MAGNESIUM RESEARCH

EFFECT OF MAGNESIUM ON GRANULOCYTE FUNCTION AND ON THE EXERCISE INDUCED INFLAMMATORY RESPONSE

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INTRODUCTION

There are some similarities between the inflammatory response caused by magnesium deficiency and exercise induced alterations in immune function. On the one hand, a number of investigations have documented that magnesium deficiency is associated with an activated state of immune cells. In magnesium deficient rodents the formation of reactive oxygen species and lipid peroxidation were enhanced [1, 2]. Levels of cytokines such as interleukin-6 which orchestrate the inflammatory response are enhanced in magnesium deficient rats [3, 4]. Moreover, examples of exacerbated responses to life bacteria and platelet activating factor were demonstrated in magnesium deficient rats [5].

Alternatively, exercise is associated with inflammatory like responses of the immune system depending on the type and intensity of exercise [6, 7]. Substantial changes in the cell counts of leucocyte subtypes and their function can be found. After an exhaustive exercise test granulocytes increased during and after the exercise test, while lymphocytes showed a biphasic behaviour with increasing counts during exercise and decreasing counts after termination of exercise [8, 9]. Cell functions like oxidative burst or cell proliferation are usually decreased suggesting a slight immune suppression after exhaustive exercise [10].

There is evidence that athletes are prone to alterations in mineral status, especially magnesium deficiency, since exercise can induce substantial mineral losses via urine and sweat [11-13]. Shifts of magnesium between extra- and intracellular compartment during exercise have been reported [14, 15]. Several studies indicate that during and after strenuous exercise plasma magnesium is decreased and that during a season of training hypomagnesemia can occur [16-19]. Therefore it has been supposed that exercise associated alterations in magnesium homeostasis may contribute to changes of immune status after exercise [20-23].

The aim of the present study was therefore (1) to determine the *in vitro* effect of various magnesium concentrations on calcium signalling, oxidative burst and phagocytosis of granulocytes (2) to study the effects of a two month magnesium supplementation on human granulocyte signalling and function and (3) to investigate whether the two months magnesium supplementation period was able to modulate the exercise associated alterations in immune cell counts and functions in young athletes.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN AND SUBJECTS

The study was split into two parts, an *in vitro* part and a clinical investigation.
First, in the *in vitro* study granulocytes were isolated from healthy donors and treated as described below.
Second, in the clinical study twenty male healthy volunteers between 21 and 30 years of age were recruited from the University of Muenster sports student population. They did not take any kind of medication. After a medical check-up subjects were tested for maximal oxygen uptake (V_{O2max}) during a continuous, progressive exercise test on a treadmill ergometer (Ergo XELG90 Spezial, Woodway, Weil am Rhein, Germany). The initial velocity was 8 km/h increasing every 3 min by 2 km/h. Respiration parameters were analyzed using Oxygen record (E. Jaeger, Wrzburg, Germany).
For the exhaustives exercise test – 9-12 days after the ergometer test – participants performed an exercise test at an intensity corresponding to about 80% of the V_{02max} until exhaustion early in the morning. Subjects were not

allowed to exercise 2 days prior to the test. After canulation of the cubital vein blood samples were taken before exercise and 1 hour after exercise.
Next, participants were randomised for either the magnesium supplementation group or the placebo group. Participants of the magnesium supplementation group received 5 mmol magnesium three times a day while the placebo group received glucose (Magnesium Verla, Verla-Pharm, Tutzing, Germany). After magnesium/placebo supplementation for two months a second exercice test at the same intensity was performed until exhaustion. Again, test blood samples were taken before and one hour after the exercise.

MAGNESIUM STATUS

Magnesium status was determined by measurement of extracellular and intracellular total and free magnesium values. Free extracellular magnesium levels ($[Mg^{2+}]_e$) were determined by an ion-sensitive microelectrode (AVL) immediately after the blood sample was taken. Total extracellular magnesium concentration ($[Mg]_e$) was determined in serum photometrically (Roche-Böhringer, Mannheim, Germany). The intracellular free magnesium concentration ($[Mg^{2+}]_i$) was determined in erythrocytes using the magnesium-sensitive fluorenscent dye Magfura-2 (Excitation wavelength 340 and 380 nm, emission wavelength 509 nm). Magfura-2 was loaded at a concentration of 5 mmol/l for 40 min at 37° C. Signals were calibrated as previously described [34]. Briefly, Rmax was obtained after equilibration of cells in high magnesium buffer containing 17 mM digitonin followed by addition of 33 mmol EGTA for determination Rmin (for calibration formula see below). Measurements in the UV range are difficult to perform with erythrocytes because of their high hemoglobin content leading to an increased autofluorescence. However, control measurements using lymphocytes demonstrated a similar response of the fluorescent dye to varying magnesium concentrations in both cell types, erythrocytes and lymphocytes.
Finally, total intracellular magnesium concentration ($[Mg]_i$) was determined in erythrocytes by atomic absorbance spectroscopy.

LEUKOCYTE COUNTS

Blood cell counts, hemoglobin and hematocrit determinations were performed on plasma anticoagulated with ethylenediaminetetraacetate (EDTA) using an semi-automated hematology analyzer (F-820, Sysmex, Norderstedt, Germany).

IMMUNPHENOTYPING OF LYMPHOCYTE SUBPOPULATIONS BY FLOW CYTOMETRY

Lymphocyte phenotyping (two-color mode) was performed on erythrocyte-lysed whole blood using mouse anti-human monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) from Coulter (Miami, Florida, USA). Briefly, 100 µl whole blood anticoagulated with EDTA were incubated with the different antibody combinations according to the CDC (Center for Disease Control) nomenclature (CD45-FITC + CD14-PE; CD3-FITC + CD19-PE; CD3-FITC + CD4-PE; CD3-FITC + CD8-PE; CD3-FITC + CD16/56-PE) for 10 min at room temperature followed by an automatical lysing procedure using Q-Prep (Coulter, Miami, Florida, USA). An isotypic control was performed with MsIgG-FITC and MsIgG-PE mouse monoclonal antibody. Samples were analyzed by a Coulter EPICS XL flow cytometer. Gating of lymphocytes was achieved by forward scatter and sideward scatter parameters and simultaneous staining of leukocytes (CD45+) and moncytes (CD14+) to assure a purity of lymphocyte population > 95%. Reliability of measurements was checked by comparing total percentage of CD3+ CD4+ and CD3+ CD8+ cells with the percentage of CD3+ cells, which should not deviate more than 10%. The absolute numbers of each lymphocyte subpopulation was calculated from its percentage multiplying by the total number of lymphocytes obtained from the automated count.

CELL ISOLATION PROCEDURE

For spectrofluorometric measurements granulocytes were prepared by density gradient centrifugation and hypotonic lysis of erythrocytes from EDTA-blood obtained by venipuncture of the cubital vein. Briefly, 5 ml of a 50:50 mixture of whole blood anticoagulated with EDTA and 0.9% NaCl solution was carefully layered upon 3ml of Lymphoprep (Nycomed, Oslo, Norway) and then centrifuged at 400 g for 35 min at room temperature. After centrifugation the lymphocyte band between the sample layer and the Lymphoprep solution was removed [24]. After washing the remaining cell suspension containing granulocytes and erythrocytes, the latter were removed by two 30 second incubation periods in cold hypotonic buffer. After centrifugation for 10 min at 2000U/min cells were resuspended in buffer of the following composition: NaCl 140 mM, KCl 3 mM, H-Hepes 10 mM, Na_2HPO_4 0.4 mM, $MgCl_2$ 1 mM, $CaCl_2$ 0.8 mM, Glucose 5.5 mM adjusted to pH 7.4. Cell viability was about 98% as demonstrated by trypan blue exclusion, while purity was about 95% as checked by flow cytometry in the forward and sideward scatter mode.
For flow cytometric measurements EDTA-blood was incubated with lysis buffer containing NH_4Cl 155 mM,

$NaHCO_3$ 20 mM, and 0.5 M EDTA adjusted to pH 8.0 for 5 min at room temperature. Finally cells were centrifugated two times at 2 200 U/min for 5 min and resuspended in phosphate buffered saline.

DETERMINATION OF OXIDATIVE BURST AND PHAGOCYTOSIS

The dihydrorhodamine (DHR) oxidation assay was performed as described previously [22, 24]. Briefly, isolated granulocytes (2 × 106/ml) were loaded with 50 µM DHR for 5 min at 37 °C together with catalase (1000 U/ml). After flow cytometric determination of basal fluorescence level cells were stimulated with chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate (PMA) or buffer (control) for 15 min at 37 °C and than again analyzed for oxidation induced fluorescence changes.
For determination of phagocytosis cells were incubated with fluorescein-(FITC)-conjugated beads for 1 hour and than analyzed by flow cytometry. Prior incubation of cells in sodium acid served as a negative control.

DETERMINATION OF CYTOPLASMIC CALCIUM CONCENTRATION ($[CA^{2+}]_I$)

For determination of $[Ca^{2+}]_i$ cells were loaded with the membrane permeant derivative (Fura-2-AM, acetoxy-methlyester) of the calcium sensitive fluorescent dye Fura-2 (5 µM for 25 min at room temperature). Excess dye was removed by two step centrifugation (400 g for 10 min) and resuspension of cells with buffer B. Cells were stored on ice until usage.
Measurements were performed on a cuvette spectrometer (Deltascan, PTI, New Jersey, USA) at excitation wavelengths of 340 and 380 nm, while emitted light was monitored at 509 nm. During recording cells were continuously stirred at 28° C. While autofluorescence of cells was negligible, autofluorescence of cuvette, solution etc. was determined before the experiment and substracted automatically. For every single experiment about 10^6 cells/ml.
$[Ca^{2+}]_i$ was calculated according the following equation [25]:
$[Ca^{2+}]_i = (R-R_{min})/(R_{max}-R) * K_d * F$
with a K_d of Fura-2 for calcium of 220 nmol/l and where R is the ratio of fluorescence of the sample at 340 and 380 nm. R_{max} and R_{min} are the ratios for Fura-2 at these wavelengths in the presence of saturating Ca^{2+} (after application of 10 µM digitonin) and under Ca^{2+} free conditions (after addition of EGTA, 10 mM final concentration), respectively; F is the ratio of fluorescence intensity at 380 nm under Ca^{2+} free conditions to the fluorescence intensity at 380 nm under Ca^{2+} saturating conditions.

STATISTICAL ANALYSIS

The *in vitro* data shown in the figures are representative of 4 or more individual experiments in each group. Wherever applicable, data points represent means ± standard error of the mean (SE). Differences between groups were compared using the Student's t-test and p values < 0.05 were considered significant.

MATERIALS

Magfura-2-AM, Fura-2-AM, and DHR were purchased from Molecular Probes, Eugene, Oregon, USA. All other chemicals were of the highest chemical grade available and were obtained from Sigma Chemical, St. Louis, USA.

RESULTS

IN VITRO STUDY – MAGNESIUM AND GRANULOCYTE SIGNALLING AND FUNCTION

First we investigated the effects of different magnesium concentrations on granulocyte calcium signalling and function. Basal intracellular calcium concentrations did not change significantly after incubating neutrophile granulocytes for one hour in calcium containing media of different magnesium concentrations (figure 1A left). However, omitting calcium in the incubation media resulted in an enhanced basal calcium concentration under magnesium depleted conditions (figure 1A right) Stimulation of cells with the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) resulted in a mean increase of cytosolic calcium of about 148 ± 11 nmol/l. After an incubation of cells for 1 hour in magnesium enriched buffer (5 mmol/l) the calcium transients were significantly decreased, while magnesium depleted buffer (0.2 mmol/l) had no effect (figure 1B left). Performing these experiments under calcium free conditions resulted in a slightly decreased calcium transient in magnesium depleted buffer while there was no difference between control and magnesium enriched conditions (figure 1B right).

The basal production of free radicals was not affected by different magnesium concentrations. However, stimulating cells with fMLP was enhanced if stimulation was performed under magnesium depleted conditions (0.2 mmol). Similar results were obtained when neutrophils were stimulated with phorbol-myristate-acetate (PMA). In contrast, incubation in high magnesium concentration had no significant effect on the stimulus induced oxidative burst (figure 1C). Under calcium free conditions the effect of different magnesium concentrations was reversed. Low magnesium decreased the fMLP induced production of free radicals (figure 1D). Spontaneous and stimulated (fMLP, PMA) phagocytosis of granulocytes was not affected by the extracellular magnesium concentration (data not shown).

CLINICAL STUDY

Next we investigated whether a two month period of magnesium supplementation had an effect on immune cell counts and function and on exercise induced alterations of the immune system.

MAGNESIUM STATUS

Magnesium status was similar in both verum and placebo group at the beginning of the study. The two month period of magnesium/placebo supplementation had no effect on the blood magnesium concentrations, both free and total magnesium, and on the free magnesium concentration in erythrocytes. In both groups the total intracellular magnesium content of erythrocytes decreased slightly. At the end of the study, magnesium status of both groups was statistically not different from each other (table I).

Table I. Total extracellular ($[Mg]_e$), free ionized ($[Mg^{2+}]_e$), total intracellular ($[Mg]_i$) and free ionized ($[Mg^{2+}]_i$) magnesium concentration (mmol/l) in the magnesium supplemented and the placebo group before (day 1) and after (day 60) supplementation.

	Magnesium		**Placebo**	
	Day 1	**Day 60**	**Day 1**	**Day 60**
$[Mg]_e$	0.87 ± 0.02	0.85 ± 0.02	0.88 ± 0.01	0.89 ± 0.02
$[Mg^{2+}]_e$	0.56 ± 0.01	0.57 ± 0.01	0.57 ± 0.01	0.59 ± 0.01
$[Mg]_i$	1.84 ± 0.05	1.60 ± 0.06*	2.09 ± 0.04	1.67 ± 0.06*
$[Mg^{2+}]_i$	0.84 ± 0.05	0.82 ± 0.05	0.76 ± 0.06	0.77 ± 0.13

* indicates $p < 0,05$ compared to day 1 values.

EXERCISE, MAGNESIUM AND IMMUNE CELL COUNTS

Magnesium supplementation had no effect on the distribution of leucocyte and lymphocyte subsets under resting conditions. After the exhaustive exercise test a characteristic shift in the immune cell counts was observed. One hour after the test leucocyte and granulocyte counts increased by 19% and 35%, respectively. In contrast, lymphocyte count decreased by about 17% (table II). Of the lymphocyte subsets the decrease was most prominent for the natural killer cells and the cytotoxic T cells. Neither the granulocyte increase nor the decrease of lymphocytes and lymphocyte subsets after the exhaustive exercise test was affected by the two month magnesium supplementation.

Table II. Exercise induced alterations in leucocyte and lymphocyte subsets (in% of pre-test values) in the magnesium supplementation and the placebo group before (1. exercise test, day 1) and after (2. exercise test, day 60) supplementation.

	Magnesium		**Placebo**	
	1. Exercise test – day 1	**2. Exercise test – day 60**	**1. Exercise test – day 1**	**2. Exercise test – day 60**
Leukocytes	+ 19%	+ 22%	+ 22%	+ 27%
Granulocytes/Monocytes	+ 35%	+ 66%	+ 35%	+ 60%
Lymphocytes	– 17%	– 32%	– 28%	– 34%
B-cells	± 0%	– 18%	– 7%	– 13%
T-cells	– 15%	– 29%	– 25%	– 28%
CD4+ -cells	– 20%	– 26%	– 24%	– 27%
CD8+ -cells	– 11%	– 31%	– 28%	– 27%

NK cells	– 47%	– 55%	– 62%	– 67%
Cytotoxic T cells	– 39%	– 39%	– 45%	– 39%

Statistical analysis revealed no significant difference between 1. and 2. exercise test in either group.

EXERCISE, MAGNESIUM AND GRANULOCYTE FUNCTION

Basal intracellular calcium levels of granulocytes were 105 ± 14 nmol/l and 103 ± 11 nmol/l for the magnesium and the placebo group, respectively, and they were not affected by the supplementation. Likewise the calcium transients of granulocytes before and after the supplementation period were not significantly different.
After the exhaustive exercise tests fMLP induced calcium transients were enhanced. Calcium transients after exercise were about two times higher than the pre-test signals. The enhanced post-exercise calcium transients, however, were not significantly affected by the two month period of magnesium supplementation (figure 2).
Oxidative burst and phagocytosis under resting conditions were not different before and after the supplementation period. After the exhaustive exercise both oxidative burst and phagocytosis of neutrophils were reduced. The exercise-induced decrease of both parameters was not different in the two groups after the supplementation period (table III, data for phagocytosis not shown).

Table III. Exercise induced decrease in granulocyte oxidative burst capacity in the magnesium supplemented group and the placebo group before (1. exercise test, day 1) and after (2. exercise test, day 60) supplementation.

	Magnesium	**Placebo**
1. test, day 1	– 34.9 ± 8.6	– 9.2 ± 37.0
2. test, day 60	– 49.5 ± 8.3	– 7.5 ± 10.5

Oxidative burst capacity is expressed in% of the pre-test values. There was no significant difference in either the magnesium or the placebo group between 1. and 2. test.

DISCUSSION

There are numerous reports about the effects of magnesium on intracellular signalling processes and cellular functions. In most cases low magnesium levels result in enhanced cellular functions while high magnesium levels decrease cellular functions [5, 26-31]. In the present study, fMLP-induced oxidative burst was enhanced by a low magnesium concentration while high magnesium concentration was not able to decrease oxidative burst. Similar results were reported recently by Bussiere *et al.* [30, 31] for superoxide anion production by human leucocytes. There is evidence that the oxidative burst triggered by fMLP depends on both the release of intracellular calcium and the entry of extracellular calcium [32, 33]. As indicated by the Fura-measurements, our results could be explained partly by altered intracellular calcium transients. High magnesium concentration was able to decrease cellular calcium transients in calcium containing buffer to a level comparable to calcium free conditions. This suggests that magnesium is acting on the membraneous calcium entry channel. This inhibitory role of magnesium on membranous calcium channels has been described in several cell types [34, 35]. However, low extracellular magnesium concentration did not enhance the cellular calcium entry. The data on calcium free conditions furthermore support the view that magnesium is acting on the intracellular calcium stores, too. The fMLP-induced calcium transients under magnesium low and calcium free conditions were significantly decreased suggesting a decreased loading of cellular calcium stores. The depletion of cellular calcium stores is most likely caused by a reduced activity of the calcium sequestering ATPases since the resting calcium levels were enhanced under there conditions. For this type of ion pump a magnesium dependency has been shown [36]. But magnesium seemed to act on other signalling pathways, too. This is suggested by the enhanced effects of low magnesium concentration on the PMA-induced oxidative burst. PMA is known to activate proteine kinase-C while it is not releasing intracellular calcium [37].
The next point the study addressed was the effect of an oral magnesium supplementation on cellular immune status and granulocyte function. There were no differences in the magnesium status before and after the supplementation period in both the magnesium and the placebo group. Several reasons could be responsible for this finding. One reason could be the length of the supplementation period. Weller *et al.* [38] found no effect on magnesium concentration in serum or any cellular compartment after a three week magnesium supplementation period similar to our study. However, they found an increased magnesium clearance in the magnesium supplementation group which might indicate an equilibrated or well-balanced magnesium status. Likewise, data of Feillet-Coudray *et al.* showed no increase in the mass of the exchangeable magnesium body pool in healthy

women after an 8 week magnesium supplementation while urinary magnesium excretion increased [39]. Unfortunately, in the present study it was not possible to determine urine magnesium secretion due to compliance reasons. Finally, it is known that both serum and erythrocyte magnesium concentration do not reflect body magnesium status very well [40, 41]. It cannot be excluded from the present data that magnesium was enhanced in other cellular compartments during the supplementation period.

We found no evidence that the magnesium supplementation had any effect on the immune function parameters under resting conditions. Pre-exercise immune cell counts and functions were not affected by magnesium supplementation. Moreover, magnesium supplementation failed to affect exercise-induced immune cell activation. Exhaustive exercise resulted in an acute activation of the immune system [6, 7]. This was indicated by the different immune cell counts before/after exercise as well as by the altered cellular calcium signalling [8, 10]. Other investigators reported an increase of acute phase proteins and changes of inflammatory cytokines such as interleukin-6 or TNF-α during and after exercise [42-44]. The changes in the cytokine network are probably responsible for the changes in exercise-induced cell counts [43]. Moreover, apoptosis seems to be involved in this process [45]. For all of these parameters a magnesium-dependency has been described either under *in vitro* conditions or in animal experiments [46, 47]. However, magnesium failed to have any effect on the exercise-induced inflammatory response in the present study. These results were similar to recent investigations about other nutritional supplements like zinc, vitamin C and other antioxidants. None of these nutrients has emerged as an effective countermeasure to exercise-induced immunosuppression [48].

Most probably the alterations in magnesium status in the athletes group before and after supplementation were too small to have a significant impact on the regulation of the immune function. The majority of studies on the effect of magnesium on immune function have been performed in animals, which allowed vigorous changes in magnesium homeostasis by different magnesium diets [1, 3, 4, 49, 50]. Substantial changes in the hormonal and immunological network, for example substance P or inflammatory cytokines, have been reported in magnesium deficient rats [3, 50]. Moreover, changes in the cellular immune function like ingestion of bacterias and production of free radicals have been described [30]. On the transcriptional level, magnesium deficiency has been shown to activate of a number of genes involved in immune cell activation [51].

While changes in Mg status in athletes especially during long and high intensity periods may occur it is still questionable whether these changes are effective and responsible for the modulation of the immune response. Recently, Kimura *et al.* reported a decreased activity of natural killer cells in athletes compared to non-athletes which was reversible upon magnesium application to the *in vitro* assay [52]. However, they did not report any data about the athlete's magnesium status. The participants of the present study were not magnesium deficient. Therefore, they did not benefit from a magnesium supplementation. This is similar to studies about magnesium effects on exercise performance since magnesium supplementation failed to enhance performance in athletes with normal magnesium homeostasis [23, 38, 53]. Furthemore it has to be considered that magnesium is only a single nutrient factor in the most likely multifactorial genesis of exercise-induced immune modulation.

CONCLUSIONS

The results of the present study suggest that while magnesium has significant impact on immune cell function under *in vitro* conditions, a magnesium supplementation is unable to affect resting immune status and function as well as to prevent exercise-associated alterations of immune cell functions in athletes with balanced magnesium homeostasis. These findings are compatible with prior studies on magnesium supplementation and exercise performance.

Athletes often acquire infections after intensive training periods and competitions of high intensity, which are known to affect the magnesium balance as well. Thus, it cannot be excluded that magnesium may be one cofactor in the multifactorial network of exercise associated alterations in immune function. Further studies, therefore, should be performed with a focus on athletes with a magnesium deficiency to elucidate a possible role of magnesium in the exercise induced inflammatory response.

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