymous, 1977). [^{3}H]-proline (sp. radioactivity 8 Ci/mmole) and [^{3}H]-thymidine (sp. radioactivity 17 Ci/mmole) were purchased from Bhabha Atomic Research Centre, Bombay, India. Fine chemicals were from Sigma Chemical Company, Missouri, U.S.A.

Table 1. Composition of rat diet

Cellulose/glucose	50.0 g
Casein lactate	20.0 g
Corn starch	15.0 g
Cellulose	5.0 g
Fat; corn oil	5.0 g
American Institute of Nutrition Vitamin Mix	1.0 g
American Institute of Nutrition Mineral Mix	3.5 g
Dimethionine	0.3 g
Choline bitartrate	0.2 g

Sprague–Dawley rats (M:F 1:1) weighing 100–110 g were pair-fed a Mg-sufficient (0.051%, age-matched control) or Mg-deficient (0.0008%) diet for the duration indicated in the Tables. At sacrifice, cardiac tissue was collected for determination of Mg and Ca levels by atomic absorption spectrophotometry (IL 551, U.S.A.).

MEASUREMENT OF LIPID PEROXIDATION

Lipid peroxidation was assessed by the thiobarbituric acid reaction, following the method of Chandra *et al.* (1994). Cardiac tissue was homogenised and ice-cold trichloroacetic acid (TCA) was added to the homogenate to a final concentration of 10%. After keeping in ice for 10 min, the samples were spun at 600 *g* and the supernatants were collected. Thiobarbituric acid, 1 ml of 0.67% (w/v) pH 7.5, was added to an equal volume of the TCA supernatants, the tubes were shaken well, heated on a boiling waterbath for 10 min and cooled under tap water. Absorbance was then measured at 535 nm. The amount of malondialdehyde formed was calculated from the molar extinction coefficient of malondialdehyde (1.56×10^5/M per cm) and expressed as nanomoles per gram tissue.

MEASUREMENT OF RATES OF COLLAGEN SYNTHESIS

Rates of cardiac collagen synthesis were measured *in vivo* exactly as described by Mays *et al.* (1991), following intraperitoneal administration of [^{3}H]-proline at 0.1 uCi/g body weight along with non-radioactive proline (1.4 mmole/100 g body weight) 1 hr before sacrifice. Fractional rates of collagen synthesis were calculated using the equation:

$$ks = Sb/Sa\ t \times 100$$

where Sb is the specific radioactivity of total hydroxyproline both in the proteinaceous material and in the tissue free pool, Sa is the specific radioactivity of proline in the tissue free pool for protein synthesis and t is the time in days between injection and death. Degradation of newly synthesized collagen (%) was

Table 2. Changes in cardiac tissue magnesium and calcium levels in magnesium-deficient rats

	Magnesium (ug/mg dry tissue)		Calcium (ug/mg dry tissue)	
Day	Control	Deficient	Control	Deficient
28	1.082 ± 0.06 (4)	1.088 ± 0.06 (4)	0.806 ± 0.06 (4)	0.801 ± 0.06 (4)
60	1.144 ± 0.07 (4)	1.168 ± 0.02 (4)	0.780 ± 0.01 (4)	0.956 ± 0.15 (4)
80	1.131 ± 0.01 (4)	1.055 ± 0.09 (4)	0.798 ± 0.02 (4)	1.058 ± 0.13 (4) *

Values are expressed as Mean SD. The number of animals used is indicated in parenthesis. Control vs. Deficient-* $p < 0.001$

calculated from the relative proportions of hydroxyproline in the tissue free pool and proteinaceous fractions. Collagen deposition rates were obtained by subtracting the proportion of collagen degraded rapidly after synthesis from the fractional synthesis rate, as described by Bishop *et al.* (1994).

MEASUREMENT OF RATES OF DNA SYNTHESIS

DNA synthesis was measured by the method of Tan *et al.* (1991). [^{3}H]-thymidine was administered intraperitoneally at 0.1 uCi/g body weight 1 hr before sacrifice. The heart was excised, weighed, minced and homogenised in 2 ml ice-cold phosphate-buffered saline. Ice-cold TCA (2 ml) was added to the homogenate to precipitate the protein, which was then washed three times with ice-cold TCA and rinsed with 2% sodium acetate in 90% alcohol to remove unincorporated thymidine. After centrifugation, the supernatant was discarded and the precipitates were resuspended in 2 ml of 5% TCA. DNA was extracted by heating the samples at 90°C for 80 min. Following centrifugation, aliquots of the supernatants were used for determination of radioactivity. DNA content was determined by the method of Burton (1956).

DATA ANALYSIS

Statistical analysis was done using Student's t-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Cardiac tissue levels of Mg and Ca are shown in Table 2. Mg levels remained unchanged throughout the study period. A small, though not statistically significant, rise in myocardial Ca was observed by day 60. However, the elevation in myocardial Ca was more marked by day 80. A fall in serum Mg and rise in serum Ca in response to dietary deficiency of Mg were observed by us (K. Shivakumar and B. Prakash Kumar, communicated) and others (Jones *et al.*, 1980; Shi *et al.*, 1995).

Levels of thiobarbituric acid reactive substances (TBRS) in the heart were elevated significantly on day 28 of deficiency but a more marked increase (39%) was observed on day 60 (Table 3).

Studies on collagen metabolism *in vivo* were earlier hampered by methodological problems associated with measurement of collagen synthesis rates (see review by Laurent, 1987). The development of the flooding dose method for measuring protein synthesis rates (McNurlan *et al.*, 1979; Garlick *et al.*, 1980) and its application to collagen (Laurent, 1982) paved the way for investigations on collagen metabolism in a variety of species and tissues. Applying the flooding dose method, the present study demonstrated significant changes in collagen metabolism *in vivo* in Mg-deficient rats (Table 4). These changes included a 12% increase in the fractional rate of synthesis, a 49% increase in the hydroxyproline-associated radioactivity per gram tissue and a 24% increase in the collagen deposition rate on day 60. There was no change in the rate of collagen degradation. On day 28, there was no difference between the groups in the fractional synthesis rate or hydroxyproline-associated radioactivity in the heart. However, there was a marked fall in collagen deposition rate in the deficient animals on day 28. This prompted us to look for changes in collagen degradation at this point of

Table 3. Changes in the levels of TBRS in the cardiac tissue of magnesium-deficient rats

Day	Control	Deficient
28	271.99 ± 9.8 (4)	291.28 ± 5.2* (4)
60	312.68 ± 46.0 (10)	434.66 ± 58.0** (10)

Values are expressed as Mean ± SD. The number of animals used is indicated in parenthesis. Values represent nmoles of malondialdehyde/g tissue, wet weight. Control vs. Deficient-*$p < 0.05$; **$p < 0.001$

Table 4. Effect of magnesium deficieny on collagen metabolism in rat heart

Parameter	Day 28		Day 60	
	Control	Deficient	Control	Deficient
Fractional synthesis rate (%/day)	21.01 ± 0.52 (4)	20.88 ± 0.18 (4)	22.64 ± 1.56 (4)	25.30 ± 1.24(4)*
Hydroxyproline-associated radioactivity in collagen (cpm/g tissue wet wt.)	6251 ± 794 (4)	5975 ± 960 (4)	6474 ± 474 (4)	9649 ± 479 (4)*
Degradation rate (%age)	73.1 ± 1.1 (4)	89.3 ± 0.5 (4)**	74.4 ± 2.0 (4)	71.6 ± 0.7 (4)*
Deposition rate (%/day)	5.66 ± 0.3 (4)	2.33 ± 0.2 (4)**	5.79 ± 0.2 (4)	7.19 ± 0.3 (4)**

Values are expressed as Mean ± SD. The number of animals used is indicated in parenthesis Control vs. Deficient-*$p < 0.05$; **$p < 0.001$

time. As shown in Table 4, there was a nearly 22% increase in the degradation rate. Collagen concentration in the heart in the two groups of animals is shown in Table 5. In keeping with the observations cited above, there was a significant reduction in cardiac collagen concentration on day 28 of deficiency while there was an increase on day 60.

Incorporation of [^{3}H]-thymidine *in vivo* is taken as a measure of fibroblast proliferation in the heart. As shown in Table 6, there was no increase in thymidine incorporation into cardiac fibroblasts on day 28 or 60. However, with increasing severity of the deficiency, stimulation of fibroproliferation was evident by day 80. A 20% increase in the DNA content of the heart was also observed on day 80 (Table 7).

DISCUSSION

The increase in TBRS in the cardiac tissue of Mg-deficient rats observed in this study (Table 3) is consistent with the postulation of oxidative damage to the myocardium in Mg deficiency (Weglicki *et al.*, 1994). Significant elevations in serum or plasma TBRS levels in Mg-deficient animals have been observed by us (K. Shivakumar and B. Prakash Kumar, communicated) and Weglicki *et al.* (1994). Moreover, the observation of Freedman *et al.* (1990) that anti-oxidant intervention limits the size of the cardiomyopathic lesions of Mg deficiency supports the concept of free radical-mediated cardiac injury.

The present study also revealed that dietary deficiency of Mg produces marked alterations in collagen metabolism in the heart. While an increase in the fractional rate of synthesis and rates of degradation and deposition was observed on day 60 of deficiency, the changes in the Mg-deficient animals on day 28 were marked by a significant drop in collagen deposition rate following an increase in the rate of degradation of newly synthesised collagen. Fractional synthesis rates remained unchanged on day 28 (Table 4). Consistent with these findings, the concentration of collagen in the heart was found to be higher on day 60 but lower on day 28 in the test group (Table 5). Further, although the aforementioned changes in collagen metabolism were not accompanied by proliferation of fibroblasts on day 28 or even day 60, a fibroproliferative response to dietary deficiency of Mg was observed by day 80 (Table 6), a finding corroborated by an increase in the DNA content (Table 7). A rise in collagen synthesis, initially mediated by existing fibroblasts and subsequently by fibroblast proliferation, has been reported to occur also in response to other stimuli, such as suprarenal abdominal aortic banding (Morkin and Ashford, 1968). The changes in collagen turnover reported here follow different patterns at the two time-points studied and may represent a process of remodelling in the heart in this model. Increased collagen deposition would lead to increased myocardial stiffness with its

Table 5. Effect of magnesium deficiency on concentration of collagen in rat heart

Day	Collagen concentration in the heart (mg/g tissue wet weight)	
	Control	Deficient
28	7.436 ± 0.56 (4)	5.013 ± 0.69* (4)
60	8.205 ± 0.46 (4)	9.752 ± 0.26* (4)

Values are expressed as Mean ± SD. The number of animals used is indicated in parenthesis. Control vs. Deficient-*$p < 0.01$

Table 6. Effect of magnesium deficiency on rates of DNA synthesis in the cardiac tissue

Day	Incorporation of [^{3}H]-thymidine into DNA in the heart (cpm/g tissue)	
	Control	Deficient
28	626 ± 50 (4)	617 ± 25 (4)
60	580 ± 48 (5)	582 ± 67 (5)
80	783 ± 71 (4)	1049 ± 87* (4)

Values are expressed as Mean ± SD. The number of animals used is indicated in parenthesis. Control vs. Deficient-*$p < 0.01$

Table 7. Effect of magnesium deficiency on DNA content of rat heart

	Content of DNA (mg DNA/g tissue)
Control	1.380 ± 0.09 (4)
Deficient	1.663 ± 0.15* (4)

Values are expressed as Mean ± SD. The number of animals used is indicated in parenthesis. Rats were maintained on a Mg-sufficent or -deficient diet for 80 days. Control vs. Deficient-*$p < 0.01$

functional sequelae but this aspect has not hitherto been probed in relation to Mg deficiency. Our observations, in conjunction with those of Freedman *et al.* (1990), suggest that cardiac fibrogenesis associated with Mg deficiency may be reparative in nature, upon oxidative damage to the myocardium.

It may be noted that levels of myocardial Mg remain unchanged in response to acute dietary Mg deficiency (Table 2) but a marked drop in serum Mg has been observed by us (K. Shivakumar and B. Prakash Kumar, communicated) and others (Shi *et al.*, 1995). It is in fact difficult to achieve a reduction in the concentration of intracellular free Mg, the biologically active form of the element, by changing the extracellular Mg concentration (Murphy *et al.*, 1989; Headrick, 1991). It appears therefore that biochemical changes in the heart in Mg deficiency are mediated through a mechanism not involving a reduction in myocardial Mg. Admittedly, the mechanisms remain unclear at present but a few possibilities, as discussed below, are noteworthy.

Using an experimental model that is nearly identical to the one employed in this study, Weglicki *et al.* (1992, 1994) have shown that Mg deficiency produces a pro-oxidant, pro-inflammatory state marked by elevated circulating levels of factors like TNF Substance P, which are known to trigger free radical generation. A role for these factors in promoting oxidative injury to the heart in Mg deficiency seems plausible and is supported by the observation that inhibition of Substance P receptor resulted in the attenuation of indices of oxidative stress *in vivo* (Weglicki *et al.*, 1994). Speculating on an alternative mechanism of free radical production, it was postulated by one of us (Shivakumar, 1995) that, in the absence of a reduction in myocardial Mg, hypomagnesemia may, through effects on vascular smooth muscle, induce mild episodes of ischemia and reperfusion, which can result in free radical production (Bolli, 1990). Such changes could exacerbate the oxidative stress imposed by the cytokines. Though speculative, the possibility that pathological changes in the myocardium in response to dietary Mg deficiency are secondary to hypomagnesemia-induced vascular changes warrants further study.

Increased rates of collagen synthesis, as observed in the present study, reflect increased fibroblast activity. Transforming growth factor-β is a potent mitogen that has a stimulatory effect on cardiac fibroblasts (Heimer *et al.*, 1995). It is up-regulated in tissue injury and is considered to be the cytokine most implicated in fibrosis (Border and Noble, 1994; Waltenberger *et al.*, 1993) but there is no evidence, at present, of its involvement in cardiac fibrogenesis associated with Mg deficiency. On the other hand, the concentration of Substance P is reported to be elevated not only in the plasma but also at the sites of lesions in the cardiac muscle in this model of Mg deficiency. Further, up-regulation of Substance P receptors in target cells has been suggested (Weglicki *et al.*, 1994). As Substance P is known to stimulate fibroblast proliferation and collagen synthesis (Nilsson *et al.*, 1985), it is possible that the neuropeptide triggers the changes in collagen metabolism reported in this communication.

Thus, it is tempting to postulate that induction of pro oxidant and mitogenic factors in hypomagnesemia may trigger not only oxidative damage but also subsequent fibrogenesis in the myocardium, even without a fall in tissue Mg levels.

To conclude, there has been a surge of interest in the cardiovascular consequences of Mg deficiency in view of its high prevalence and its suspected role in ischemic heart disease (Altura and Altura, 1985; Karppanen, 1990). There are several studies on the possible relationship between Mg deficiency and increased vascular tone, hypercoagulability of blood, atherogenesis and cardiac arrhythmias (Arsenian, 1993). However, there are very few reports on biochemical changes in the heart that could provide insights into the pathogenesis of cardiac muscle damage and fibrosis associated with Mg deficiency. This communication reports alterations in lipid peroxidation and collagen metabolism in the heart *in vivo* in response to dietary deficiency of Mg. Importantly, it has been proposed that a fall in serum Mg, without any change in tissue Mg, would suffice to promote oxidative injury to the myocardium and trigger reparative cardiac fibrogenesis. Future studies should address the interaction

between cardiac fibroblasts, Substance P and TGF-β, besides the cellular origin of these mitogens, in order to gain insights into the pathogenesis of myocardial fibrosis in Mg deficiency.

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