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EXACERBATED IMMUNE STRESS RESPONSE DURING EXPERIMENTAL MAGNESIUM DEFICIENCY RESULTS FROM ABNORMAL CELL CALCIUM HOMEOSTASIS

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Summary

The aim of this study was to assess the potential mechanism underlying the enhanced inflammatory processes during magnesium deficit. In this study, exacerbated response to live bacteria and platelet activating factors was shown in rats fed a magnesium-deficient diet. Peritoneal cells from these animals also showed enhanced superoxide anion production and calcium mobilising potency following in vitro stimulation. The latter effect occurred very early in the course of magnesium deficiency. These studies first showed that an abnormal calcium handling induced by extracellular magnesium depression in vivo may be at the origin of exacerbated inflammatory response.

Key Words: magnesium deficit, immune stress, calcium homeostasis

Magnesium plays an important role as co-factor in many biochemical reactions (1) leading to speculation concerning its role in the aetiology of degenerative diseases including cardiovascular diseases, diabetes, hypertension, or atherosclerosis (2). In a variety of animal models, magnesium deficiency was shown to affect the immune system. Pioneer works of Kruse et al. (3) in 1932 have established that peripheral vasodilatation with hyperaemia was the most obvious consequence of inflammatory syndrome associated with Mg deficiency. This was later confirmed by greater degranulation of mast cells and release of histamine and inflammatory mediators in Mg-deficient animals (4). Using rats having decreased magnesium status and their peritoneal cells, we explored the effect of immune enhancing factors both in vivo and in vitro. A hypothesis on abnormal calcium homeostasis during magnesium deficiency was developed to account for the abnormal reactivity to immune stress.

Methods

Animals and diets: Male Wistar rats (Iffa-Credo, L'Arbresle, France), 3 weeks old and weighing 65-70 g were used in this study. They were randomly housed 4 rats per wire-bottomed transparent cage, in a temperature controlled room (22°C) with a 12 h light-dark cycle.

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They were pair-fed for 2 or 8 days semi-purified diets containing 980 mg magnesium/kg diet (control) or 30 mg/kg (deficient). The institution's guidelines for the care and use of laboratory animals were followed.

Bacterial challenge: An E. coli strain (serotype O153:K-:H-) isolated from calf septicaemia was used. Six rats from control and Mg-deficient groups received 1.9 x 10^8 or 1.9 x 10^7 bacteria per rat, by intravenous injection into caudal vein. The mortality rate was followed for up to 2 days following injection.

Platelet activating factor (PAF) injection: PAF in absolute methanol was evaporated under nitrogen, then was dissolved in saline. The rats were injected intraperitoneally with PAF at the indicated doses and were monitored for mortality. Results are expressed as percentages of surviving animals during 100 to 150 minutes following PAF injection.

Peritoneal cells harvesting: Peritoneal resident cells from rats fed the appropriate diets for 2 or 8 days were obtained aseptically by peritoneal lavage with 15 ml of cold Hank's balanced salt solution (HBSS). The resulting cell suspension was centrifuged at 500 x g for 15 min, the cell pellet was washed by RPMI 1640 supplemented with 5 % foetal bovine serum (FBS) and 10 mM Hepes at pH 7.4 and finally the cells were resuspended in the latter medium at a concentration of 8.0 x 10^6/ml. Cell suspensions contained 60 % macrophages as determined by differential cell counts.

Measurement of intracellular calcium concentration ($[Ca^{2+}]i$): The cells were loaded with Fura 2/AM (5 μM) and kept at 23°C in the dark for 20 min. After washing to remove the extracellular dye, the cells were resuspended in HBSS containing 10 mM Hepes, pH 7.4, at a concentration of 3.2 x 10^6 cells/ml. For each sample, fluorescence was simultaneously recorded at 340 and 380 nm and the $[Ca^{2+}]$i was estimated as described previously (5). PAF (10 nM) was added after resting $[Ca^{2+}]$i was measured.

Measurement of superoxide anion production: Two different experiments were done for this assay: one with resident peritoneal cells obtained from control and Mg-deficient rats, the other with peritoneal cells from standard chow fed rats in a medium supplemented with serum from either control or Mg-deficient rats. In the first case, the cell suspensions in HBSS were added to a medium containing 80 μM cytochrome c (type III; Sigma). The final concentration of cells was 2.0 x 10^6/ml. After 5 minutes incubation at 37°C, the cells were stimulated with 100 ng/ml of phorbol myristate acetate (PMA) and absorbency changes were recorded continuously during 5 minutes, at 550 nm, against a blank consisted of incubation medium without cells. The specificity of cytochrome c reduction was assessed by the presence of 75 μg (350 UI) of superoxide dismutase. The amount of superoxide anion was calculated using an extinction coefficient of 21 mM^{-1}. cm^{-1}. The initial rate was determined during the first 2 minutes after initiation of the reaction. In the second case, we tested the effect of serum from control and magnesium deficient rats on cells collected from chow fed rats. The cells re-suspended in HBSS with Ca^{2+} and without Mg^{2+} were added in microtiter plates to the same medium containing 80 μM cytochrome c, 100 ng/ml PMA and 10 % serum from either control or Mg-deficient rats. The final volume of the reaction mixture was 200 μl. Microtiter plates were incubated at 37°C and absorbency was recorded during 120 minutes at 550 nm. Results are expressed as differences between absorbency of samples and absorbency of incubation medium without cells.

Statistics: Students' t-test was used, with statistical significance representing $P<0.05$.

Results and discussion

The weaning rats used in that study showed a number of clinical modifications after eight days on Mg-deficient diet. The most characteristic feature is the development of redness of the ears, the limbs, and the tail. Mg-deficient rats also had a slight but significant decrease of body weight (91 *vs* 95 g, $P<0.05$), a sharp decrease in plasma magnesium (0.22 *vs* 0.82 mmol/L, $P<0.01$) and an increase of spleen relative weight (7.1 *vs* 5.7 mg/g body weight, $P<0.05$). These observations together with an increased level in plasma nitric oxide recently evidenced in this model (6) clearly show that inflammatory syndrome occurs in Mg-deficient animals. Most of these clinical signs can be reversed by feeding rats with diet containing adequate level of magnesium (7).

Rats were challenged with $1.9x10^8$ or $1.9x10^7$ of live E. coli and mortality was determined. With $1.9x10^8$ bacteria all 6 Mg-deficient animals dead within 4 hours whereas 5 of 6 control animals dead after 18 hours. With $1.9x10^7$ bacteria, 5 among 6 Mg-deficient rats dead 6 hours after the challenge whereas all control animals were still alive after 48 hours (Figure 1).

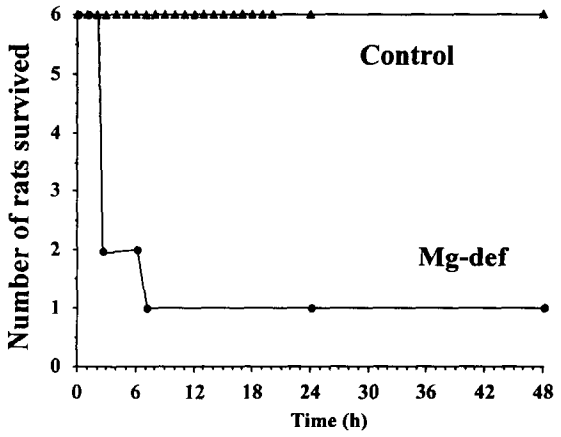

Fig. 1

Survival of control and Mg-deficient rats after E. Coli challenge. Live bacteria-induced mortality in 6 rats of each group was followed up to 48 hours. Controls (triangle) and Mg-deficient (circle) rats were challenged 1.9×10^7 bacteria per animal.

These data, obtained with live E. coli, confirm the greater sensitivity to septic shock of Mg-deficient rats previously evidenced by bacterial wall components i.e. lipopolysaccharide from E. coli (8,9,10). Similarly, we studied the mortality using platelet-activating factor (PAF) as a model for anaphylaxis (11) (Figure 2). A lethal dose of 100 µg PAF per kg b.wt. (i.p.) killed the animals after 20 minutes and 40 minutes after the challenge in Mg-deficient and control animals, respectively. A lower dose of PAF (25 µg PAF per kg b.wt.) led to 30 % mortality in control animals after 150 minutes following PAF injection whereas during the same time, 70 % mortality was observed in Mg-deficient animals. These data show the greater sensitivity to anaphylaxis of Mg-deficient rats and are in agreement with those previously obtained in ovalbumin-sensitised

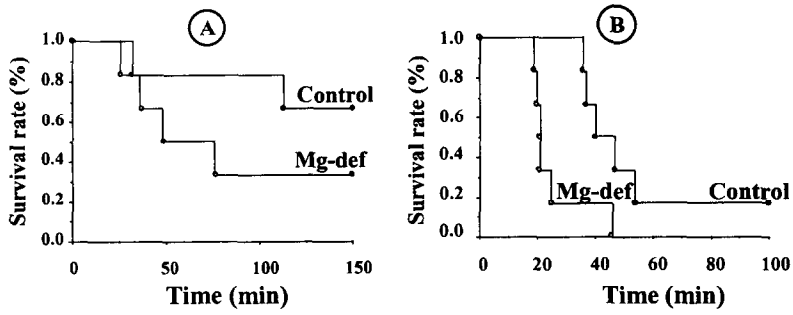

Fig. 2

Survival rate after PAF challenge of control and Mg-deficient rats. Twelve rats per group were injected with 25 μg/kg i.p. (A) and 100 μg/kg i.p. PAF (B).

Mg-deficient guinea-pigs (12). Together, these results also extend the concept that Mg deficiency greatly enhanced the acute response to immune stress whatever the stimuli used for that activation.

The reaction of the immune system depends on the reaction of immune cells including macrophages, polymorphonuclear leukocytes or mast cells. To investigate the effects of Mg deficiency on cell function, we studied superoxide anion production resulting from respiratory burst induced by phorbol myristate acetate (PMA).

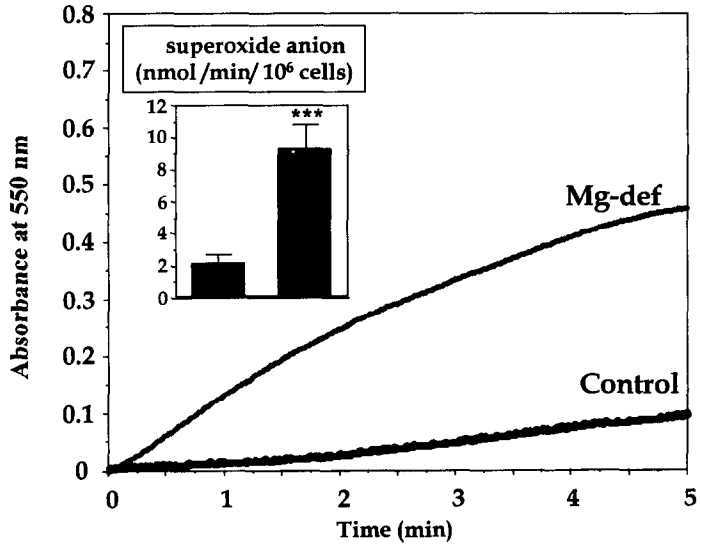

Fig. 3

Superoxide anion production in peritoneal cells from control and Mg-deficient rats. Shown is a representative experiment on absorbance changes of cells from control or Mg-deficient rats activated by 100 ng/ml of PMA (See Materials and Methods). The initial rate of superoxide anion production was illustrated in the inset; results are expressed as means ± SE for 9 rats per group. *** : $p<0.001$.

The data (Figure 3) showed that addition of PMA led to 3 to 4 fold increase of superoxide anion production in cells from Mg-deficient animals as compared with controls. Enhanced production of superoxide anion by stimulated cells was coupled to 5 fold increase of the rate of production (Figure 3, inset). These data clearly show that the cells from Mg-deficient animals possess the potential to be more activated by immune stimulators and likely at the origin of exacerbated response leading to mortality.

This enhanced activation could result from pre-activated or primed cells which are known to release high amount of oxygen metabolites during stimulation (13). The following experimental approach has been made to assess this hypothesis. Peritoneal cells from standard chow-fed rats were incubated with 10 % serum obtained from Mg-deficient or control rats. The data showed that the presence of serum of Mg-deficient rats lead to significantly higher level of superoxide anions production after PMA stimulation (Figure 4).

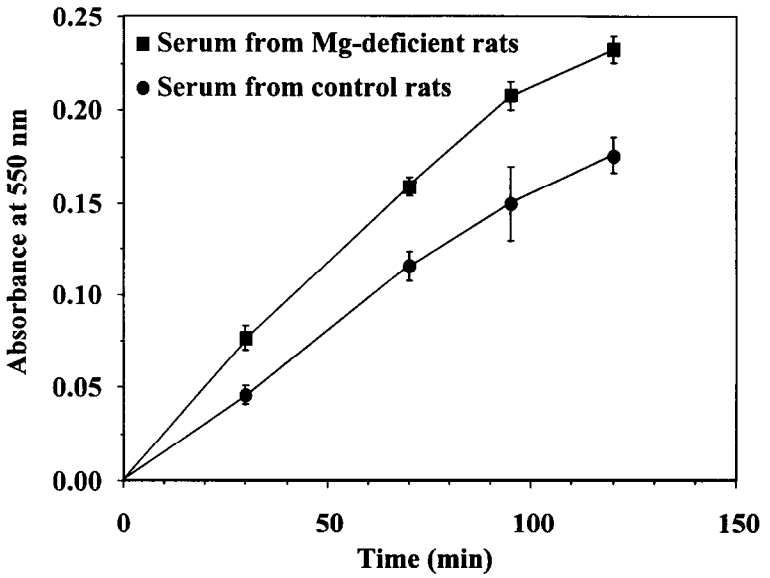

Fig. 4

Effect of control and Mg-deficient rats serum on superoxide anion production by peritoneal cells of chow-fed rats. Cells were stimulated with 100 ng/ml of PMA (See Materials and Methods) in the presence of 10 % pooled serum obtained from four control or Mg-deficient rats. Results are expressed as means ± SE for 4 experiments.

This data confirmed that priming factors, such as cytokines or substance P, are present in serum of magnesium deficient rats (14). However, it was noticed that this increase is about 25 % of control activity which is much lower than 300 to 400 % difference observed between the cells from control and Mg-deficient animals (Figure 3). Also, these experiments on cell activity strongly suggest that intrinsic cell modifications occurring during Mg depletion in vivo, rather than serum factors known to be released during inflammation, predominantly affected their

functions. To further assess the modifications induced by hypomagnesemia in vivo, we investigated intracellular calcium changes, as a second messenger in transmembrane signal transduction enhancing NADPH oxidase activity (15). Using peritoneal cells from control and Mg-deficient animals, basal level and changes of $[Ca^{2+}]i$ induced by PAF were thus investigated after fura2-AM loading (Figure 5).

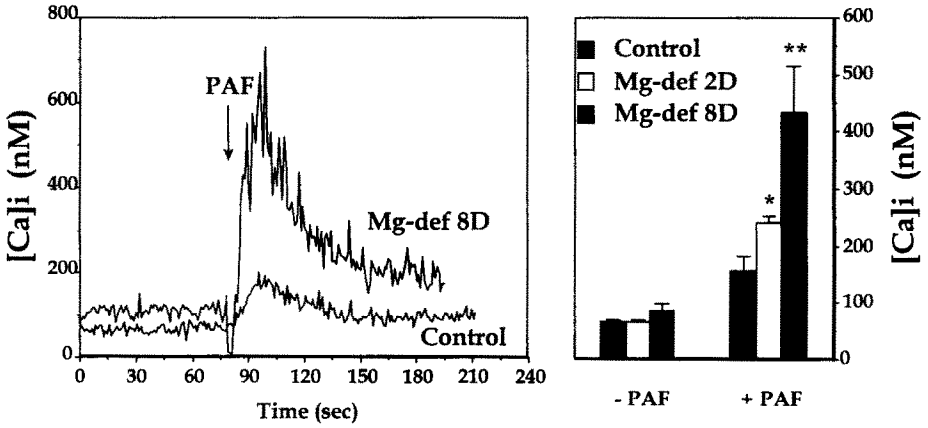

Fig. 5

PAF-induced calcium changes in peritoneal cells from control and Mg-deficient rats. (*Left*) representative traces of PAF-induced intracellular calcium transients in peritoneal cells from rats fed control or Mg-deficient diet for 8 days. (*Right*) Basal and PAF-stimulated calcium changes in peritoneal cells from 10 controls and 5 rats fed for 2 days (2D) or 8 days (8D) Mg-deficient diet respectively. *: $p<0.05$; **: $p<0.01$.

Addition of PAF induced the expected rise of $[Ca^{2+}]i$; however, as compared to controls, 2.7 fold increase was observed at the peak level in cells from Mg-deficient animals (435 nM *vs* 158 nM for controls). Studies performed with macrophages indicated that intracellular as well as extracellular calcium is involved in that elevation. However, calcium mobilisation in murine peritoneal macrophages by PAF has been shown to occur in the absence of extracellular calcium (16,17) and the data from Randriamampita and Trautmann (18) firmly established that the peak of calcium rise, in PAF-induced macrophage activation, derived mainly from intracellular calcium pool. Another important observation is that basal level of calcium is not significantly enhanced in peritoneal cells of Mg-deficient rats. This observation did not account for priming processes which usually lead to rise in the resting $[Ca^{2+}]i$ level (13). To verify this point, we hypothesised that in an earlier phase of magnesium deficiency, circulating factors involved in cell priming should be lower. Experiments were thus undertaken with peritoneal cells obtained from rats fed Mg-deficient diet for 2 days where i) plasma magnesium dropped only to 60 percent of control values and ii) no clinical signs still appeared. As shown in Figure 5, these cells also showed an abnormal rise in $[Ca^{2+}]i$ induced by PAF (242 nM *vs* 158 nM for controls) indicating that transduction signal mechanism was affected at very early stage of Mg deficiency. However, the basal level of $[Ca^{2+}]i$ is 64 nM for both control and Mg-deficient cells suggesting that priming process could be secondary to other mechanisms involved in the activation process.

Together these data may suggest that alterations of signal cascade might occur in the course of magnesium deficiency, including those of PAF receptors, phosphorylation processes and breakdown of phosphoinositides. However, the reverse effect of acute magnesium replacement on endotoxin-induced mortality of magnesium deficient animals (8) lead to another hypothesis taking into account the calcium-magnesium antagonism. Such an hypothesis could be made from the effects of extracellular magnesium already described in various cell types and isolated tissues. In contrast with studies showing that rise of extracellular magnesium usually blocked cell function by decreasing intracellular calcium (19), withdrawal of extracellular Mg^{2+} increased either intracellular calcium (20,21,22) or calcium dependent stimuli (23,24,25), including enhanced NADPH oxidase activity (26,27). The in vivo effects of magnesium deprivation on the response to immune stress, as stated in the present study, are in agreement with these latter in vitro experiments. However, no sustained intracellular calcium level was observed in cells from rats deprived of dietary magnesium either for 2 or 8 days, despite of higher calcium peak levels observed following immune stimuli. Together these data showed that abnormal cell calcium homeostasis occurred during magnesium deficiency may account for the exacerbated immune stress response at the body level. Assuming that calcium is mobilised from intracellular pools, other studies should be performed to verify whether the higher PAF-induced calcium release occurring in the peritoneal cells of Mg-deprived animals may result from modulation of calcium release system or from higher pre-loaded calcium level induced by extracellular magnesium decrease.

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