Effects of long-term dietary intake of magnesium on oxidative stress, apoptosis and ageing in rat liver

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Abstract. In the present study, we investigated the effect of long-term dietary Mg intake on the rate of oxidative stress, apoptosis and ageing in rat livers. To address this issue, rats were fed diets containing either a moderately deficient (0.15 g Mg/kg diet), a standard (0.8 g Mg/kg diet) or a high (3.2 g Mg/kg diet) Mg dose for two years. It is noteworthy that a higher percentage of animal mortality was observed in the lowest Mg diet, as compared to the other groups. Oxidative stress and antioxidant status were evaluated by measuring different enzyme activities, among which glutathione peroxidase activity was significantly reduced when Mg content was decreased in the diet. Moreover, we obtained an activation of caspase-3 and a higher lipid peroxidation in the Mg-deficient group, as compared to the Mg standard group, while no changes in Mg-supplemented group were observed, in accordance with our previously published data in primary cultures of rat hepatocytes (Martin et al., J Nutr 2003). Telomere shortening was measured in rat livers, as a marker of ageing. We found that telomere length was decreased in old animals, as compared to young animals confirming that telomere shortening correlated well with ageing events. Moreover, in old animals, we obtained a decrease of telomere length in the Mg-deficient group, as compared to the other groups. Taken together, our results show that a long-term chronic Mg deficiency led to oxidative stress, apoptosis and an acceleration of ageing in rat livers.

Key words: magnesium, long-term, chronic, rat liver, oxidative stress, apoptosis, telomere, ageing

In most industrialized countries, hypomagnesaemia is frequent among the general population [1, 2], since dietary behaviors have changed with industrialized food. Indeed, food such as whole grains and vegetables which are naturally magnesium (Mg)-rich, is largely processed before being consumed, leading to an important loss of Mg content. Consequently, a chronic dietary inadequacy of Mg is commonplace. One other reason for the prevalence of hypomagnesaemia is due to disturbances in the intestinal Mg absorption and/or to increased renal Mg excretion. Hypomagnesaemia is well recognized as an important human health problem, since Mg is involved in many enzymatic reactions and hypomagnesaemia...
plays an important role in the pathogenesis of numerous diseases, including ischemic heart disease, sudden cardiac death, hypertension, stroke, atherosclerosis and cancer (for review, see [3]).

Several in vivo and in vitro studies dealing with the effects of Mg intake, have concluded that oxidative stress was involved in the response to Mg deficiency in different tissues and organs [4-11]. This was evidenced either by an impairment of the defenses against oxidative stress or by an accumulation of oxidation products which was frequently associated with an increase in lipid peroxidation. In our previous studies, we showed that extracellular Mg deficiency has a negative effect on the survival of cultured rat and human hepatocytes by inducing apoptosis involving oxidative stress; however, supplementation of extracellular Mg did not reduce spontaneous apoptosis occurring over time in hepatocyte cultures [12, 13]. Other studies have evidenced that apoptosis was induced in different rat tissues, following Mg deficiency [8, 14, 15]. However, most of the publications aiming to study Mg effects consisted of short-term or acute exposure, which is not representative of the in vivo chronic human situation. Moreover, such experiments do not allow evaluation of the possible impact of Mg intake on the ageing and mortality rate. Nevertheless, Mg deficiency could have an important role in the acceleration of cellular senescence. Indeed, epidemiological studies have suggested that Mg intake may be beneficial in the prevention of ageing, since Mg deficiency is a significant risk factor of ageing [16, 17]. Recently, Ferrè et al. have shown that Mg deficiency induced senescent features in cultured human endothelial cells, the cdk inhibitor p21 being upregulated [18]. Moreover, Killilea and Ames have evidenced that Mg deficiency accelerated cellular senescence in cultured human fibroblasts, by the increased p16(NK4a) and p21(WAF1) protein expression and an increased telomere attrition [19].

In the present study, we evaluated the effect of long-term dietary Mg intake on the rate of oxidative stress, apoptosis and ageing in rat livers. To address this issue, rats were fed diets containing either a moderately deficient (0.15 g Mg/kg diet), a standard (0.8 g Mg/kg diet) or a high (3.2 g Mg/kg diet) Mg dose for two years, which corresponds to the average life time of this species.

Materials and methods

In-life Experiment

All animals were treated in compliance with applicable guidelines formulated by the European Union for the care and use of laboratory animals (L 358-86/609/ECC). Male Sprague-Dawley rats four weeks old were obtained from Charles River (St Germain sur l’Arbresle, France). They were randomly divided into three groups and housed in standard animal laboratory cages (4 rats per cage, 5 cages per group) with free access to distilled water and food. They were kept under constant temperature (23°C), constant humidity (50-60%), and a daily 12h light-dark cycle. The rats were pair-fed a 0.15 g, 0.8 g or 3.2 g Mg/kg diet for 90 weeks. The synthetic diets contained the following (%): casein 20, starch 40, sucrose 21, cellulose 6, groundnut oil 2.5, corn oil 2.5, mineral mixture 7, and vitamin mixture 1. Mg was given in the form of magnesium monooxide and Mg concentrations of the diets were verified by atomic absorption spectrophotometry (Perkin Elmer 3300, Saint Quentin en Yvelines, France). At the end of the treatment period, the terminal body weights were recorded. The animals were anesthetized with sodium pentobarbital (40 mg/kg b.w.) and exsanguinated by abdominal artery puncture. Blood samples were collected in heparinized tubes for subsequent plasma Mg measurement by atomic absorption spectrophotometry (Perkin Elmer 3300). The livers were rapidly removed and weighed. Small parts of liver of approximately 1g were washed in cold phosphate-buffer and immediately flash-frozen in liquid nitrogen and kept frozen until used for analysis.

Oxidative stress measurements

Liver homogenates were prepared as previously described [20, 21]. All the procedures to quantify the different enzyme activities and the reduced glutathione (GSH) content have been previously described by Binda et al. [20] and Nicod et al. [21]. The enzyme activities that we tested in the present study were the following: catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities. CAT activity was expressed as EU/mg protein, SOD, GPx and GR activities were expressed as nmol/min/mg protein. GSH content was expressed as nmol/mg protein. For thiobarbituric acid reactive substances (TBARS) concentration determination, liver homogenates were precipitated with trichloacetic acid and centrifugated at 1600g for 15 min. The supernatants were mixed with TBA reagent (0.067%) and the mixtures were kept at 100°C for 15 min. The fluorescent reaction product was extracted with n-butanol and the fluorescence was measured in the organic phase using a fluorescence spectrophotometer (excitation: 535nm and emission: 555 nm). TBARS concentra-
tions were calculated relative to a standard preparation of 1,1,3,3-tetra-ethoxypropane and expressed as nmol malondialdehyde (MDA) per mg protein.

**Apoptosis measurement**

Approximately 100 mg of frozen rat livers were homogenised in the lysis buffer provided in the kit (Interchim, Montluçon, France). After centrifugation at 15,000 rpm for 15 minutes at 4 °C, the supernatants were collected and used to quantify caspase-3 activity according to the manufacturer’s instructions. Standard curves were obtained using 7-amino-4-methylcoumarin (AMC). Caspase-3 activity was determined using a fluorescence spectrophotometer (excitation at 340 nm and emission at 450 nm) and expressed as pmol AMC/min/mg protein.

**Protein concentration determination**

The protein concentration of liver homogenates was evaluated by the bicinchoninic acid protein assay kit, according to the manufacturer’s instructions (Sigma Chemicals, St Louis, MO, USA) and BSA was used as a standard.

**Telomere length measurement**

Hepatocytes from frozen rat livers were isolated by mechanical disruption using a 35 μm Medicon (DAKO, Glostrup, Denmark) followed by a cell filtration on a 50 μm filter (DAKO). Hepatocytes were then mixed with 1301 cell line (2.10⁶ cells of each type). The 1301 cell line [22] was used as an internal control because it is near-tetraploid and has very long telomeres (> 25 kb), and can therefore be distinguished from the cell types used in the assay [23]. After DNA denaturation, hybridization with the fluorescein (FITC)-conjugated peptide nucleic acid (PNA) probe (DAKO) was performed overnight. Negative control allowing the detection of cell auto-fluorescence was also prepared by omitting the PNA probe in the mixture. After washing, cells were stained with propidium iodide (PI) to identify G0/G1 cells and calculate the DNA index of hepatocytes and 1301 cell line. Determination of telomere length was performed by flow cytometry [23, 24]. FC500 flow cytometer (Beckman Coulter, USA) was used to quantify FITC- and PI-staining cells. The Relative Telomere Length (RTL) expressed in % was calculated according to the manufacturer’s instructions (DAKO).

**Statistical analysis**

Statistical comparisons among experimental animal groups were performed by one-way analysis of variance, using Tukey’s test. The level of statistical significance was set at 0.05.

**Results and discussion**

With the aim of evaluating a chronic human in vivo situation of Mg deficiency and/or supplementation, the present experiments were designed to study the effects of either a Mg deficiency or a Mg supplementation on the liver. Rats were therefore fed different diets over their entire lifespan, i.e. approximately 2 years for this species, with Mg dose levels that led respectively to diets being Mg moderately deficient, standard and Mg supplemented.

As shown in Table 1, the standard Mg diet we used (0.8 g of Mg/kg diet) led to a plasma Mg concentration of 0.7 ± 0.02 mmol/L, that defined the standard “Std” rat group. The Mg moderately deficient diet (0.15 g of Mg/kg diet) we used, which corresponds to “Def” group, resulting in a plasma Mg concentration of 0.52 ± 0.03 mmol/L, has been preferred to a Mg severe deficient diet for the maintenance of body weight gain of animals during the 2 years of treatment. Moreover, this allowed us to achieve low Mg plasma levels clinically relevant to Mg-deficiency in humans. Finally, a Mg supplemented group (3.2 g of Mg/kg diet), which corresponds to “Suppl” group, led to a plasma Mg concentration of 0.86 ± 0.02 mmol/L.

By using these experimental conditions, we noted no

<table>
<thead>
<tr>
<th>Group</th>
<th>Mg diet (g Mg/kg diet)</th>
<th>Plasma Mg concentration (mmol/L)</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>Def</td>
<td>0.15</td>
<td>0.52 ± 0.03</td>
<td>605.0 ± 23.9</td>
<td>15.6 ± 0.8</td>
</tr>
<tr>
<td>Std</td>
<td>0.8</td>
<td>0.70 ± 0.02</td>
<td>648.1 ± 20.4</td>
<td>16.0 ± 0.8</td>
</tr>
<tr>
<td>Suppl</td>
<td>3.2</td>
<td>0.86 ± 0.02</td>
<td>632.9 ± 16.9</td>
<td>16.1 ± 0.7</td>
</tr>
</tbody>
</table>

Means ± SEM (n = 13-17 animals/group). Means without a common letter differ, p < 0.05.

Table 1. Effects of dietary magnesium on plasma Mg concentration, body and liver weights in rat groups after 2 years treatment.
differences in the body weights and liver weights of the rats from the three groups at the end of the treatment period (table 1). However, it is noteworthy that a higher percentage of animal mortality was observed in the lowest Mg diet, as compared to the other groups. During the first 50 weeks of the experiment no death was noted in the three animal groups, while at the end of experiments, the percent of dead animals corresponded to 38%, 25% and 11% of the initial animal number in Def, Std and Suppl groups, respectively (figure 1). The mortality trend was evaluated using a log-rank test and was found significant (p = 0.011).

Oxidative stress was evaluated by the determination of several enzyme activities: CAT, SOD, GPx and GR activities, as well as GSH content. Figure 2 showed that Mg diets significantly impacted on GPx activity, since a statistically significant lower GPx activity was measured in the Def group. This result is in accordance with data obtained after short-term exposures, showing a decrease in GPX activity in rats kept on a Mg-deficient diet during 22 days or 8 weeks [25, 26]. In the present study, no other significant changes between the three animal groups were noted on the other enzyme activities tested (data not shown). Several studies reported the impact of Mg deficiency on SOD and glutathione transferase gene expression in rat thymocytes [8] and on CAT, SOD,
GR and glutathione S-transferase activities in red blood cells [26]. But these studies were performed with acute exposure (i.e. no more than 22 days). Consequently, discrepancies between our results and other published data could be explained by the duration of treatment (short- vs long-term exposure). Moreover, we can hypothesize that GPx activity revealed the most prominent alteration among antioxidant enzymes, as earlier noted in studies dealing with the effect of physical exercise [27, 28] and as already observed in our laboratory. Finally, we can also suggest that a different pattern could be obtained among various tissues, i.e. thymocytes or red blood cells vs liver cells.

Lipid peroxidation, as assessed by TBARS concentration measurement, was induced in rat liver by Mg deficiency, in agreement with our in vitro data [12] and with several publications [5, 6, 9], confirming that a chronic Mg deficiency induced oxidative stress and subsequent production of oxidative compounds in rat livers. However, no differences between the Mg-standard and the Mg-supplemented groups were obtained. This is most probably not related to the only marginal increase in plasma Mg concentration in the latter group (0.86 ± 0.02 mmol/L plasma Mg) compared to animals receiving a standard diet, since the same observation was made when extracellular Mg was increased up to 2 mmol/L (as compared to 0.8 mmol/L for standard conditions) in primary cultures of rat hepatocytes [12]. This suggests not only that rats under a chronic Mg supplementation regimen regulate their blood Mg concentrations, but also that hepatocytes regulate Mg uptake, therefore keeping cell homeostasis constant.

Moreover, we observed a statistically significant activation of caspase-3 in the Mg-deficient group, as compared to the Mg-standard group. To our knowledge, this is the first demonstration that a chronic Mg deficiency induced apoptosis in rat liver. This result, in total agreement with our in vitro data on rat hepatocytes [12], is also in accordance with other publications showing an induction of caspase-3 in neutrophils [14] and in heart [15] of rat depleted in Mg. The involvement of reactive oxygen species and related secondary oxidant species such as lipid hydroperoxides are now well documented in apoptosis [29-31]. Thus, the results of the present in vivo study with livers from rats after a two-year chronic Mg-deficient diet, confirm our previous in vitro observations in rat hepatocytes cultured for 3 days in a Mg-deficient culture medium [12], i.e. that extracellular Mg deficiency has a negative effect on the survival of rat hepatocytes by inducing apoptosis, probably as a result of an oxidative stress-related mechanism.

By using a long-term exposure that corresponds to the average lifespan of rats, we were able to consider if Mg dietary intake and subsequent plasma Mg concentration, can have a role in cellular senescence. For this purpose, telomere shortening was measured as a marker of ageing. Telomeres are unique DNA/protein structures that contain noncoding TTAGGG repeats and telomere-associated proteins [32]. They are present at the end of chromosomes as protective chromosomal caps, since they protect against chromosomal end-to-end fusions, which could lead to telomere dysfunction. In somatic cells, telomere length is very heterogeneous but typically declines with age, posing a barrier to tumor growth but also contributing to loss of cells with age [33].

Telomeres have been reported to shorten during ageing in various tissues [34]. To confirm these data using rat liver cells, we completed our experiment with young animals (four weeks old), which were fed only for one month with the same standard diet rather than "old" rats. Relative Telomere Length (RTL) was evaluated in hepatocytes by cytofluorimetric analysis using a telomere specific PTC-conjugated PNA probe. An internal control of the analysis was added by using the 1301 leukemic cell line characterized by a stable telomere length [22]. RTL was expressed as a percentage of the length of the 1301 cells. The mean RTL was 54.5 ± 1.1% and 57.5 ± 1.1% in "old" and "young" animals, respectively. These data showed that telomere length was decreased in old animals, fed a physiological Mg dose, as compared to young animals, i.e. animals fed a physiological Mg dose for only one month, confirming that telomere shortening well correlated with ageing events. Moreover, in old animals, telomere length was significantly reduced in Mg-deficient group, as compared to the other animal groups. Indeed, the mean RTL was 46 ± 2.2, 54.5 ± 1.8 and 54.5 ± 1.1% in Def, Std and Suppl groups, respectively. Accordingly, other authors reported that Mg deficiency induced cellular senescence, in cultured human endothelial cells and fibroblasts [18, 19]. This result is consistent with the higher percentage of animal mortality that we observed with the Mg-deficient group. Finally, this is in accordance with the up-regulation of dyskeratosis congenita 1, dyskerin (dkc1) expression we found in a recent work when Mg decreased in the diet of animals [35], since it is known that Dkc cells have a premature telomere shortening, leading to accelerated ageing [36].

The present work should be linked to our recently published observations on the impact of dietary Mg
intake on the rat liver transcriptome after a long-term exposure [35]. Among genes that were differentially expressed following Mg deficiency, 32% of them belonged to “the homeostasis family” that corresponded to genes involved in oxidative stress, DNA damage, apoptosis and cellular ageing. The present study therefore confirms on the protein level the changes previously reported at the gene levels. More studies are needed to assess underlying mechanisms involved in the detrimental effect of Mg deficiency. However, recent studies suggest that a systemic inflammation may play a role in the pathophysiology of Mg deficiency. Indeed, Mg deficiency induced a clinical inflammatory syndrome with excessive production of free radicals (for review, see [37, 38]). Moreover, in humans, Mg intakes below the recommended daily allowance are associated with elevated C-reactive protein, a marker of inflammation, suggesting that Mg deficiency may be involved in the development of the low-grade chronic inflammatory syndrome [39, 40]. We demonstrate for the first time that, while Mg supplementation had no beneficial effect on the physiological decline in rat liver, a moderate Mg-deficient diet was able to chronically accentuate cellular ageing induced by oxidative stress and apoptosis processes. These deleterious effects were associated with a higher mortality in rats. It is noteworthy that these observations occurred with a deliberately chosen moderate Mg deprivation, more relevant to clinically low plasma levels.

References


