Pergamon

Int. J. Biochem. Cell Biol. Vol. 29, No. 11, pp. 1273–1278, 1997

Printed in Great Britain

PII: S1357-2725(97)00068-X

1357-2725/97 $17.00 + 0.00

Magnesium Deficiency Enhances Oxidative Stress and Collagen Synthesis *In Vivo* in the Aorta of Rats

K. SHIVAKUMAR*, B. PRAKASH KUMAR

Division of Cellular and Molecular Cardiology, Sree Chitra Tirunal Institute for Medical Sciences & Technology, Trivandrum-695 011, India

Magnesium deficiency has been shown to produce vascular lesions in experimental animals, but the underlying mechanisms of vascular injury are not clear. It has been reported that in rodents, magnesium deficiency enhances circulating levels of factors that promote free radical generation and are mitogenic. In pursuance of these observations, the present study tested the hypothesis that magnesium deficiency may enhance oxidative stress and trigger an accelerated growth response *in vivo* in the aorta of rats. Oxidative stress was evaluated in terms of levels of thiobarbituric acid-reactive substances in the serum and aorta and activity of superoxide dismutase and catalase in the aorta; fractional rates of collagen synthesis were assessed using [^{3}H]-proline. Serum and tissue levels of magnesium and calcium were determined by atomic absorption spectrophotometry. The present study demonstrated for the first time that magnesium deficiency significantly ($P < 0.001$) increases levels of thiobarbituric acid-reactive substances in the aorta of rats. Other changes in the aorta of animals on the Mg-deficient diet included a significant reduction (54%, $P < 0.001$) in the activity of superoxide dismutase and catalase (37%, $P < 0.01$) and a 19% increase in net fractional rates of collagen synthesis ($P < 0.05$). While serum magnesium was significantly reduced in these animals ($P < 0.001$), aortic tissue levels of magnesium in these animals remained unaltered throughout the duration of the study, suggesting the existence of other control mechanisms, apart from reduced tissue levels of magnesium, mediating the observed effects. These findings suggest that magnesium deficiency may trigger a wound healing response, involving oxidative injury and growth stimulation, in the vascular system.

Keywords: Aorta Magnesium deficiency Lipid peroxidation Superoxide dismutase Catalase Collagen synthesis DNA Synthesis

Int. J. Biochem. Cell Biol. (1997) **29**, 1273–1278

There is considerable evidence to suggest that magnesium deficiency may produce pathological lesions in the vascular system (Bloom, 1988; Arsenian, 1993; Shivakumar, 1995), but the underlying mechanisms of vascular injury remain unclear. Recent studies on rats established that Mg deficiency induces a pro-inflammatory, pro-oxidant state marked by elevated circulating levels of factors like endothelin, TNF-α and Substance P that promote free radical generation and are mitogenic (Weglicki *et al.*, 1992, 1994; Komuro *et al.*, 1988; Villablanca *et al.*, 1994; Nilsson *et al.*, 1985). Consistent with these observations, the results presented in this communication support the hypothesis that Mg deficiency may enhance lipid peroxidation and trigger an accelerated growth response in the vessel wall. These observations are important in view of the high prevalence of Mg deficiency and its strong association with cardiovascular diseases (Altura and Altura, 1985; Prakash Kumar *et al.*, 1996).

*To whom all correspondence should be addressed.

Abbreviations: Mg, magnesium, Ca, calcium, SOD, superoxide dismutase, TBARS, thiobarbituric acid, TCA, trichloroacetic acid, MDA, malondialdehyde, TNF, tumour necrosis factor.

Received 2 December 1996; accepted 9 June 1997.

MATERIALS AND METHODS

Animal feed, Mg-sufficient and -deficient, was procured from Zeigler Bros (PA, U.S.A.). Feed composition was based on the nutrient requirements for rats proposed by the American Institute of Nutrition (Anonymous, 1977). The feed contained, per 100 g, cerelose/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 and choline bitartrate 0.2 g. The Mg-sufficient diet contained 0.051% Mg and 0.5188% Ca. The Mg-deficient diet contained 0.0008% Mg and 0.5188% Ca.

[^{3}H]-thymidine (specific radioactivity 17 Ci/mmol) and [^{3}H]- proline (specific radioactivity 3 Ci/mmol) were from Bhabha Atomic Research Centre (Bombay, India). Fine chemicals were purchased from Sigma Chemical Company (MO, U.S.A.).

Sprague–Dawley rats (M:F 1:1) weighing 100–110 g were used for the experiments. The animals were pair-fed a Mg-sufficient or Mg-deficient diet for the duration indicated in the tables. After killing, blood and aorta were collected for determination of magnesium and calcium levels by atomic absorption spectrophotometry (IL 551, MA, U.S.A.). The thoracic aorta was aseptically excised and the adhering fat was removed with forceps.

Measurement of lipid peroxidation

Lipid peroxidation was assessed by the thiobarbituric acid reaction, following the method of Chandra *et al.* (1994). Briefly, ice-cold trichloroacetic acid (TCA) was added to the tissue homogenate or serum to a final concentration of 10%. The samples were kept in ice for 10 min, centrifuged at 600 **g** and the supernatant was collected. One millilitre of 0.67% (w/v) thiobarbituric acid (pH 7.5) was added to an equal volume of the TCA supernatant. The tubes were shaken well, heated on a boiling waterbath for 10 min and cooled under tap water. The absorbance was read at 535 nm and the amount of malondialdehyde (MDA) formed was calculated from its molar extinction coefficient ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$) and expressed as nanomoles of MDA per gram tissue wet weight or micromoles MDA per 100 ml serum.

Measurement of SOD activity

The tissue was homogenized at 0–4°C in phosphate buffer (pH 7.0) and centrifuged at 10 000 **g** for 10 min. The supernatant was centrifuged again at 18 000 **g** for 10 min. To the supernatant obtained following this step, ammonium sulphate was added to a final concentration of 90%, kept in ice for 10 min and centrifuged at 10 000 **g** for 10 min. The supernatant was dialysed overnight at 4°C against 0.2 M Tris–HCl with three changes of buffer and the dialysate was used for the assay of SOD at 30°C by the method of Kakkar *et al.* (1984). Protein was estimated by the method of Lowry *et al.* (1951) and enzyme activity was expressed as units per milligram of protein. One unit was the amount of enzyme required to inhibit the rate of chromogen formation by 50%.

Measurement of catalase activity

Catalase activity in the tissue was assayed according to the method of Sinha (1972). Reaction with catalase was followed by disappearance of H_2O_2 at 240 nm. Enzyme activity was expressed as $\times 10^{-3}$units/mg protein (unit = velocity constant/sec).

Measurement of rates of collagen synthesis

Aortic collagen synthesis rates were measured *in vivo* as described earlier (Mays *et al.*, 1991), following intraperitoneal administration of [^{3}H]-proline at 0.1 μCi/g body wt along with non-radioactive proline (1.4 mmol/100 g body wt) 1 hr before killing. Fractional rates of collagen synthesis were calculated using the equation: $k_s = S_b/S_a t \times 100$, where S_b is the specific radioactivity of total hydroxyproline both in the proteinaceous material and in the tissue free pool, S_a is the specific radioactivity of proline in the tissue-free pool (precursor pool for protein synthesis) and t is the time in days between injection and death.

Measurement of rates of DNA synthesis

Aortic DNA synthesis was measured *in vivo* following the method of Tan *et al.* (1991). Briefly, [^{3}H]-thymidine was administered intraperitoneally at 0.1 μCi/g body wt 1 hr before killing and its incorporation into TCA-insoluble material from aorta was determined using a liquid scintillation spectrophotometer (Wallac 1409, Sweden). Protein was estimated

Table 1. Changes in serum magnesium and calcium levels in magnesium-deficient rats

Day	Magnesium (mmol/l)		Calcium (mmol/l)	
	Control	Deficient	Control	Deficient
12	0.83 ± 0.25 (8)	0.38 ± 0.09* (8)	1.99 ± 0.05 (5)	2.54 ± 0.13* (10)
28	0.94 ± 0.13 (5)	0.35 ± 0.11* (6)	1.81 ± 0.14 (6)	2.95 ± 0.29* (6)
60	0.88 ± 0.14 (6)	0.42 ± 0.09* (6)	2.06 ± 0.20 (6)	2.85 ± 0.22* (6)

Values are expressed as mean ± SD. The number of animals used is indicated in parentheses.
Control vs deficient: *$P < 0.001$.

Table 2. Changes in the levels of magnesium and calcium in the aorta of magnesium-deficient rats

Day	Magnesium (μg/mg tissue dry wt)		Calcium (μg/mg tissue dry wt)	
	Control	Deficient	Control	Deficient
12	1.06 ± 0.08 (4)	1.11 ± 0.11 (4)	10.80 ± 0.63 (4)	10.97 ± 0.98 (4)
28	1.19 ± 0.10 (5)	1.24 ± 0.06 (5)	12.0 ± 1.04 (5)	12.17 ± 1.62 (5)
60	1.28 ± 0.18 (6)	1.21 ± 0.19 (6)	13.24 ± 0.91 (4)	17.72 ± 1.97* (5)

Values are expressed as mean ± SD. The number of animals used is indicated in parentheses.
Control vs deficient: *$P < 0.005$.

by the method of Lowry *et al.* (1951) and the results were expressed as cpm/mg protein.

Data analysis

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

RESULTS

Rats on Mg-sufficient and Mg-deficient diets were compared with respect to: (1) levels of Mg, Ca and TBARS in the serum and aorta; (2) activity of SOD and catalase in the aorta; and (3) rates of collagen and DNA syntheses in the aorta. Animals on the Mg-deficient diet had significantly lower serum levels of Mg from day 10, compared to the corresponding control values (Table 1). Serum Ca was elevated in these animals (Table 1), which is consistent with the fact that in rats, hypomagnesemia leads to hypercalcemia (Jones *et al.*, 1980). There was no change in the tissue levels of Mg on days 10, 28 or 60 (Table 2). However, tissue Ca was significantly higher on day 60 but not on days 10 or 28 (Table 3).

The results presented in Table 3 show a marked elevation in the amount of TBARS in the serum and aorta of animals in the test group, compared to the control, at all the time-points examined. As MnSOD activity is not detectable in rat aorta (L'Abbe *et al.*, 1991), the SOD activity reported here is the activity of the other form, namely, CuZnSOD.

Table 3. Changes in the levels of TBARS in the serum and aorta of magnesium-deficient rats

Day	Serum		Aorta	
	Control	Deficient	Control	Deficient
10	1.45 ± 0.02 (3)	1.74 ± 0.09* (4)	34.7 ± 0.60 (4)	44.20 ± 1.00** (4)
28	1.55 ± 0.23 (4)	3.06 ± 0.35** (4)	34.3 ± 4.12 (4)	66.98 ± 4.66** (4)
60	1.79 ± 0.10 (4)	2.98 ± 0.20** (4)	35.8 ± 2.66 (5)	53.50 ± 6.30 **(6)

Values are expressed as mean ± SD. The number of animals used is indicated in parentheses.
Values represent nanomoles of malondialdehyde per gram tissue wet weight or micromoles of malondialdehyde per 100 ml of serum.
Control vs deficient: *$P < 0.01$, **$P < 0.001$.

Table 4. Effect of magnesium deficiency on SOD and catalase activity in the aorta of rats

	Control	Deficient
SOD (units/mg protein)	3.74 ± 0.32 (4)	1.72 ± 0.13* (4)
Catalase ($\times 10^{-3}$ units/mg protein)	5.15 ± 0.66 (4)	3.25 ± 0.75** (4)

Values are expressed as mean ± SD. The number of animals used is indicated in parentheses. Rats were maintained on Mg-deficient or Mg-sufficient diet for 21 days.
Control vs deficient: *$P < 0.001$; **$P < 0.01$.

Table 5. Effect of magnesium deficiency on fractional rates of collagen synthesis in the aorta of rats

Control	12.67 ± 0.98 (4)
Deficient	15.13 ± 1.05* (4)

Values are expressed as mean ± SD. The number of animals used is indicated in parentheses. Rats were maintained on a Mg-sufficent or -deficient diet for 28 days.
*$P < 0.05$.

The activities of SOD and catalase decreased by about 54 and 37%, respectively, in the aorta in these animals (Table 4).

Table 5 shows fractional rates of collagen synthesis. The net fractional rate was about 19% higher in the Mg-deficient rats compared to the control on day 28. Rates of incorporation of [^{3}H]-thymidine into vascular cells were slightly, but significantly, higher in the test group (Table 6).

DISCUSSION

The present study furnishes evidence of increased oxidative stress *in vivo* in the aorta of Mg-deficient rats, which is in agreement with an earlier observation that Mg deficiency enhances free radical-induced intracellular oxidation and cytotoxicity *in vitro* in aortic endothelial cells (Dickens *et al.*, 1992). The reduction in activity of the anti-oxidant enzymes in the aorta in Mg deficiency (Table 4) may exacerbate the oxidative stress imposed by augmented free radical generation in this condition (Weglicki *et al.*, 1996). Interestingly, neither SOD nor catalase contains Mg and hence the mechanism whereby their activity is lowered in Mg deficiency is not clear. However, it has been reported that copper deficiency lowers the activity of not only cuproenzymes but also of enzymes such as glutathione peroxidase that do not contain copper (Prohaska, 1991); it has been suggested that glutathione peroxidase may be inactivated by superoxide radicals (Blum and Fridovich, 1985). The possibility that such a mechanism may operate in Mg deficiency warrants further investigation.

The marked increase in rates of collagen synthesis reported here, together with the small increase in DNA synthesis, indicate that Mg deficiency elicits a growth response in the vessel wall. Moreover, elevation in rates of DNA synthesis is in line with earlier histological evidence of proliferating vascular smooth muscle cells in Mg-deficient animals (Bloom, 1988; Vitale *et al.*, 1963). It is not clear from this study whether the accelerated growth response indicates a direct effect of Mg deficiency or a reparative process following oxidative damage to the vessel in Mg deficiency. It is also not clear if a causal relationship exists between the observed increase in lipid peroxidation and increased growth response. However, oxidized LDL and superoxide have been shown to stimulate collagen synthesis in cultured arterial smooth muscle cells (Jimi *et al.*, 1995) and fibroblasts (Chandrakasan and Bhatnagar, 1991), respectively. Moreover, hydrogen peroxide stimulates DNA synthesis *in vitro* in vascular smooth muscle cells (Rao and Berk, 1992). Taken together, the findings reported in this communication suggest a mechanism of vascular injury in Mg deficiency based on oxidative stress and enhanced matrix synthesis.

We have recently shown that reparative fibrogenesis in the heart in response to dietary deficiency of Mg is not associated with alterations in myocardial Mg levels (Prakah Kumar *et al.*, 1997). Interestingly, in the present study, biochemical changes in the vessel were observed even as vascular tissue levels of Mg, unlike serum Mg (Table 1), remained unchanged over the entire 2-month period of the study in both groups of animals (Table 2). Further, elevation in the tissue levels of Ca did not correlate with these changes at all the time-points studied, implying that even Ca may not be a factor in the observed effects of Mg deficiency, at least in the early stages. These findings are significant as they point to

Table 6. Effect of magnesium deficiency on rates of DNA synthesis in the aorta of rats

	Day 12	Day 60
Control (cpm/mg protein)	1075 ± 20.9 (5)	1744 ± 168.9 (7)
Deficient (cpm/mg protein)	1503 ± 149.4* (7)	2363 ± 123.1* (5)

Values are expressed as mean ± SD. The number of animals used is indicated in parentheses.
Control vs deficient: *$P < 0.001$.

the existence of other control mechanisms, apart from altered tissue content of Mg and Ca, mediating the vascular effects of Mg deficiency. Earlier work on a rat model of Mg deficiency, nearly identical to the one employed here, had demonstrated elevations in plasma and vascular tissue levels of mitogens like Substance P, which are also known to promote free radical generation (Weglicki *et al.*, 1992, 1994). Though speculative, the possibility that these vasoactive factors trigger vascular changes in hypomagnesemia, without a reduction in the tissue levels of Mg, merits scrutiny. Summarizing, the link between dietary deficiency of Mg, fall in serum Mg levels without any change in tissue Mg levels and the observed changes in the tissue in this model, as postulated by us, is best depicted by the following scheme:

Dietary deficiency of Mg → fall in serum Mg levels without a fall in aortic tissue Mg levels → induction of pro-oxidant, mitogenic factors*→increased oxidative stress/accelerated growth response in the aorta

The results presented in this communication are significant for the reasons cited below. To our knowledge, this is the first demonstration of the influence of a nutritional deficiency on lipid peroxidation or expression of a matrix protein in vascular cells *in vivo*, and its implications need to be seen in the light of the high prevalence of Mg deficiency (Altura and Altura, 1985) whose etiology could be congenital, dietary, drug-induced or metabolic. While oxidative stress and increased matrix synthesis may represent a mechanism of vascular injury in Mg deficiency, the pathophysiologic significance of these observations is difficult to comment upon at present. However, as oxidative processes and an accelerated growth response in the vessel wall are considered important steps in the pathogenesis of atherosclerosis (Freubis *et al.*, 1995; Daemen *et al.*, 1991), the observations presented here appear to be in line with the postulated relationship between Mg deficiency and atherosclerosis (Arsenian, 1993; Altura *et al.*, 1990).

Acknowledgements—This work was supported by a research grant to Dr K. Shivakumar from the Department of Science and Technology, New Delhi. The authors thank Dr R. Renuka Nair for statistical analysis of data.

*(Weglicki *et al.*, 1996).

REFERENCES

Anonymous (1977) American Institute of Nutrition *ad hoc* Committee on Standards for Nutritional Studies. *J. Nutr.* **107,** 1340–1348.

Arsenian M. A. (1993) Magnesium and cardiovascular disease. *Progr. Cardiovasc. Dis.* **35,** 271–310.

Altura B. M. and Altura B. T. (1985) New perspectives on the role of magnesium in the physiology of the cardiovascular system—clinical aspects. *Magnesium* **4,** 226–244.

Altura B. T., Brust M., Bloom S., Barbour R. L., Stempak J. G. and Altura B. M. (1990) Magnesium dietary intake modulates blood lipid levels and atherogenesis. *Proc. Natl Acad. Sci. U.S.A.* **87,** 1840–1844.

Bloom S. (1988) Magnesium deficiency cardiomyopathy. *Am. J. Cardiovasc. Path.* **2,** 7–17.

Blum J. and Fridovich I. (1985) Inactivation of glutathione peroxidase by superoxide radical. *Arch. Biochem. Biophys.* **240,** 500–508.

Chandra M., Chandra N., Agrawal R., Kumar A., Ghatak A. and Pandey V. C. (1994) The free radical system in ischemic heart disease. *Int. J. Cardiol.* **43,** 121–125.

Chandrakasan G. and Bhatnagar R. S. (1991) Stimulation of collagen synthesis in fibroblast cultures by superoxide. *Cell. Mol. Biol.* **37,** 751–755.

Daemen M. J. A. P., Lombardi D. M., Bosman F. T. and Schwartz S. M. (1991) Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. *Circ. Res.* **68,** 450–456.

Dickens B. F., Weglicki W. B., Li Y. S. and Mak I. T. (1992) Magnesium deficiency *in vitro* enhances free radical-induced intracellular oxidation and cytotoxicity in endothelial cells. *FEBS Lett.* **311,** 187–191.

Freubis J., Carew T. E. and Palinski W. (1995) Effect of vitamin E on atherogenesis in LDL receptor-deficient rabbits. *Atherosclerosis* **117,** 217–224.

Jimi S., Saku K., Uesugi N., Sakata N. and Takebayashi S. (1995) Oxidised low density lipoprotein stimulates collagen production in cultured arterial smooth muscle cells. *Atherosclerosis* **116,** 15–26.

Jones J. E., Schwartz R. and Krook L. (1980) Calcium homeostasis and bone pathology in magnesium-deficient rats. *Calc. Tiss. Int.* **31,** 231–238.

Kakkar P., Das B. and Vishwanath P. N. (1984) A modified spectrophotometric assay of superoxide dismutase. *Ind. J. Biochem. Biophys.* **21,** 130–132.

Komuro I., Kurihara H., Sugiyama T., Takaku F. and Yazaki Y. (1988) Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. *FEBS Lett.* **238,** 249–252.

L'Abbe M. R., Trick K. D. and Beare-Rogers J. L. (1991) Dietary $(n-3)$ fatty acids affect rat heart, liver and aorta protective enzyme activities and lipid peroxidation. *J. Nutr.* **121,** 1331–1340.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

Mays P. K., McAnulty R. J., Campa J. and Laurent G. J. (1991) Age-related changes in collagen synthesis and degradation in rat tissue. *Biochem. J.* **276,** 307–313.

Nilsson J., Euler A. M. V. and Dalsgaard C. J. (1985) Stimulation of connective tissue cell growth by Substance P and Substance K. *Nature* **315,** 61–63.

Prakash Kumar B., Shivakumar K. and Kartha C. C. (1997) Magnesium deficiency-related changes in lipid peroxidation and collagen metabolism *in vivo* in rat heart. *Int. J. Biochem. Cell Biol.* **29,** 129–134.

Prakash Kumar B., Shivakumar K., Kartha C. C. and Rathinam K. (1996) Magnesium deficiency and cerium promote fibrogenesis in rat heart. *Bull. Environ. Contam. Toxicol.* **57,** 517–524.

Prohaska J. R. (1991) Changes in Cu,Zn-superoxide dismutase, cytochrome *c* oxidase, glutathione peroxidase and glutathione transferase activities in copper-deficient mice and rats. *J. Nutr.* **121,** 355–363.

Rao G. N. and Berk B. C. (1992) Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ. Res.* **70,** 593–599.

Shivakumar K. (1995) Magnesium deficiency and the cardiovascular system. *Curr. Sci.* **68,** 1221–1226.

Sinha A. K. (1972) Colorimetric assay of catalase. *Anal. Biochem.* **47,** 389–394.

Tan L. B., Jalil J. E., Pick R., Janicki J. S. and Weber K. T. (1991) Cardiac myocyte necrosis induced by angiotensis II. *Circ. Res.* **69,** 1185–1195.

Villablanca A. C., Murphy C. J. and Reid T. W. (1994) Growth promoting effects of Substance P on endothelial cells *in vitro*. *Circ. Res.* **75,** 1113–1120.

Vitale J. J., Velez H., Guzman C. and Correa P. (1963) Magnesium deficiency in the cebus monkey. *Circ. Res.* **12,** 642–650.

Weglicki W. B., Mak T. and Phillips T. M. (1994) Blockade of cardiac inflammation in magnesium deficiency by Substance P receptor inhibition. *Circ. Res.* **74,** 1009–1013.

Weglicki W. B., Mak I. T., Kramer J. H., Dickens B. F., Cassidy M. M., Stafford R. E. and Phillips T. M. (1996) Role of free radicals and Substance P in magnesium deficiency. *Cardiovasc. Res.* **31,** 677–682.

Weglicki W. B., Phillips T. M., Freedman A. M., Cassidy M. M. and Dickens B. F. (1992) Magnesium deficiency elevates circulating levels of inflammatory cytokines and Substance P. *Mol. Cell. Biochem.* **110,** 169–173.