Eur J Nutr 41 : 197–202 (2002)
DOI 10.1007/s00394-002-0376-0

ORIGINAL CONTRIBUTION

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Protective effect of calcium deficiency on the inflammatory response in magnesium-deficient rats

Summary *Background* Previous studies indicated that dietary Mg-deficiency in rats results in a marked pro-inflammatory effect. Since magnesium (Mg) frequently acts as a natural calcium (Ca) antagonist, the possibility exists that the pro-inflammatory effect of Mg-deficiency may be a consequence of a reduced extracellular Mg^{2+}/Ca^{2+} antagonism. *Aim of the study* Thus, the aim of the study was to assess whether dietary Ca-deficiency improves the abnormal inflammatory response of Mg-deficient rats. *Materials and methods* Weaning male Wistar rats were randomly divided into 4 groups according to the dietary Mg and Ca as follows: Mg-adequate Ca-adequate (control), Mg-adequate Ca-deficient, Mg-deficient Ca-adequate, Mg-deficient Ca-deficient. Animals were fed the appropriate diets for 8 days. *Results* Mg-deficient Ca-adequate rats as compared to controls displayed the usual decrease in plasma Mg, whereas the plasma Ca concentration was unaffected. The classical symptoms of inflammation including hyperemia, increased number of blood leukocytes and increased spleen weight were observed. In addition, these animals also showed an increase in heart lipid peroxidation and in plasma triglyceride concentration. In Mg-deficient rats, Ca-deficiency induced hypocalcemia and offered a significant protection against the pro-inflammatory effect of Mg-deficiency. This was evidenced by lower inflammation scores, prevention of leukocytosis and of spleen enlargement. The protective effects of Ca-deficiency on the inflammatory response in Mg-deficiency was accompanied by significant reduction in lipid peroxidation and by a normalization of plasma triglyceride concentration. *Conclusion* All together, the results suggest that Ca is implicated in the inflammatory response of experimental Mg-deficiency and that oxidative stress and hypertriglyceridemia are the results of the acute phase response following Mg-deficiency in rats.

Key words magnesium – calcium – inflammation

Received: 3 June 2002
Accepted: 14 August 2002

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Abbreviations

Ca	calcium
Mg	magnesium
TBARS	thiobarbituric acid reactive substances
TGRLP	triglyceride rich lipoprotein

Introduction

Multiple alterations in inflammatory and immunological functions have been demonstrated in experimental magnesium (Mg)-deficiency [1–4]. Dietary Mg-deficiency in rats gives rise after a few days to a characteristic inflammatory response. This inflammatory response has been proposed to be responsible for oxidative damages in Mg-deficiency and for metabolic disturbances including modifications of lipid metabolism [5]. How-

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ever, the underlying mechanism for the activation of inflammatory cells in Mg-deficient animals is unknown. The pathophysiological response to immune stress includes activation of several processes which are dependent on cytosolic Ca^{2+} elevation [6] and Mg frequently acts as a natural calcium antagonist [7]. Thus, it may be hypothesized that a reduced extracellular Mg^{2+}/Ca^{2+} antagonism results in a pro-inflammatory effect [8]. Hypocalcemia is a manifestation of Mg-deficiency when rats are fed a Ca-deficient diet [9]. Thus, we used this peculiarity to assess whether hypocalcemia induced by a Ca-deficient diet affects the inflammatory response in Mg-deficient rats.

Materials and methods

Experimental design

Weaning outbreed male Wistar rats, 26 d old, obtained from the Comparative Nutrition Unit (INRA: National Institute of Agricultural Research, Clermont-Ferrand-Theix, France) were used. Animals were housed in wire-bottomed cages under constant temperature (20–22 °C) and humidity (45–50 %) in rooms with fixed 12 hour artificial light-dark cycles. Rats were selected for uniform body weight (67 ± 2 g) from a larger population and randomly assigned to dietary treatments without an acclimatization period. Rats were randomly divided into 4 groups according to the dietary Mg and Ca as follows: group 1: Mg-adequate Ca-adequate (8 rats), group 2: Mg-adequate Ca-deficient (8 rats), group 3: Mg-deficient Ca-adequate (6 rats), group 4: Mg-deficient Ca-deficient (6 rats). Animals were fed the respective diets for 8 days. The semi-purified diets contained (in g/kg diet): 650 sucrose, 200 casein, 50 corn oil, 50 alphacel, 3 D,L-methionine, 2 choline bitartrate, 35 modified AIN–76 mineral mix formulated in our laboratory to omit MgO and 10 AIN-76A vitamin mix (ICN biomedicals, Orsay, France). Mg content of the diet was 30 mg/kg diet (Mg-deficient diets) and Mg-adequate diets were prepared by adding MgO to produce a final concentration of 950 mg Mg/kg. The Ca-deficient diets were formulated by adding NaH_2PO_4 and KH_2PO_4 in place of $CaHPO_4$. Ca contents of the diets were 5.020 g/kg and 0.029 g/kg, respectively, for the Ca-adequate and the Ca-deficient diets. All rats were fed *ad libitum* with distilled deionized drinking water. Non-fasted animals were killed after being anesthetized with pentobarbital sodium (40 mg/kg body weight i. p.). Blood was collected into heparinized tubes and plasma was obtained by low speed centrifugation. The spleen was removed and weighed; the heart was rapidly removed and placed in liquid nitrogen and stored at –80 °C. All procedures were in accordance with the institute's guide for the care and use of laboratory animals.

Clinical signs of inflammation

The redness of the ears was recorded once a day to evaluate the occurrence and intensity of the clinical signs of inflammation. We used the score of Nishio et al. [10] based on the following criteria: scores from 0 to 4 (score 0: no hyperemia, score 1: hyperemia at the base of the ears, score 2: hyperemia over half of the ears, score 3: hyperemia at over three quarters of the ears, score 4: hyperemia over the entire ears).

Analytical procedures

Mineral analysis: Mg and Ca were determined in plasma with Perkin Elmer 800 atomic absorption spectrophotometer. The number of total white blood cells was determined by a cell counter (Cobas, Hoffmann, La Roche). Heart tissue was chosen to assess the effect of experimental diet on lipid peroxidation. Thiobarbituric acid-reactive substances (TBARS) were determined in BHT-free tissue homogenates after lipid peroxidation induced by $FeSO_4$ (10 µmol/l)-ascorbate (250 µmol/l) for 30 minutes in a 37 °C water bath in an oxygen free medium, using a standard of 1,1,3,3-tetraethoxypropane [11]. Plasma triglycerides were determined using a colorometric assay (Biomérieux, Marcy l'Etoile, France).

Statistical analysis

Statistical analysis was conducted using Statview (Statistical Software, France). Results are presented as means with their standard errors. Two-way analysis of variance (ANOVA) was used to determine the main effects (Mg, Ca levels) and interaction (Mg × Ca). When $P < 0.05$, means were compared by using the PLSD Fisher post test. The unpaired student t test was used for statistical analysis when results were obtained in only two experimental groups. The differences were considered to be statistically significant when the P value was less than 0.05.

Results

In rats fed Mg-adequate diet, Ca-deficiency was associated with a significant decrease in plasma Ca concentration and a significant increase in plasma Mg concentration. Parameters concerning inflammation, oxidative stress, triglyceride plasma levels were unaffected as compared to rats fed the control diet (Tables 1 and 2). Mg-deficient Ca-adequate rats as compared to control rats suffered from a small weight reduction and displayed the usual decrease in plasma Mg, whereas plasma

Table 1 Effect of dietary calcium and magnesium on body weight, plasma concentration of Mg, Ca, number of blood leukocytes and spleen weight[1]

	Mg-Adequate		Mg-Deficient		Two-Way ANOVA[2]		
	Ca-Adequate	Ca-Deficient	Ca-Adequate	Ca-Deficient	Mg	Ca	Mg x Ca
Body weight (g)	117 ± 2.0^a	110 ± 3.0^a	102 ± 2.0^b	94 ± 4.0^b	< 0.0001	< 0.05	NS
Plasma Mg (mmol/L)	0.74 ± 0.02^a	0.95 ± 0.06^b	0.16 ± 0.01^c	0.21 ± 0.05^c	< 0.0001	< 0.01	NS
Plasma Ca (mmol/L)	2.65 ± 0.02^a	2.33 ± 0.10^b	2.61 ± 0.03^a	1.87 ± 0.10^c	< 0.01	< 0.0001	< 0.05
Leukocytes (10^6 cells/mL)	4.1 ± 0.2^a	4.0 ± 0.4^a	14.5 ± 1.9^b	6.0 ± 1.0^a	< 0.0001	< 0.0001	< 0.001
Relative spleen weight (g/100 g body weight)	0.54 ± 0.03^a	0.47 ± 0.03^a	0.87 ± 0.12^b	0.43 ± 0.03^a	< 0.05	< 0.001	< 0.01

[1] Mean values with their standard errors for eight rats in Mg-adequate groups and for six rats in Mg-deficient groups
[2] *P* values, two-way ANOVA. *NS* not significant. Means in the same row with different superscripts are significantly ($P < 0.05$) different (PLSD Fisher post-ANOVA)

Table 2 Effect of dietary calcium and magnesium on plasma triglycerides and heart lipid peroxidation[1]

	Mg-Adequate		Mg-Deficient		Two-Way ANOVA[2]		
	Ca-Adequate	Ca-Deficient	Ca-Adequate	Ca-Deficient	Mg	Ca	Mg x Ca
TBARS[3] (nmol/g wet weight)	32 ± 2.0a	29 ± 3.0^a	47.0 ± 5.0^b	34.0 ± 2.0^a	< 0.05	< 0.05	NS
Triglycerides (mmol/L)	1.4 ± 0.1^a	1.2 ± 0.3^a	2.6 ± 0.4^b	1.1 ± 0.3^a	NS	< 0.01	NS

[1] Mean values with their standard errors for eight rats in Mg-adequate groups and for six rats in Mg-deficient groups
[2] *P* values, two-way ANOVA. *NS* not significant. Means in the same row with different superscripts are significantly ($P < 0.05$) different (PLSD Fisher post-ANOVA).
TBARS[3] thiobarbituric acid reactive substances

Ca concentration was unaffected. The classical symptoms of inflammation including hyperemia, increased number of blood leukocytes and increased spleen weight were observed in these rats (Table 1 and Fig. 1). In addition, these animals presented increased lipid peroxidation of heart tissue as shown by the TBARS concentration, and plasma triglyceride concentrations were also found significantly increased (Table 2). These consequences of Mg-deficiency greatly differed when rats were also fed a Ca-deficient diet. As expected, although body weight and blood plasma Mg were found similar, plasma Ca concentration was significantly decreased (Table 1). The time course of the clinical signs of inflammation was reported in Fig. 1. Hyperemia of the ears became apparent in Mg-deficient Ca-adequate rats from day 4 and increased from day 4 to day 6 and the inflammation score remained elevated until the end of the experimental period. In Mg-deficient Ca-deficient rats, hyperemia appeared more slowly and the inflammation score remained significantly lower than in the Mg-deficient Ca-adequate group. Thus, at the end of the experimental period, inflammation scores were 1.9 ± 0.2 *vs* 0.8 ± 0.4 A. U. ($P < 0.05$) in Mg-deficient Ca-adequate *vs* Mg-deficient Ca-deficient rats, respectively. Mg-deficient rats fed the Ca-deficient diet had a normal number of blood leukocytes and normal relative spleen weight (Table 1). Moreover, heart lipid peroxidation was lower in rats fed the Mg-deficient Ca-deficient diet compared to animals fed Mg-deficient Ca-adequate diet as shown by TBARS concentration before induction (Table 2) or after exposure of tissue homogenates to iron-induced lipid peroxidation (351 ± 14 *vs* 412 ± 17 nmol/g wet weight; $P = 0.02$). In addition, Mg-deficient rats fed the Ca-deficient diet were protected from hypertriglyceridemia (Table 2).

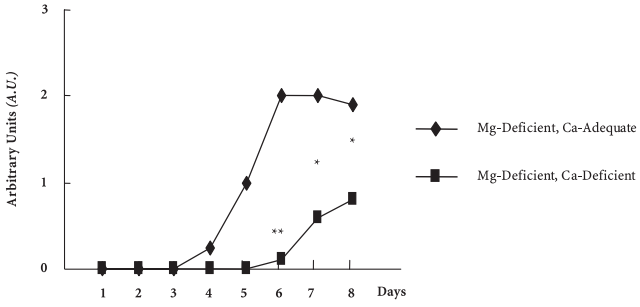

Fig. 1 Inflammation score (hyperemia of the ears) in Mg-deficient rats fed adequate or low Ca level. Each point represents the mean with their standard errors of six rats per group. *p < 0.05; **p < 0.01

Discussion

The data indicate that Ca-deficiency offers significant protection against the pro-inflammatory effect of Mg-deficiency. This was evidenced by a lower inflammation score, prevention of leukocytosis and prevention of spleen enlargement which are well-known consequences of inflammatory response in Mg-deficiency. A characteristic allergy-like crisis occurs spontaneously in Mg-deficient rats. As a consequence, pronounced ery-

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thema, hyperemia and edema appear simultaneously and predominantly at the ears. The blood leukocyte response as shown in the present study is also a consequence of Mg-deficiency [12] and the larger spleen size in Mg-deficient rats is believed to be due to infiltration of the spleen with polymorphonuclear leukocytes and macrophages [13]. In the present study, all Mg-deficient rats exhibited similar blood plasma Mg concentration regardless of Ca content of the diet. However, the inflammatory response was dramatically reduced when blood plasma Ca concentration decreased in agreement with a previous observation showing the attenuation of erythema formation in Mg-deficient rats following Ca-deficiency [14]. Mg is known to influence the formation or secretion of hormones that regulates Ca homeostasis [15]. Mg is thus essential for the normal function of the parathyroid gland, vitamin D metabolism, adequate sensitivity of target tissues to parathyroid hormone and to active vitamin D metabolites. Therefore, hypocalcemia is a prominent manifestation of severe Mg-deficiency in man as in most other species [15]. Unlike in other species, blood plasma Ca concentration has been found unchanged or even increased in Mg-deficient rats. This peculiarity has been attributed to an increased Ca absorption since the response depends on dietary Ca [9, 16]. The Ca adequate diet used in the present experiment contained 5.020 g/Kg in agreement with the AIN standard for nutritional studies [17]. Blood plasma Ca concentration was found unchanged in Mg-deficient rats after 8 days on the experimental diet. However, with the same diet, hypercalcemia was observed in experiments of longer duration [18]. Other studies indicate that hypercalcemia is an early event in Mg-deficient rats when calcium content in the diet was higher than in our experimental conditions [19]. When there is a reduction of Ca absorption as a result of low Ca diet, rats respond to Mg-deficiency by becoming hypocalcemic. By contrast, rats fed a Mg-adequate diet have the ability to maintain Ca homeostasis during Ca depletion by increasing bone resorption. In the present study, the slight decrease in blood plasma Ca concentration and the increase in blood plasma Mg concentration in rats fed the Mg-adequate, Ca-deficient diet as compared to rats fed the control diet are indicative of this response whereas severe hypocalcemia occurred in Mg-deficient rats fed the Ca-deficient diet.

As a consequence of phagocytic cell activation during inflammatory response of Mg-deficiency, there is synthesis and release of numerous mediators (toxic oxygen species, cytokines, lipid mediators, etc.) which may produce generalized inflammation and tissue damage in the body [2–4] but the underlying mechanism for the activation of inflammatory cells in Mg-deficient animals remains unclear. Recent studies indicated that the activated state of immune cells is an early event occurring after a few days of Mg-deficiency [20]. Since the cellular Mg content is tightly regulated and changes only slightly even when the extracellular concentration is drastically decreased, total intracellular Mg is unaffected in short-term deficiency [21] suggesting that the effect of Mg-deficiency might be induced by the reduction of the extracellular Mg concentration. In the present study, it was remarkable that the decrease of plasma Ca concentration was associated with a significant reduction of the inflammatory response in Mg-deficiency even if there is no difference in Mg plasma concentration. This result supports the suggestion that disturbances in Ca cellular regulation are responsible or contribute to the inflammatory response during Mg-deficiency.

Ca has been implicated in many aspects of inflammatory response. For instance, Ca is recognized as an important second messenger in the signaling process of leukocyte oxidative burst and is involved in eicosanoid formation. Ca ions are key mediators in regulation of oxidant formation by polymorphonuclear leukocytes and in maintaining normal functions of these cells [6]. Pharmacological stimulation of Ca entry through the neutrophil membranes by means of Ca ionophore has been shown to induce a burst of metabolic activity [22] whereas Ca antagonist can suppress chemiluminescence activity [23]. Moreover, the production of leukotrienes is Ca dependent *via* phospholipase A_2 and lipooxygenase activation [24]. Ca has also been implicated in the pathophysiology of immune stress. In animal models, increased ionized Ca progressively increased endotoxin lethality whereas hypocalcemia lowered endotoxin-induced mortality [25]. Finally, at the cellular level, the response to many agents is transducted by changes in cytosolic Ca which involves both mobilization of cellular pools and entry of extracellular Ca through membrane channels. Thus, as previously suggested [8], a possible explanation for the inflammatory response to Mg-deficiency and for the vulnerability of Mg-deficient rats to endotoxins may be a net influx of extracellular Ca ions into the cells. In Mg-deficiency a lower plasma Ca could be beneficial by decreasing Ca entry and external Ca availability and thus intracellular Ca concentration. Ca-deficient rats fed the Mg-adequate diet presented a slight decrease in plasma Ca levels compared to Ca-deficient/Mg-deficient rats. The effect of hypocalcemia when the inflammatory response is not related to Mg deficiency is unknown. Nevertheless, the present experiment suggests that the pathological mechanism of the inflammatory response in Mg deficiency may consist of reduced extracellular Mg^{++} Ca^{++} antagonism. Thus the possibility exists that above the threshold of extracellular Mg concentration the potential effect of Ca deficiency alone can be compensated for and do not reach significance.

Experimental evidence provided support for the notion that oxidative stress occurs during Mg-deficiency. Tissues of Mg-deficient rats have a greater tendency to

undergo lipid peroxidation than do tissues of control animals as shown by TBARS measurements [11] and electron spin resonance studies [26]. Moreover, various antioxidants provide protection against lesions of the myocardium of Mg-deficient rodents [4]. The origin of the oxidative stress in Mg-deficiency may be related to free radical production, to enhanced vulnerability of cellular components to free radical attack or to decreased tissues antioxidant content. Given the consequences of Mg-deficiency on cardiovascular risk [5], heart tissue was chosen to assess the effect of experimental diets on lipid peroxidation. Results suggest that oxidative stress in Mg-deficiency is clearly related to inflammation since the protective effect of Ca-deficiency on the inflammatory response in Mg-deficiency is accompanied by a significant reduction in lipid peroxidation of heart tissue. This suggestion is supported by previous results showing that experimental Mg-deficiency induces phagocyte activation providing a potential source of free radical production [12, 27]. The depletion of antioxidant defences which limit free radical detoxification [4, 27] may be a consequence of that increased inflammatory response.

The most obvious consequence of Mg-deficiency on plasma lipids is a marked increase in triglyceride levels [5]. A characteristic hyperlipidemia associated with Mg-deficiency is an accumulation of triglyceride-rich lipoprotein (TGRLP) and a decrease in the concentration of high density lipoproteins [28]. Recent data show a complex pattern of alterations in lipid hepatic metabolism and apolipoprotein gene expression in Mg-deficient rat and suggest a defect in the catabolism rather than in the secretion of TGRLP as a major factor underlying the altered lipoprotein profile [29]. Moreover, triglyceride-rich lipoprotein isolated from Mg-deficient rats was more susceptible to *ex vivo* oxidation with copper than lipoproteins isolated from control animals [11]. Since a large body of evidence implicates lipoprotein oxidation at an early stage of atherosclerosis [30], oxidative modifications of lipoproteins could play a significant role in the pathogenesis of vascular lesions following Mg-deficiency [5]. Similarly, several studies demonstrated that inflammation, whatever its origin, rapidly increases serum triglyceride levels by stimulating hepatic very low density lipoprotein production and by decreasing triglyceride clearance. Moreover, inflammation is a potent stimulation for inducing oxidation of serum lipoproteins [31]. On the basis of this observation, it was reasonable to hypothesize that inflammation that occurs during experimental Mg-deficiency could be the mechanism that induces proatherogenic changes in lipoprotein metabolism. This hypothesis is supported by the normalization of plasma triglycerides concentration when the inflammatory response of Mg-deficient rats is improved by Ca-deficiency. All together, the results of the present experiment suggest that Ca is implicated in the inflammatory response of Mg-deficiency in rats and that oxidative stress and hypertriglyceridemia are the result of the acute phase response following Mg-deficiency.

Two limitations of this study are noteworthy. First, compared with the situation in humans, the degree of Mg deficiency in this experimental model was extreme. However, a mild degree of Mg deficiency might occur frequently in humans [32]. Second, rats present some peculiarities concerning the inflammatory response and the calcium metabolism disturbances following experimental Mg deficiency [33]. In addition, future studies are needed to compare several degrees of Mg/Ca-deficiency and to observe the effect of Ca/Mg-deficiency on inflammatory response for a longer period. Although the relevance of these findings to the disease processes in humans remains to be established, these results are consistent with recent findings showing the influence of extracellular Mg on human leukocyte activation and suggesting that extracellular magnesium might diminish leukocyte activation by its calcium antagonist deficiency [33, 34].

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