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Magnesium Deficiency Affects Mammary Epithelial Cell Proliferation: Involvement of Oxidative Stress

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Low Mg availability reversibly inhibited the growth of mammary epithelial HC11 cells by increasing the number of cells in the G0/G1 phase of the cell cycle. Because low Mg has been reported to promote oxidative reactions, we considered that low Mg-dependent growth arrest was mediated by oxidative stress. Surprisingly, both dichlorofluorescein-detectable reactive oxygen species and hydrogen peroxide-induced oxidative DNA damage were found to be lower in cells cultured in low Mg than in cells grown under control or high-Mg conditions. Gene expression profiling of low- and high-Mg cells showed the modulation of several genes, some regulating cell proliferation. In addition, low Mg cells also displayed overexpression of glutathione S-transferase (GST), leading to increased enzymatic activity. Of note, GST has been shown to modulate cell growth; therefore, we suggest that in low-Mg cells, GST upregulation might have a dual role in protecting against oxidative stress and in modulating cell proliferation.

INTRODUCTION

It has been known for decades that magnesium (Mg; referring to its free or bound form) availability is essential for cell growth (1,2). In fact, Mg deprivation inhibits cell proliferation to different extents depending on the cell type (3,4). Increased Mg availability in turn stimulates cell growth (5). In a murine model of experimental tumor, the readdition of Mg to a Mg-deficient diet accelerated the growth of solid tumors, which became larger than those grown in mice under a normal diet (6).

We have shown that Mg deprivation inhibits cell growth by upregulating cell cycle inhibitory factors as p27 in HC11 mammary epithelial cells and p21 in human endothelial cells (7,8). However, the molecular mechanisms upstream of the expression of these cyclin dependent kinase inhibitors have not been identified yet.

Low Mg has been associated with oxidative stress in different pathologic conditions, among which are diabetes, hypertension, atherosclerosis, and neuronal injury (9–11). Because low Mg availability is known to promote or potentiate oxidative stress (11–13), Mg-dependent inhibition of cell growth might be due to reactive oxygen species (ROS)-mediated DNA damage that, via p53, could lead to the upregulation of p21 and p27. In mice injected with Lewis lung cancer (LLC) cells and fed with either a control or a Mg-deficient diet causing hypomagnesemia (6), we have demonstrated that the significant decrease of tumor growth in Mg-deficient mice paralleled the increase of oxidative DNA damage. We therefore hypothesized that low Mg increases ROS production and, consequently, oxidative DNA damage that might promote the upregulation of p21 and p27 (14).

On the bases of the abundant data in literature describing the influence of Mg on oxidative events, we evaluated the role of oxidative stress on low Mg-mediated inhibition of cell proliferation in mammary epithelial cells grown in vitro. To this aim, we set up an experimental model allowing us to compare cells adapted to grow at nonphysiological Mg concentrations, namely, 0.05 and 45 mM (7). Although this model amplifies the conditions of pathological Mg imbalance found in the aforementioned diseases or in severely reduced dietary Mg intake, it has proved useful for studying the role of Mg in the control of cell proliferation (3). In this study, we investigated the effects of both chronic and acute Mg deprivation on the relationship between cell growth and intracellular ROS in order to shed some light on the mechanisms linking Mg, cell cycle regulation, and oxidative stress.

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MATERIALS AND METHODS

Cell Culture

HC11 mouse mammary epithelial cells were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin, streptomycin, and l-glutamine. High-Mg (H-HC11) and low-Mg (L-HC11) cells were obtained from HC11 cells by stepwise chronic adaptation to grow at nonphysiological Mg concentrations (45 and 0.05 mM $MgSO_4$, respectively) following (7). The low-Mg conditions were obtained using a Mg-free RPMI (Gibco, Carlsbad, CA) supplemented with dialyzed Mg-free serum as described previously (7). All the data were obtained from subconfluent cultures.

Cell Cycle Analysis

Cell pellets were treated with 0.2 mg/ml propidium iodide in Hank's balanced salt solution containing 0.6% NP-40 and RNAse (1 mg/ml) and incubated in the dark at room temperature for 30 min. Upon filtration, cell suspensions (10^6/ml) were analyzed for DNA content on a Coulter EPICS 753 flow cytometer (Beckman Coulter, Miami, FL). The percentage of cells in each phase of the cell cycle was determined by using a Multicycle software version 2.53 (Phoenix Software, San Diego, CA).

Intracellular ROS Measurements

ROS were evaluated by intracellular dichlorofluorescein (DCF) fluorescence. Cells were seeded on 6-well plates at a density of 40,000/well. After experimental treatments (e.g., acute Mg deprivation), cells were rinsed in PBS and loaded with 5 μM H_2DCF-DA (Molecular Probes, Leiden, The Netherlands) at 37°C in the dark for 20 min. Addition of 100 μM H_2O_2 in the last 10 min served as positive controls. The blank was made by incubating cells without DCF. Trypsinized and PBS-resuspended cells (10^6/ml) were analyzed on a Coulter EPICS 753 flow cytometer for DCF fluorescence (excitation 488 nm, emission 530 nm).

Comet Assay for DNA Strand Breaks

Detection of DNA strand breaks by single cell microgel electrophoresis (comet assay) was performed basically following (15). Data are reported as the ratio between tail and nucleus areas, evaluated by Optimas 5 software (Bioscan, Washington, DC). At least 50 randomly selected representative comets were calculated for each blind sample.

cDNA Array

RNA was extracted by the cesium chloride method from control, H-HC11, and L-HC11. Human cDNA expression array membranes consisting of 1,176 known genes (Atlas™ Mouse 1.2 Array and Atlas™ Mouse Cancer 1.2 Array; Clontech, BD Biosciences, Clontech Palo Alto, CA) were used according to the manufacturer's instructions. The hybridization data were collected with PhosphoImager (Molecular Dynamics, Sunnyvale, CA). The AtlasImage 1.0 (Clontech) software was used to compare gene expression. Signal intensities between the compared arrays were normalized using the global mode (to develop a normalization coefficient) that uses an average value based on all the expressed genes as previously described (16).

Glutathione S-Transferase (GST) Activity Assay

Subconfluent cells were scraped and lysed in 50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1% Triton, and 150 mM NaCl. GST activity was measured by using the GST activity Assay Kit (Cayman, Ann Arbor, MI) according to the manufacturer's instructions on 80–100 μg protein. GST activity was expressed in nmol/min/mg protein. Protein contents were assayed by the Bradford method.

Statistical Analyses

Data are expressed as mean ± SD of triplicates from at least 3 separate experiments. To assess the significance, unpaired Student's t-test was calculated and difference considered significant when $P < 0.05$. Multifactorial 2-way analysis of variance (ANOVA) was performed to assess differences between multiple sets of data. When significant values were found ($P < 0.05$), post hoc significance of means was made by the Tukey's multiple comparison test.

RESULTS

Magnesium Regulates Epithelial Cell Proliferation

Figure 1 shows cell cycle distribution of control, H-HC11, and L-HC11 cells (0.8, 45, and 0.05 mM $MgSO_4$, respectively). Although H-HC11 showed a cell cycle distribution similar to control HC11 cells, L-HC11 showed an increased number of cells in the G0/G1 phase and a decreased number of cells in the S phase of the cell cycle. To confirm this effect was due to Mg deprivation, we restored the normal Mg concentration in L-HC11

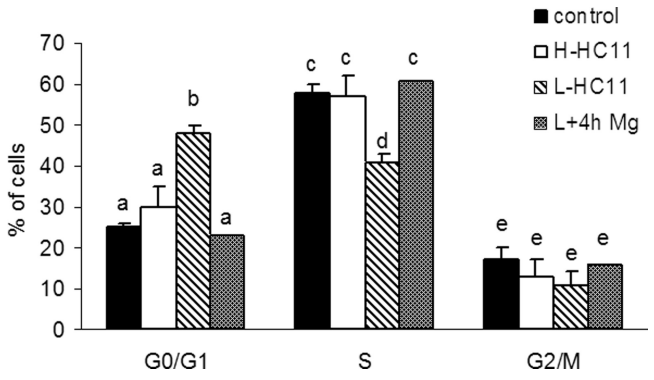

FIG. 1. Cell cycle distribution in H-HC11, L-HC11, and control HC11 cells and the effect of 0.8 mM Mg readdition to L-HC11. Data are mean ± SD of at least three separate experiments. Data sharing the same letter are not statistically different as assessed by Tukey's multiple comparison test.

cells. Four hours after the addition of 0.8 mM $MgSO_4$, cells in G0/G1 phase decreased and cells in S phase increased compared to L-HC11 cells, approaching the values of the controls.

Intracellular ROS in H-HC11 and L-HC11 cells

In many circumstances, decreased Mg availability has been correlated with increased oxidative stress (11–13). To investigate this possibility, we measured intracellular ROS by DCF fluorescence in basal conditions and upon treatment with H_2O_2 in L-HC11 and H-HC11 compared to control cells.

Figure 2 shows that the basal level of DCF fluorescence was similar in control and H-HC11, whereas it was significantly lower in L-HC11 cells. Exposure to H_2O_2 increased DCF fluorescence in all the cells tested. However, DCF fluorescence after challenge with H_2O_2 was lower in L-HC11 cells than in controls and H-HC11 cells. When Mg was supplemented to L-HC11 medium to reach the physiological concentration (0.8 mM), the levels of DCF fluorescence increased approaching within 4 h the amounts observed in control cells.

H_2O_2-Induced DNA Damage in H-HC11 and L-HC11 Cells

Oxidative DNA damage may modulate proliferation by increasing p53 level and, consequently, p21 and p27. To investigate whether Mg availability affected H_2O_2-induced DNA damage, which could be responsible for inhibiting cell cycle progression, we measured oxidative DNA damage by comet assay. Figure 3 shows that H_2O_2-induced DNA damage was significantly lower in L-HC11 cells than in control or H-HC11 cells. These results are consistent with the levels of intracellular ROS described in Fig. 2.

The Effects of Acute Mg Deprivation on HC11 Cells

Because H-HC11 and L-HC11 cells have been chronically adapted to nonphysiological Mg concentrations, we reasoned that these cells might have activated an adaptive response to

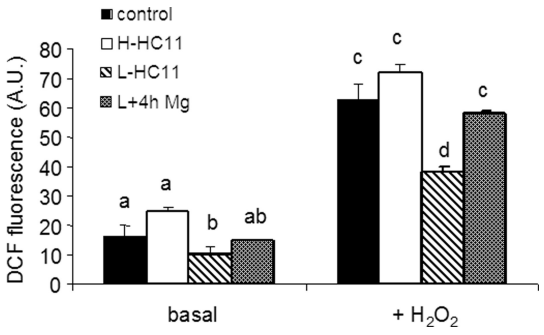

FIG. 2. DCF-detectable ROS in H-HC11, L-HC11, and control HC11 cells and the effect of 0.8 mM Mg readdition to L-HC11. DCF fluorescence was assayed in basal conditions and upon treatment with 100 μM H_2O_2 for 15 min. Data are mean ± SD of at least 3 separate experiments. Data sharing the same letter are not statistically different (Tukey's multiple comparison test).

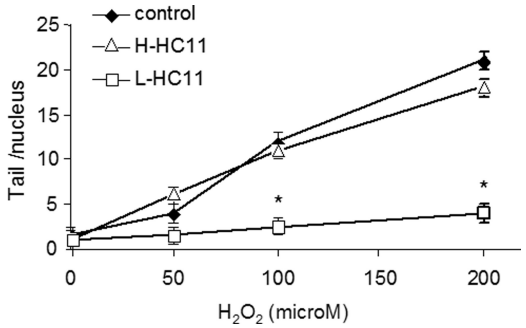

FIG. 3. H_2O_2-induced oxidative DNA damage in H-HC11, L-HC11 and control HC11 cells. DNA damage is expressed as tail/nuclear ratio. Data are mean ± SD of at least three separate experiments. $*P < 0.05$ (unpaired Student's t-test) compared to control cells at the same time point.

nonphysiological Mg availability as previously described for intracellular Mg contents (3).

To investigate whether differences occur between chronic and acute exposure to low Mg, we studied the effect of an acute Mg depletion on endogenous and H_2O_2-stimulated intracellular ROS production. Switching control cells to a low-Mg medium (0.05 mM) did not determine any significant variation of DCF-detectable ROS over a time ranging from 30 min to 24 h either in basal or in H_2O_2-stimulated conditions. Interestingly, and consistent with the results obtained in L-HC11 cells, after 24 h of Mg depletion, DCF fluorescence decreased and remained lower than in the controls (Fig. 4).

Gene Profiling in H-HC11 and L-HC11 Cells

To unravel the molecular mechanisms of Mg-mediated control of cell proliferation, we examined gene expression profiles by cDNA array. Of 1,176 known genes examined, we found 35 genes clearly down or overexpressed in H-HC11 or L-HC11 cells compared to the controls (Table 1). These genes encode proteins involved in several cell functions. Among those directly involved in the control of cell proliferation, L-HC11 upregulated p53, cyclin dependent kinase 7, and jumonji, whereas

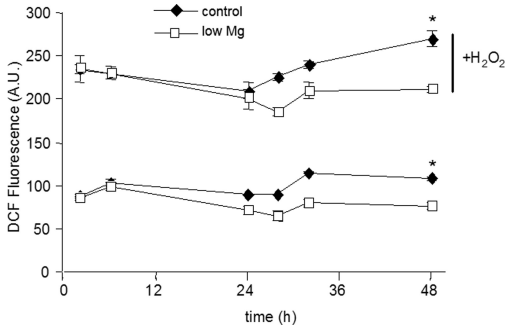

FIG. 4. DCF-detectable ROS in HC11 cells under acute Mg deprivation. Time course of basal and H_2O_2-induced (100 μM for 15 min) DCF fluorescence. Data are mean ± SD of at least three separate experiments. $*P < 0.05$ (unpaired Student's t-test) compared to control cells at the same time point.

TABLE 1
Gene expression profile of H-HC11 and L-HC11 cells vs. controls[a]

Gene	H-HC11	L-HC11
40S ribosomal protein SA	=	↑
78-kDa glucose regulated protein	↑	=
Activating transcription factor 2	↓	↓
Butyrate response factor 1	↑	↑
Calcyclin binding protein	↓↓	↓
Cellular tumor antigen p53	=	↑↑
Crk adaptor protein	=	↓
Cyclin dependent kinase 7	=	↑
Cyclin F	↑↑	=
Cytoplasmid dynein light chain	=	↓↓
Dystroglycan1	=	↓
ETs-related transcription factor	↑↑	=
Glutathione S-transferase	=	↑
GADD153	↑	=
HSP84	↑	=
Inhibin β A subunit precursor	↑	=
Jumonji	=	↑↑
Laminin $\alpha 5$ subunit precursor	↓	=
Max protein	↑↑	↑
Numblike	=	↑↑
Octamer-binding transcription factor	=	↑↑
Osteoblast-specific factor 2	=	↓↓
Protein phosphatase 2C α isoform	↓	=
P-selectin glycoprotein ligand 1 precursor	↑	=
Ser/Thr protein kinase receptor	=	↑↑
Stra14 basic-helix-loop-helix protein	=	↑
TGF β receptor	↓	↑↑
Thymosin β	=	↓
Transcription termination factor 1	=	↑↑
Transferrin receptor protein	=	↓
Transcription factor E2F	=	↓
Tyrosine protein kinase receptor	=	=
Tyrosine protein kinase precursor	=	↑↑
v-erbA related proto-oncogene	↑	=

[a]Abbreviations are as follows: H, high magnesium (Mg); L, low Mg; Crk, CT10 sarcoma oncogene cellular homolog; Ets, E26 avian erythroblastosis virus transformation specific protein; GADD, growth arrest and DNA damage inducible protein; HSP, heat shock protein; TGF, transforming growth factor; ↓ down-expression (each ↓ corresponds to 50% decrease vs. control HC11 cells), ↑ over-expression (each ↑ corresponds to twofold increase vs. control HCl1 cells).

transcription factor E2F and the CrK adaptor protein were downregulated. On the contrary, H-HC11 cells downregulated calcyclin binding protein and overexpressed cyclinF as well as some transcription factors, such as Max, that among its functions, also governs cell proliferation. Interestingly, we observed the downregulation of TGF-beta receptor in H-HC11 and its overexpression in L-HC11.

In addition, among the scavenger enzymes involved in the detoxification of free radical species, we found that glutathione S-transferase was overexpressed in L-HC11 compared to control cells.

GST Activity and Mg Availability in HC11

Following indications derived from gene expression profile and to investigate the observed strict correlation between DCF-detectable ROS and Mg availability, we assayed GST activity in L-HC11 vs. control cells. Table 2 shows that GST activity was increased by about 2fold in L-HC11 cells compared to controls. Furthermore, when control cells were switched to a low-Mg medium (0.1 mM Mg), as in the model of acute Mg deprivation, the GST activity promptly increased as shown by the level reached after 48 h of Mg deprivation, approaching that of L-HC11 cells. It is worth noting that the increase of GST activity after 48 h of Mg depletion parallels the decrease of DCF-detectable ROS (cf. Fig. 4). The readdition of Mg to L-HC11 cells confirmed these results because after 4 h from the addition of 0.8 mM Mg, the GST activity was found significantly decreased and comparable to that of control cells.

DISCUSSION

In this work, we investigated whether oxidative stress mediates the effects of Mg on the growth of HC11 mammary epithelial cells.

Our results show that L-HC11 cells display G0/G1 cell cycle arrest, which can be reverted by readdition of Mg. Inhibition of cell cycle is not linked to oxidative stress because L-HC11 cells have lower levels of DCF-detectable intracellular ROS compared to controls or H-HC11 cells. Accordingly, also the levels of H_2O_2-induced oxidative DNA damage are lower in L-HC11 than in controls or H-HC11 cells.

In previous studies with mice under Mg-deficient diet, we found that low Mg availability similarly inhibited tumor growth;

TABLE 2
GST activity in HC11 cells as a function of Mg availability[a]

	GST Activity nmoles/min/mg Protein	Statistics
Control	4.35 ± 0.1 (4)	a
Control – Mg (48 h)	6.75 ± 0.4 (4)	b
L-HC11	9.79 ± 1.9 (4)	b
L-HC11 + Mg (4 h)	4.10 ± 0.9 (3)	a
L-HC11 + Mg (24 h)	5.15 ± 1.1 (3)	a

[a]Abbreviations are as follows: GST, glutathione S-transferase; Mg, magnesium; L, low Mg; Data are mean ± SEM (number of experiments). The data groups with different letters are statistically different (Tukey's multiple comparison test).

however, such an effect was accompanied by biochemical indexes of oxidative stress such as, for example, increased 8-OHdG levels, the major product of oxidative DNA damage (14). We believe that the discrepancy between those studies and our present findings is only apparent. Experimental Mg deficiency in rodents induces after a few days a clinical inflammatory syndrome characterized by leukocyte and macrophage activation, release of inflammatory cytokines and acute phase proteins, and excessive production of free radicals; moreover, magnesium deficiency induces a systemic stress response by activation of neuro-endocrinological pathways (17,18). Thus, Mg deficiency in vivo contributes to an exaggerated response to immune stress, and oxidative stress might be the consequence of the low Mg-induced inflammatory response rather than a direct effect of Mg deficiency. It is still not clear whether the conclusions drawn from animal models can be applied to humans in which pathological conditions characterized by Mg imbalance are complicated by several concurrent factors.

Our findings should be reconciled also with reports that demonstrated an upset of oxidative damage in a variety of cell types exposed to low Mg availability in vitro (e.g., endothelial cells, hepatocytes, erythrocytes) (13,19,20). We suggest that a comparison of those reports with our present findings would be inappropriate; in fact, low Mg-dependent oxidative stress was described in cells that had already been subjected to dihydroxyfumarate/ADP-Fe^{3+}-induced lipid peroxidation (19) or serum starvation (13,20). Under such defined conditions, low Mg availability overlapped with many concurrent mechanisms of cell damage, and such a complexity casts doubts about whether low Mg availability acted as a primary or ancillary determinant of oxidative stress. We would also note that other reports have failed to characterize a link between low Mg and increased oxidative stress, whether in vitro or in vivo (21,22).

We previously demonstrated that low Mg inhibits cell cycle progression by upregulating p27 (2,7,14); here we found that this growth arrest may be independent of oxidant stress-mediated signals. Gene profiling of H-HC11 and L-HC11 cells offers some additional insight into how Mg modulates cell growth. In L-HC11 cells, the overexpression of cdk7 and p53 could be suggestive of an upregulation of p53 transcriptional activity and related growth arrest events. In the same cells, the upregulation of jumonji is consistent with its role as a negative controller of cell growth (23). Of note, jumonji upregulation was observed also in tumor-bearing, low-Mg mice and was accompanied by inhibition of tumor growth in the face of an increased oxidative stress (14). In principle, this would support the notion that low Mg inhibited cell proliferation through modulation of growth-related factors regardless of concomitant upregulation or mitigation of oxidative stress. Gene profiling of L-HC11 and H-HC11 cells showed that also the TGF-β receptor was modulated by Mg. TGF-β is considered a negative regulator of cell growth as suggested by recent evidence that the loss of the TGF-β receptor caused hyperplasia of mammary epithelium (24). Overexpression or downregulation of TGF-β receptor in L-HC11 or H-HC11 cells, respectively, is therefore consistent with a role for Mg in regulating growth-related factors.

Gene profiling revealed GST overexpression in L-HC11 cells, which also exhibited a 2-fold increase of GST activity as compared to control cells. Inasmuch as GST is a known protection against xenobiotics and ROS as well (25,26), we surmised that GST overexpression and increased activity contributed to diminishing basal or H_2O_2-induced oxidative stress in L-HC11. Two lines of evidence lent support to such a possibility: 1) Mg withdrawal increased GST activity in control cells concomitant with reduced levels of DCF-detectable ROS, and 2) Mg supplementation caused opposite effects in L-HC11 cells (cf. Table 2 and Figs. 2 and 4).

Improved antioxidant defenses may represent an adaptive response to oxidative events; therefore, we considered that acute Mg deprivation induced oxidative stress that primed the cells to adaptive events such as GST overexpression and increased activity. Unfortunately, however, there was no measurable increase of DCF-detectable ROS in mammary HC11 cells subjected to acute Mg deprivation regardless of whether the cells were probed under basal or H_2O_2-stimulated conditions. We may not rule out that an acute Mg deprivation caused ROS levels that were too low or transient to be sensed by the DCF assay or developed within the context of regulatory processes that also precluded detection by the DCF assay. Indeed, we showed that Na-dependent Mg efflux occurs over the first 30 min of Mg depletion, and this efflux can be influenced by hormones, receptor-mediated signals, and intracellular events (27).

Also, GST expression is subject to some prominent receptor-mediated signals induced by, for example, insulin (28). Overexpression of GST could, in turn, affect formation of the 15-d-PGJ(2) prostaglandin, which is known to influence cellular processes such as gene expression, differentiation, proliferation, and apoptosis (29). It is worth noting that leukocytes from GST-deficient mice displayed increased proliferation coupled with downregulation of negative pathways of cell proliferation like JNK and Janus kinase/STAT (30). Conversely, hepatocytes overexpressing GST displayed increased doubling times and delayed G1-S phase transition (31). All such findings underscore a link between GST expression and regulation of cell growth, lending support to our suggestion that Mg depletion could modulate cell growth through GST expression/activity and related intracellular signals that will require ad hoc characterizations.

Whether oxidative stress plays a role in low Mg-dependent cell growth arrest clearly remains a matter of debate. There are conditions when immuno-inflammatory responses and concurrent oxidative reactions might cooperate with Mg deficiency in inducing growth arrest in vivo. Other Mg-deficiency conditions, such as those described in our in vitro studies, may cause growth arrest in the face of concomitant overexpression of GST and downmodulation of measurable ROS.

GST may have complex direct effects on cell growth. The possible relation between Mg availability and oxidative

damage will therefore need to be incorporated in a framework that accommodates also GST and proves to be more complex than previously believed.

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