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Reduced Cellular Mg^{2+} Content Enhances Hexose 6-Phosphate Dehydrogenase Activity and Expression in HepG2 and HL-60 Cells

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Abstract

We have reported that Mg^{2+} dynamically regulates glucose 6-phosphate entry into the endoplasmic reticulum and its hydrolysis by the glucose 6-phosphatase in liver cells. In the present study, we report that by modulating glucose 6-phosphate entry into the endoplasmic reticulum of HepG2 cells, Mg^{2+} also regulates the oxidation of this substrate via hexose 6-phosphate dehydrogenase (H6PD). This regulatory effect is dynamic as glucose 6-phosphate entry and oxidation can be rapidly down-regulated by the addition of exogenous Mg^{2+}. In addition, HepG2 cells growing in low Mg^{2+} show a marked increase in hexose 6-phosphate dehydrogenase mRNA and protein expression. Metabolically, these effects on hexose 6-phosphate dehydrogenase are important as this enzyme increases intra-reticular NADPH production, which favors fatty acid and cholesterol synthesis. Similar effects of Mg^{2+} were observed in HL-60 cells.

These and previously published results suggest that in an hepatocyte culture model changes in cytoplasmic Mg^{2+} content regulates glucose 6-phosphate utilization via glucose 6 phosphatase and hexose-6 phosphate dehydrogenase in alternative to glycolysis and glycogen synthesis. This alternative regulation might be of relevance in the transition from fed to fasted state.

Keywords

magnesium; hepatic Mg^{2+} homeostasis; glucose 6 phosphate; hexose 6 phosphate dehydrogenase; HL-60

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Introduction

Magnesium (Mg^{2+})[1], the second most abundant cation within mammalian cells after potassium, is highly compartmentalized within the cytoplasm, the nucleus, the mitochondria, and the endoplasmic reticulum of the hepatocyte [1,2]. This cellular compartmentalization is maintained through a combination of buffering and transport mechanisms [1,2] that maintain free $[Mg^{2+}]_i$ at ~0.7-1 mmol/L within the cytoplasm [3] and the mitochondrial matrix [1,2]. These concentrations remain relatively stable even when total cellular Mg^{2+} content increases or decreases by an estimated 2-3 mmol/L [4] following hormonal or metabolic stimuli [1,2]. Such a tight regulation suggests that maintenance of Mg^{2+} homeostasis is essential to regulate the activity of specific Mg^{2+}-dependent enzymes.

In the hepatocyte, experimental evidence indicates that Mg^{2+} homeostasis and transport across the cell membrane is coupled to glucose metabolism. Catecholamine and glucagon, hormones that stimulate glycogenolysis and glucose output from the liver to maintain euglycemia, also elicit Mg^{2+} extrusion from liver cells [5,6]. Conversely, hormones promoting glucose entry into the hepatocyte (e.g. insulin) promote Mg^{2+} accumulation within the cell [7]. At the organellar level, however, limited information is available about Mg^{2+} redistribution following these hormonal stimuli and its regulatory role on selected enzymes.

In the case of the endoplasmic reticulum, Mg^{2+} has been reported to regulate protein synthesis [8] and Ca^{2+}-cycling [9]. More recently, we have reported that cytoplasmic Mg^{2+} level regulates glucose 6-phosphate (G6P) entry and hydrolysis via glucose 6-phosphatase (G6Pase) within the hepatic endoplasmic reticulum [10,11], in agreement with the report by Mithieux et al. [12]. The effect occurs at the level of the G6P entry mechanism into the E.R. as bypassing of this step abolishes Mg^{2+} regulation [10,11]. This regulation is dynamic and inversely proportional to Mg^{2+} concentration, and it has been observed in permeabilized hepatocytes [10] and in hepatic microsomal vesicles from rats maintained for 2 weeks on Mg^{2+} deficient diet [11].

In addition to being hydrolyzed to glucose and inorganic phosphate (Pi) by the G6Pase, following its entry into the endoplasmic reticulum G6P can be converted to 6-phosphogluconolactone by the hexose 6-phosphate dehydrogenase (H6PD, E.C. 1.1.1.47), an intra-reticular NADP-dependent enzyme [13]. This enzyme constitutes the reticular version of the glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49) [14], and catalyzes the first two reactions of the pentose shunt pathway [15]. In addition to being used in the pentose shunt pathway, the generated NADPH can be utilized in several other metabolic reactions including fatty acid and cholesterol synthesis, and glutathione homeostasis [16].

In the present study, we investigate the effect of reduced cellular Mg^{2+} content on the reticular conversion of G6P by H6PD in HepG2 cells, a cell model of hepatocytes in culture previously utilized to study H6PD [13]. HepG2 cells were maintained in culture in the presence of physiological (1 mmol/L) or reduced (0.6 or 0.4 mmol/L) extracellular Mg^{2+} ($[Mg^{2+}]o$) The results reported here indicate that G6P entry into the ER increases in HepG2 cells in a manner that is inversely proportional to the cellular and cytoplasmic Mg^{2+} content.

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This regulation is strictly Mg^{2+} dependent, and is associated with an enhanced NADPH production resulting from the increased oxidation of G6P to 6-phosphogluconolactone via H6PD [13]. In addition to the post-translational regulation of G6P oxidation by H6PD, a decrease in cellular Mg^{2+} also increases H6PD mRNA expression by ~4-fold and protein expression by about 3-fold. A similar up-regulation of H6PD activity and expression is observed in HL-60 cells, which also possess reticular H6PD [13,14].

Taken together, these results suggest that low cellular Mg^{2+} content results in an increased routing of G6P towards E.R.-based metabolic processes, namely G6Pase and H6PD, most likely for metabolic purposes. These data together with previously published results point to cellular Mg^{2+} as a regulator of glucose utilization by alternative metabolic pathway within the hepatocyte, and possibly other cell types.

Materials and Methods

Materials

HepG2 cells were a kind gift from Dr. Cederbaum (Mt. Sinai, New York). ^{3}H-2-deoxyglucose was from Amersham (GE, Pittsburgh, PA). Human promyelocytic leukemia HL-60 cells were a kind gift of Dr. Dubyak (Case Western Reserve University, Cleveland). Culture medium and bovine calf serum were from Gibco (Life Science, Grand Island, NY). All other reagents were of analytical grade (Sigma, St Louis, MO).

Methods

Cell Cultures—HepG2 cells were maintained in MEM medium (M2279, Sigma) containing 0.8 mmol/L MgSO4 and 5.5 mmol/L glucose in the presence of 5% CO_2 until 85% confluent. HL-60 cells were maintained in RPMI-1640 medium (Gibco) containing 0.4 mmol/L $MgSO_4$ and 5.5 mmol/L glucose (as per vendor) in the presence of 5% CO_2 until 85% confluent. At confluence, either cell line was divided into three groups and cultured in the presence of physiological (0.8 mmol/L) $[Mg^{2+}]o$ or under Mg^{2+} deficient conditions (0.4, and 0.2 mmol/L $[Mg^{2+}]o$). The presence of 10% bovine calf serum (BCS) increased extracellular Mg^{2+} content to 1, 0.6, and 0.4 mmol/L, respectively (measured by atomic absorbance spectrophotometry). Hence, for the remainder of this study the cells will be identified by these adjusted Mg^{2+} concentrations.

Both HepG2 and HL-60 cells were cultured until 85% confluent (~5-6 days) in the presence of 1, 0.6 0r 0.4 mmol/L $[Mg^{2+}]o$. Cells were then harvested by trypsin digestion (2 min at 37°C). Trypsin was neutralized by addition of medium containing BCS and the appropriate amount of $[Mg^{2+}]o$. Cells were sedimented at 800 rpm x 2 min, and washed twice in BCS/Mg^{2+}-medium to remove dead cells and debris. After the second sedimentation cells were used to measure Mg^{2+} content and the activity of reticular hexose 6-phosphate dehydrogenase (H6PD).

Cellular Mg^{2+} content—To measure total cellular Mg^{2+} content, normal or Mg^{2+} deficient HepG2 and HL-60 cells were harvested at confluence, washed in PBS and sedimented again (500 rpm x 1 min) in microfuge tubes. The supernatants were removed and the cell pellets dissolved in 10% HNO_3. The acid mixture was sonicated for 10 min in a

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sonicating bath at room temperature, and acid-digested overnight at 4°C. The following morning, the acid mixtures were sonicated again, vortexed, and sedimented (5000 rpm x 3 min) in microfuge tubes. The supernatants were removed and assessed for Mg^{2+} content in a Varian 210 atomic absorbance spectrophotometer (AAS) calibrated with Mg^{2+} standards.

To measure intracellular Mg^{2+} distribution, HepG2 and HL-60 cells were harvested and washed as described above. The cell pellets were then resuspended in a cytosol-like, Mg^{2+} free medium containing (mmol/L) 100 KCl, 20 MOPS, 20 NaCl, 1 KH2PO4, pH 7.2 [10], pre-warmed at 37°C, at the final concentration of 0.5 mg protein/ml. Digitonin (50 μg/ml), FCCP (2 μg/ml), and A23187 (1 μg/ml) were sequentially added to the cell mixture at 5 min intervals to measure cytoplasmic, mitochondrial, and post mitochondrial Mg^{2+} content, respectively [17]. Aliquots of the cell mixture were withdrawn in duplicate prior to the addition of any of these agents, and sedimented in microfuge tubes (5000 rpm x 3 min). The supernatants were removed and assessed for Mg^{2+} content by AAS. The pellets were digested in 10% HNO3 and assessed for Mg^{2+} content as reported above. Although indirect, this approach has been successfully used in our laboratory, with results equivalent to those reported by others with different techniques [17].

Animals on Mg^{2+} Deficient Diet—Male Sprague–Dawley rats (230–250 g body weight) were maintained for 2 weeks on a Mg^{2+}-deficient diet (Dyets, Bethlehem, PA) containing 380 mg Mg^{2+} per kg of pellet food whereas pared-age control rats received a ‘normal’ Mg^{2+} diet containing 507 mg Mg^{2+} per kg of pellet food [11]. All other minerals in the diet were at the same concentration. After 2 weeks, control and Mg^{2+} deficient rats were euthanized by i.p. administration of saturated sodium pentobarbital solution (60 mg/ml). The rat’s abdomen was opened and the liver excised for microsomal vesicle isolation. This and the subsequent protocol were in agreement with the NIH directives for the use and maintenance of experimental animals and was approved by the Institutional Animal Care and Utilization Committee (IACUC) at Case Western Reserve University.

Liver Microsomes Purification—The liver was rapidly rinsed in 250 mmol/L ice-cold sucrose, blotted on absorbing paper, and weighted. Microsomes were isolated according to the procedure of Henne and Soling [18], modified as follows. Livers were homogenized (10% w/v) in (mmol/L): 250 sucrose, 20 K-HEPES (pH 7.2) in a Thomas C potter by 4 passes with a tight-fitting Teflon pestle. Homogenate was sequentially centrifuged in a JA-20 Beckman rotor at 2,500g x 10 min to remove nuclei and cellular debris, and at 10,000g x 10 min to remove mitochondria. The post-mitochondria supernatant was sedimented at 80,000g x 45 min in a Ti 90 Beckman rotor. The glycogen pellet was discarded. The microsomal pellet was washed once with cytosol-like, Mg^{2+} free medium (incubation medium) previously described, and centrifuged at 80,000g x 45 min. The final pellet was resuspended in the described incubation medium at a concentration of 15 mg/ml, and stored in liquid nitrogen until used.

Mg^{2+} determination in Total Liver and in Microsomes—After explant, 0.5 g of liver tissue were homogenized in 250 mmol/L sucrose (10% w/v) in a Thomas C potter by 4 passes with a tight-fitting Teflon pestle, acidified by addition of HNO_3 (10% final concentration and digested overnight at 4°C. For the microsomes, after isolation, aliquots of

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microsomal vesicles (0.5 mg protein) were digested overnight in 10% HNO_3 (final concentration). The next day, total liver homogenate and microsomes denatured protein were sedimented (14,000 rpm x 5 min) in microfuge tubes, and the Mg^{2+} content of the acid extracts assessed by AAS as previously described [see also ref. 11].

Hexose 6-Phosphate Dehydrogenase Activity—Hexose 6-phosphate dehydrogenase activity was measured as reported by Senesi et al. [19]. Briefly, HepG2 and HL-60 cells at confluence were harvested and washed as previously reported. The cells were then resuspended in the cytosol-like medium described previously, and permeabilized by the addition of 50 μg/ml digitonin. Permeabilization was assessed by Trypan Blue exclusion test [5]. Cells were resuspended at the final protein concentration of ~10 mg/ml (~$4*10^6$ cells.ml). Aliquots of the cells were diluted 20-fold in a final volume of 2 ml cytosol-like incubation medium, and transferred to a Perkin Elmer 3 fluorimeter (Perkin Elmer, 3) equipped with data acquisition software, with setting at 340nm excitation wavelength and 490 nm emission wavelength [20]. Aliquots of microsomes (were also diluted 20-fold in a final volume of 2 ml/ incubation medium previously indicated (~0.5 mg protein/ml, final concentration). For all experimental samples, the reaction was started by the addition of 1mmol/L G6P, and the production of NADPH recorded for 10-15 min at room temperature. For comparison purposes, in separate sets of experiments, NADPH production was fluorimetrically recorded for a similar period of time at 37°C, or measured in an Agilent 8453 spectrophotometer as reported [19]. Calibration was carried out by adding known amounts of NADPH standards to aliquots of heat-inactivated permeabilized cells under similar experimental conditions. It has to be noted that no NADP + needed to be added to the reaction as this pyridine nucleotide is retained within the endoplasmic reticulum of the cells [21]. For the microsomes, the vesicles were mildly permeabilized by addition of alamethicin (0.1 mg/mg protein) as reported by Banhegyi et al. [14], in the presence of 100 μM $NADP^+$ to guarantee an appropriate level of coenzyme for the H6PD reaction and avoid unwarranted microsomes isolation-related intra-reticular (endo-microsomal) $NADP^+$ depletion.

Hexose 6-Phosphate Dehydrogenase Gene Expression—Hexose 6-phosphate dehydrogenase expression was assessed in HepG2 and HL-60 cells by qRT-PCR. Cells were digested in Trizol (Life Technologies, Grand Island, NY), and mRNA extracted according to the producer indications. Two different sets of primers (Table 1) were used. Gene expression was normalized against β-actin and GAPDH expression, using HotStart-IT SYBR Green One-Step qRT-PCR Master Mix Kit (Affymetrix, Santa Clara, CA)

Hexose 6-Phosphate Dehydrogenase Protein Expression—The expression of reticular H6PD was assessed byWestern blot analysis. Equal amounts of microsomal protein (20 μg/lane) were loaded onto 12% poly-acrylamide gradient gel, separated by SDS–PAGE, and transferred onto PVDF immobilization membrane (Schleicher & Schuell Inc.). Commassie Blue and Rouge Ponceau S staining were used to confirm equal protein loading and transfer, respectively. The membranes were extensively washed with PBS, and the binding of primary antibodies against H6PD (Cell Signaling, Boston, MA) was visualized using peroxidase conjugated secondary antibodies and ECL western blotting detection

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reagents (Amersham). Protein expression was evaluated by densitometry using Scion Image program (NIH), as previously reported [22].

Other experimental Procedures—Protein content was assessed according to Lowry et al. [23], using bovine serum albumin as a standard. Contaminant Mg^{2+} present in the cytosol-like incubation buffer used for microsomes resuspension and for H6PD determination was measured by AAS (ranging between 2 and 5 μmol/L) and subtracted accordingly.

Statistical Analysis—Data are reported as means + S.E. of at least four different preparations for each experimental condition, each tested in duplicate. Data were analyzed by *Student T- test* set at $p<0.05$ for significance. For experiments evaluating multiple experimental samples, data were first analyzed by one-way ANOVA. Multiple means were then compared by Tukey's multiple comparison test performed with a q value established for statistical significance of $p < 0.05$.

Results

HepG2cells cultured in the presence of 0.6 mmol/L and 0.4 mmol/L $[Mg^{2+}]o$ presented ~15% and ~35% less total cellular Mg^{2+}, respectively, than HepG2 cells maintained in the presence of physiological 1 mmol/L $[Mg^{2+}]o$ (Fig.1). Assessment of the intracellular Mg^{2+} distribution as reported under Materials and Methods confirmed that Mg^{2+} content decreased in the cytoplasm (~34% and ~51%, respectively), mitochondria (~12% and ~26%, respectively), and post mitochondrial pools (~24% and ~55% respectively) in HepG2 cells grown in 0.6 mmol/L and 0.4 mmol/L (Fig. 1).

Consistent with previous observation [**ref.**], we have reported that a decrease in cytoplasmic Mg^{2+} content enhances the rate of G6P transport into the hepatic endoplasmic reticulum and its hydrolysis by glucose 6-phosphatase [10,11]. These results were obtained in both freshly isolated hepatocytes [10] and in microsomal vesicles from livers of Mg^{2+} deficient animals [11]. Figure 2 shows a similar increase in G6P transport and hydrolysis in HepG2 cells cultured in the presence of different extracellular Mg^{2+} concentrations, and permeabilized with digitonin as previously reported [10]. Overall, these data confirmed the inverse relationship between cellular Mg^{2+} content and G6P transport and hydrolysis previously observed [10-12].

Once transported within the endoplasmic reticulum, G6P can also be oxidized to 6-phosphogluconolactone by the reticular hexose 6-phosphate dehydrogenase, in a reaction associated with the production of endo-luminal NADPH [14,19-21]. This observation also supports the presence of a segregated intra-luminal pyridine nucleotide pool within the endoplasmic reticulum of the HepG2 cells [21, and refs. therein].

We investigated whether oxidation of G6P via H6PD was also enhanced in Mg^{2+} deficient cells following the increased entry of the substrate into the endoplasmic reticulum. The production of NADPH coupled to the H6PD activity was measured in HepG2 cells in the presence of various amounts of exogenous G6P. The results reported in Fig. 3A indicate that

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HepG2 cells grown in the presence of 0.4 and 0.6 mmol/L $[Mg^{2+}]o$ produced almost three-times and two-times, respectively, the amount of NADPH generated by HepG2 cells maintained in 1 mmol/L $[Mg^{2+}]o$. To further validate that NADPH generation strictly depended on the amount of G6P entering the ER through a Mg^{2+}-controlled entry mechanism, permeabilized HepG2 cells grown in 0.6 mmol/L $[Mg^{2+}]o$ were exposed to1 mmol/L G6P in the presence of progressively increasing extra-reticular Mg^{2+} concentrations. As Fig. 3B shows, increasing extra-reticular Mg^{2+} concentration resulted in a progressive reduced NADPH generation. Qualitatively similar results were observed for HepG2 cells cultured in the presence of 0.4 and 1 mmol/L $[Mg^{2+}]o$ (not shown). This effect is similar to that reported on the amount of Pi and glucose generated by the hydrolytic activity of the G6Pase [10-12]. Consistent with what reported previously for the G6Pase [10,11], the effect of Mg^{2+} was specific in that no significant modulatory effect on NADPH production was observed when Mg^{2+} was replaced with an equimolar concentration of another divalent (e.g. Ca^{2+} or Mn^{2+}) or monovalent (Na^{+} or K^{+}) cations (not shown). The modulatory effect of Mg^{2+}, however, was observed only when the cation was added prior to, or together with G6P (Table 2). Once the H6PD catalyzed reaction was fully activated by G6P addition, exogenous Mg^{2+} failed to exert its inhibitory effect (Table 2).

At variance of the cytoplasmic glucose 6 phosphate dehydrogenase, which can assemble as a tetramer in the presence of $MgSO_4$ [21], the hexose 6-phosphate dehydrogenase is functionally active as a dimer [21]. To exclude the possibility that variations in endogenous or exogenous Mg^{2+} concentrations affected the dimeric conformation of the enzyme, NADPH production was measured for each extracellular Mg^{2+} concentration in the presence of βmercaptoethanol, which fully stabilizes the dimeric structure of the enzyme. The treatment did not affect the amplitude of NADPH production irrespective of the $[Mg^{2+}]o$ utilized for the cell growth (data not shown). Although more detailed experiments need to be carried out on the purified H6PD protein, these preliminary results suggest that Mg^{2+} is not changing the comformational state of the protein in our experimental model.

Next, we assessed whether culturing HepG2 cells in the presence of low extracellular Mg^{2+} concentration changed H6PD expression. The qRT-PCR determination attested that the enzyme expression increased by ~4-fold (~3-fold with a second set of primers, not shown) in cells cultured in 0.6 mmol/L $[Mg^{2+}]o$ (Fig.4A). A larger increase in expression was observed in cells cultured in the presence of 0.4 mmol/L $[Mg^{2+}]o$. These results were confirmed by Western Blot analysis, which indicated a 3-fold increase in H6PD protein expression (Fig. 4B).

To exclude that the increased oxidation of G6P and associated production of NADPH could be attributed to the neoplastic origin of our HepG2 cells, similar experiments were carried out in microsomal vesicles from rats maintained on a regular or a 35% deficient Mg^{2+} diet, using our previously published protocol [11]. No significant differences in body and liver weights and in protein content per g of liver between the two experimental groups were observed (Table 3). Microsomal yield and RNA content were also not statistically different between the two experimental groups. Total homogenate Mg^{2+} content and microsome Mg^{2+} content, however, were statistically lower (minus 23% and minus 27%, respectively) in the Mg^{2+}-deficient diet group as compared to the normal Mg^{2+} diet group (Table 3). As

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for the H6PD activity, the results reported in Fig. 5A were qualitatively similar to those reported for HepG2 cells, supporting the notion that the observed effect of Mg^{2+} on G6P entry and utilization via H6PD was independent of the neoplastic origin of the HepG2 cells. Figure 5B indicates that also in microsomes from Mg^{2+} deficient animals H6PD expression was ~2-fold increased as compared to microsomes from normal Mg^{2+} animals.

Reticular H6PD is reported to be expressed in neutrophils [13,14,21,24] and adipose tissue [25-26]. To exclude that our observation was hepatocyte-specific, HL-60 promyelocitic leukemia cells were tested as neutrophils representative. The results reported in Figure 6 indicate that also in these cells, reducing [Mg^{2+}]o resulted in a decrease in total and compartmentalized Mg^{2+} content (Fig. 6A), and in an increased production of NADPH via H6PD (Fig. 6B), whereas addition of exogenous Mg^{2+} inhibited NADPH production in a dose-dependent manner (Fig. 6C). H6PD protein expression (Fig. 6D) was ~2-3 fold increased in HL-60 cultured in 0.6 mmol/L [Mg^{2+}]o as compared to those culture in physiological 1 mmol/L [Mg^{2+}]o.

Discussion

The endoplasmic reticulum has been identified as one of the most abundant pool of magnesium within mammalian cells including hepatocytes [1,2]. Total Mg^{2+} concentrations ranging between 16 to 18 mmol/L have been estimated within the organelle using a variety of methodological approaches including electron probe X-ray microanalysis (EPXMA) [1,2]. Unfortunately, no current approach has been able to determine the free Mg^{2+} concentration within the organelle due to the fact that the commercially available Mg^{2+}-specific fluorescent indicators retain a higher affinity for Ca^{2+}, the concentration of which is approximately 2-3 mmol/L within the ER [1,2]. As a consequence, this methodological limit has hampered our ability to measure the free intra-reticular Mg^{2+} concentration and its role in regulating specific reticular functions.

Recently, we have reported that in vivo and in vitro changes in cellular and cytoplasmic Mg^{2+} content modulate in an inverse manner glucose 6-phosphate transport and hydrolysis within liver cells [10,11]. This is in line with reports by other laboratories [12]. Currently, two different hypotheses are available to explain the transport of G6P across the E.R. membrane and its delivery to the catalytic site of the G6Pase. According to the *substrate–* transport model, G6P enters the ER lumen via a specific transport mechanism (T1) distinct from the hydrolase [27]. According to the *conformational flexibility* substrate transport model, instead, G6P binds the hydrophilic region of G6Pase facing the cytoplasm, which changes in conformation and promotes the delivery of the substrate to the intra-luminal catalytic site [28]. In this model, the substrate-binding site and the hydrolytic site are two parts of the same protein [28]. Because of the dynamic nature of the changes in Mg^{2+} content, and the influence of chelating agents such as EDTA, we have proposed that Mg^{2+} exerts its regulatory effect at the level of the G6P entry mechanism, which represent the limiting step for G6Pase hydrolysis rate, irrespective of the model considered [10,11].

Hydrolysis of G6P to glucose and Pi (inorganic phosphate) by the G6Pase is one of the two metabolic processes G6P undergoes within the endoplasmic reticulum. The other one is the

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oxidation of G6P to 6-phosphogluconolactone via H6PD, the reticular version of glucose 6 phosphate dehydrogenase [14] that catalyzes the two reactions leading to the oxidation of G6P to 6-phosphogluonolactone [15].

Consistent with our hypothesis that Mg^{2+} regulates the G6P entry rate, cells cultured in the presence of low extracellular Mg^{2+} show a marked increase in H6PD activity, measured as NADPH production, for a given G6P concentration. The presence of an inverse relationship between Mg^{2+} content and G6PD activity is further supported by the results in HepG2 cells cultured in 0.6 mmol/L $[Mg^{2+}]o$ showing that the addition of progressively increasing concentrations of exogenous Mg^{2+} in the millimolar range progressively down regulate NADPH production. This effect is Mg^{2+} specific as it is not reproduced by the addition of equimolar concentrations of monovalent or other divalent cations, as already reported for the hydrolysis of G6P by the G6Pase [10,11]. Arguably, some of the Mg^{2+} concentrations tested in our study are significantly higher than the oscillations in Mg^{2+} concentrations observed under a variety of physiological conditions. In this respect, variations in cytoplasmic Mg^{2+} concentrations between 0.4 and 1mmol/L (i.e. the concentrations used in our cell culture system) have been reported to occur in hepatocytes [1-3], and provide a more physiological correlation for our study. Irrespective of the Mg^{2+} concentration tested, the dynamic regulation exerted by Mg^{2+} show the specificity of its effect on G6P entry into the E.R. lumen as other cations are incapable to reduplicate it [10,11]. Furthermore, these concentrations emphasize that the dynamic Mg^{2+} regulation is independent of, and in addition to the cytoplasmic level present under the various culturing conditions (i.e. 0.4 vs. 0.6 vs. 1 mmol/L $[Mg^{2+}]o$). As this effect is observed only when exogenous Mg^{2+} is added at the beginning of the reaction and not when the reaction is already in full motion, it argues against the possibility that the inhibition is due to the equilibration of the cation across the E.R. membrane. Presently, it is not clear why Mg^{2+} inhibitory effect is observed only when the cation is added together or prior to G6P. The most likely explanation is that NADPH, which represents the read-out of the reaction in our system, can only be dissipated by other reactions utilizing it (e.g. fatty acid or cholesterol synthesis) that are not taking place in our digitonin permeabilized cells as the necessary enzymes and/or substrates are not available. In both HepG2 and HL-60 cells a decrease in cellular Mg^{2+} content increases H6PD expression. A qualitatively similar increase in protein expression is also observed in microsomes from rats exposed to Mg^{2+} deficient diet for 2 weeks. Presently, it is undefined whether this effect is the results of changes in Mg^{2+} content at the nuclear level (which we cannot technically assess) or an adaptive response following a persistent substrate availability (i.e. elevated G6P entry into the reticular lumen).

By comparing the information obtained on G6Pase with the results reported here, a picture emerges whereby cellular (cytoplasmic) Mg^{2+} operates as a key regulator of glucose utilization within the hepatocyte. Under conditions in which external and internal Mg^{2+} is plenty (e.g. fed state, adequate nutrition state, or functional circulating insulin levels) Mg^{2+} enters the cells and promotes optimal conversion of glucose to glucose 6-phosphate via glucokinase. This substrate can then be used for immediate energetic purposes (glycolysis) or stored as glycogen upon proper metabolic conversion while its hydrolysis via G6Pase and oxidation by H6PD within the endoplasmic reticulum are limited by the regulatory effect of Mg^{2+} on G6P entry rate into the organelle. Conversely, under conditions in which external

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or internal Mg^{2+} is low (e.g. fasted state, nutritional defect, hypomagnesaemia, or diabetes), utilization or storage of external glucose is limited, and the hepatocyte put in place alternative NADPH-supported metabolic processes (i.e. pentose shunt, fatty acid synthesis, or cholesterol synthesis) to guarantee glucose output and energy production. Additional studies are needed to investigate in detail these aspects of liver metabolism and to validate the extent to which these processes occur in other tissues (e.g. adipose tissue), which share with the liver similar metabolic processes (H6PD expression and fatty acid synthesis, [26]). The validation of the main Mg^{2+} effects in HL-60 cells as representative of neutrophils, another cell type in which the presence of the H6PD machinery has been observed [21,24], suggests that the regulatory role of Mg^{2+} occurs in other cell types possessing the same basic biochemical machinery besides the hepatocyte. In the case of HL-60 (and neutrophils), the observed increase in H6PD expression and activity and the associated production of NADPH could be linked to the maintenance of luminal NADPH necessary for neutrophils' survival, oxidative burst, or to other immune-related responses specific for these cells [24]. Additional studies are required to further validate this hypothesis.

In the case of the hepatocytes, for which more information is available, our results are consistent with several published observations, and are suggestive of major changes in hepatic and perhaps whole body metabolism. Animals exposed to magnesium deficient diet for 2 weeks or more show increased levels of circulating triglycerides and low density lipoproteins (LDL) [29]. Similar abnormal levels of circulating triglycerides and LDL are observed in severe cases of type-1 and type 2 diabetes [30,31], conditions that are also associated with hypomagnesaemia and/or decreased hepatic Mg^{2+} content [30,31]. Noteworthy, experimental and clinical evidence associated increased G6PD expression and activity with insulin resistance and obesity [32], two conditions often linked to non-alcoholic fatty liver disease (NAFLD), diabetes, and metabolic syndrome [33,34]. Reduced glucose utilization is also observed following exposure of hepatocytes to ethanol [35], the administration of which results in Mg^{2+} loss from liver cells in a dose-dependent manner [36,37]. In all these conditions, the ultimate challenge remains to discern to which extent the observed dysmetabolisms depend on the occurring pathology or on the concomitant Mg^{2+} loss.

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- Total and compartmentalized cellular Mg^{2+} decreases in cell exposed to low $[Mg^{2+}]o$
- Decreased cytosolic Mg^{2+} favors G6P entry into the E.R. and its oxidation by H6PD
- This oxidation increases intra-reticular NADPH content for other metabolic pathways.
- H6PD gene and protein expression increase in cells grown in low $[Mg^{2+}]o$ conditions
- Cytoplasmic Mg^{2+} regulates G6P utilization by reticular H6PD.

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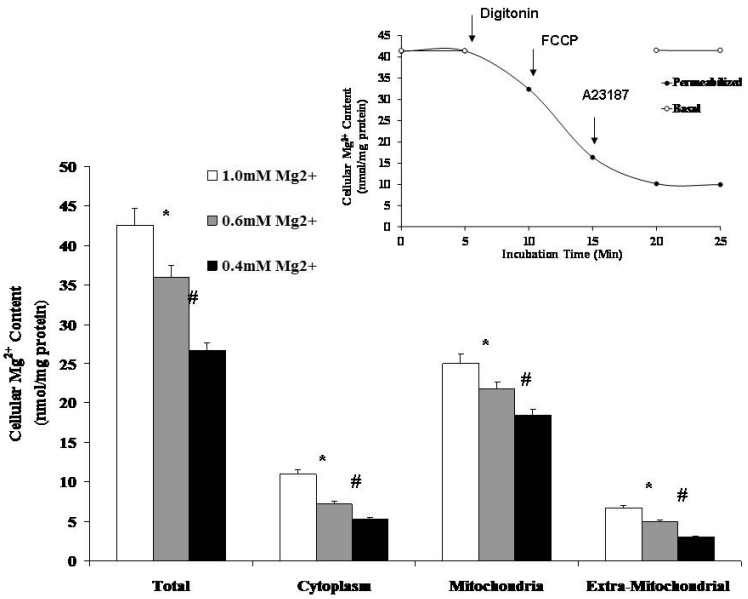

Figure 1. Total cellular Mg^{2+} content and intracellular Mg^{2+} distribution in HepG2 cells grown in the presence of different extracellular Mg^{2+} concentrations ($[Mg^{2+}]o$)

HepG2 cells maintained in culture in the presence of 1 (physiological), 0.6, or 0.4 mmol/L (Mg^{2+}-deficient) extracellular Mg^{2+} were assessed for total cellular Mg^{2+} content and intracellular Mg^{2+} compartmentalization as described under Materials and Methods. The data are means ± S.E. of 4 different cell preparations, each assessed in duplicate. *Statistical significant ($p<0.05$) versus the corresponding value in HepG2 cells grown in the presence of 1 mmol/L. #Statistical significant ($p<0.02$) versus the corresponding value in HepG2 cells grown in the presence of 1 mmol/L. Figure 1 onset reports the time points at which Mg^{2+} content was assessed and the various agents added to the cells. A typical experiment is reported.

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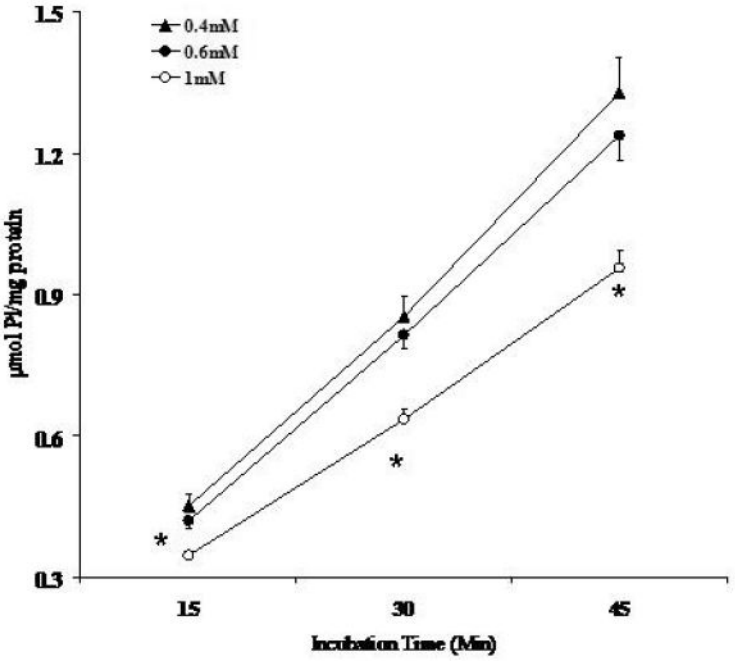

Figure 2. Hexose 6-phosphate hydrolysis in HepG2 cells

HepG2 cells were maintained in culture in the presence of 0.4, 0.6, or 1 mmol/L $[Mg^{2+}]o$. At confluence, cells were harvested, and permeabilized by digitonin addition (50µg/ml). The cells were then washed and incubated in a cytosol-like medium in the presence of 1 mmol/L G6P, at 37°C. Hydrolysis of G6P by glucose 6-phosphatase was measured as the amount of Pi released into the incubation medium at specific time points. Data are means ± S.E. of 4 different cell preparations, each carried out in duplicate, for all the experimental conditions reported. *Statistical significant versus the corresponding values in HepG2 cells maintained in the presence of 0.6 or 0.4 mmol/L $[Mg^{2+}]o$.

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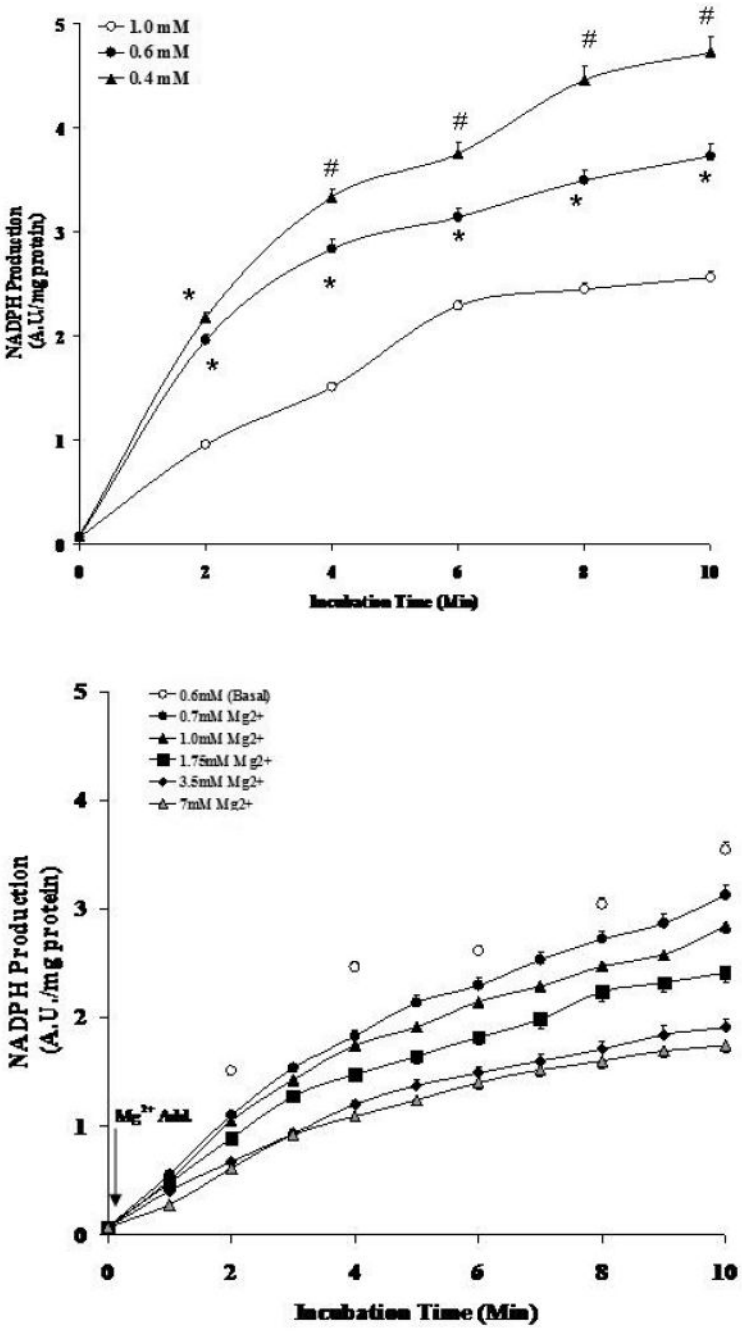

Figure 3. Hexose 6-phosphate dehydrogenase activity in HepG2 cells

HepG2 cells were maintained in culture in the presence of 0.4, 0.6, or 1 mmol/L $[Mg^{2+}]o$. At confluence, cells were harvested, and permeabilized by digitonin addition (50μg/ml). The cells were then washed and incubated in a cytosol-like medium in the presence of 1mmol/L G6P, at 37°C. H6PD-coupled NADPH production was measured fluorimetrically as described under Materials and Methods (Fig. 3A). NADPH calibration was carried out as described in the Methods section. The inhibitory effect of increasing concentrations of exogenous Mg^{2+} added prior to G6P addition to HepG2 cells grown in the presence of 0.6

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mmol/L $[Mg^{2+}]o$ is reported in Fig.3B. Data are means ± S.E. of 3 different cell preparations for each of the experimental conditions reported. Figure 3A: *Statistical significant versus the corresponding value in HepG2 cells grown in 1 mmol/L $[Mg^{2+}]o$. #Statistical significant versus the corresponding value in HepG2 cells grown in 0.6 or 1 mmol/L $[Mg^{2+}]o$. Figure 3B: all the data points for the progressively increasing Mg^{2+} concentrations are statistical significant (ANOVA) vs. the corresponding values for basal 0.6 mmol/L Mg^{2+}. Labeling is omitted for simplicity

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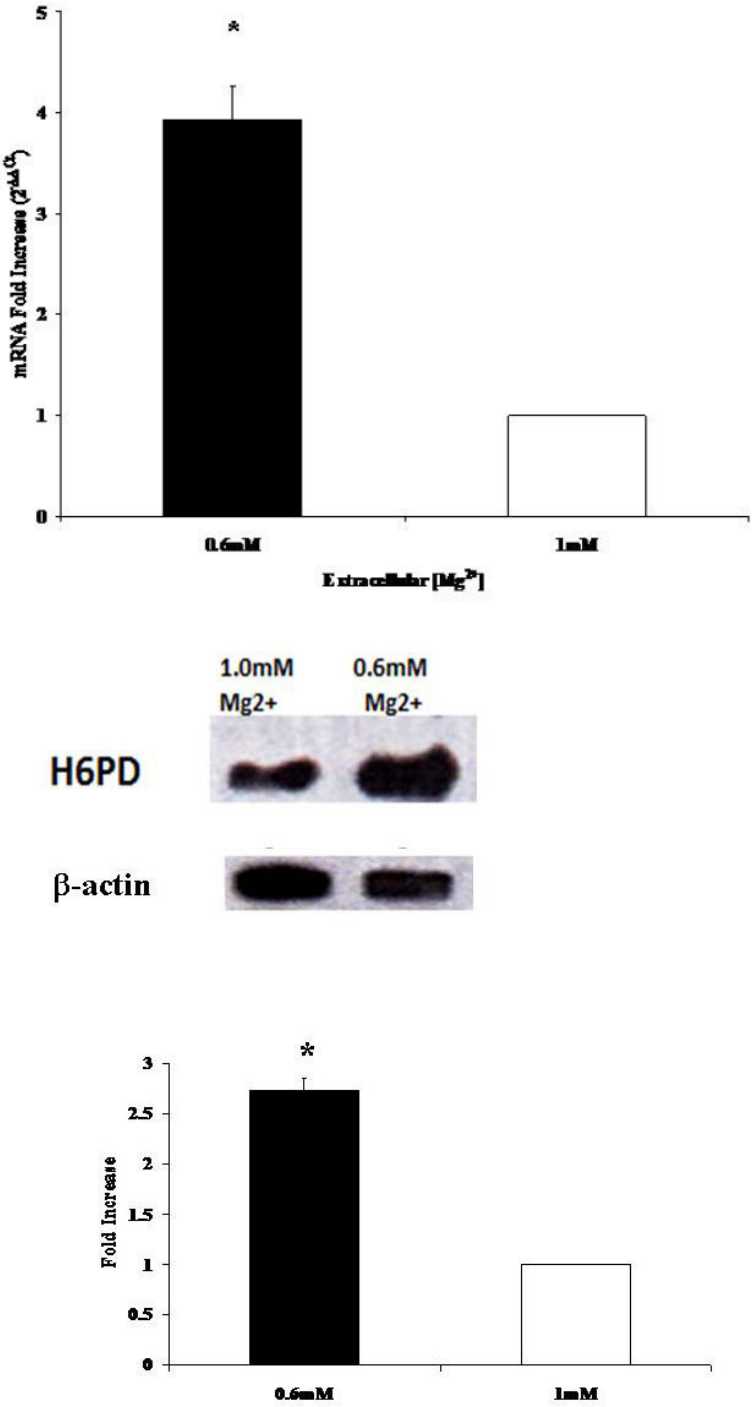

Figure 4. Hexose 6-phosphate dehydrogenase expression in HepG2 cells

HepG2 cells were maintained in culture in the presence of 0.4, 0.6, or 1 mmol/L $[Mg^{2+}]o$. At confluence, cells were harvested, and assessed for gene expression (Fig. 4A) and protein expression (Fig. 4B). For gene expression, mRNA was extracted. qRT-PCR was carried out using two different sets of primers for G6PD (H6PD, Table 1). The results, normalized for β-actin and GAPDH expression, are reported in Fig. 4A as $2^{-\Delta\Delta CT}$. Data are means + S.E. of

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3 different cell preparations for each experimental condition, each carried out in duplicate. *Statistical significant versus the corresponding value in HepG2 cells grown in the presence of 1 mmol/L $[Mg^{2+}]o$. For protein expression, cells were lysed in the presence of protease inhibitors cocktail (Sigma). Following gel separation and transfer, Western Blot analysis was carried out with commercially available antibodies (Cell Signaling, Boston, MA). Band intensity was normalized to β-actin protein expression. Data are means ± S.E. of 4 different cell preparations for each experimental condition, each carried out in duplicate. *Statistical significant versus the corresponding value in HepG2 cells grown in the presence of 1 mmol/L $[Mg^{2+}]o$.

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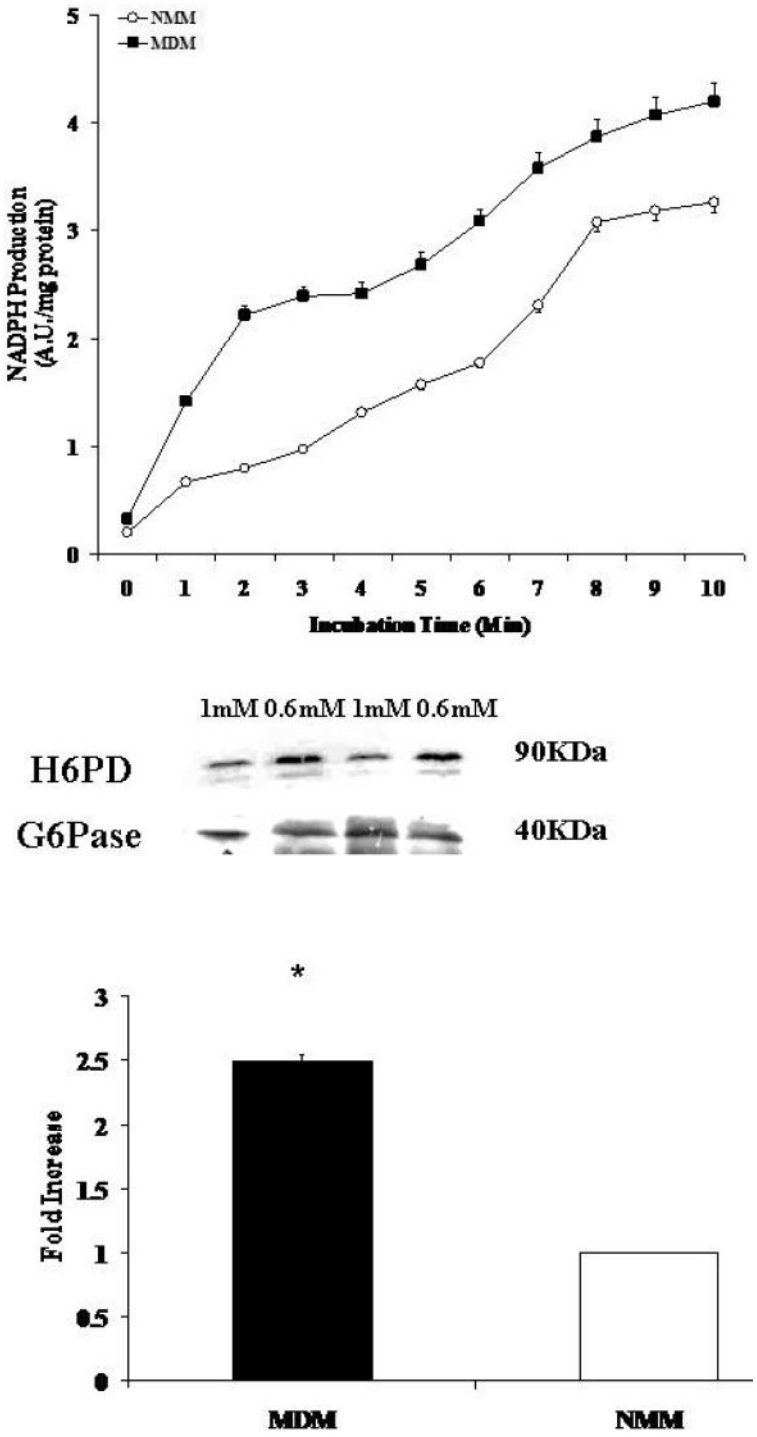

Figure 5. Hexose 6-phosphate dehydrogenase activity in microsomal vesicles

Microsomes from livers of animals maintained on normal or 35% deficient Mg^{2+} diet were isolated as reported under Material and Methods and elsewhere [11]. H6PD-coupled NADPH production was measured fluorimetrically as reported under Materials and Methods, in the presence of 1 mmol/L G6P, at 37°C. Data are means ± S.E. of 4 different

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microsomal preparations for each experimental group, each assessed in duplicate. All the data points for Mg^{2+}-deficient microsomes (MDM) are statistical significant versus the corresponding values for normal Mg^{2+} microsomes (NMM). Labeling is omitted for simplicity. For protein expression, aliquots of MDM and NMM microsomes were lysed in the presence of protease inhibitors cocktail (Sigma). Following gel separation and transfer, Western Blot analysis was carried out with commercially available antibodies (Cell Signaling, Boston, MA). Band intensity was normalized as described under Material and Methods. Band intensity of the G6Pase catalytic subunit is reported as a loading control for comparison. Data are means ± S.E. of 4 different cell preparations for each experimental condition, each carried out in duplicate. *Statistical significant versus the corresponding value in HepG2 cells grown in the presence of 1 mmol/L $[Mg^{2+}]o$.

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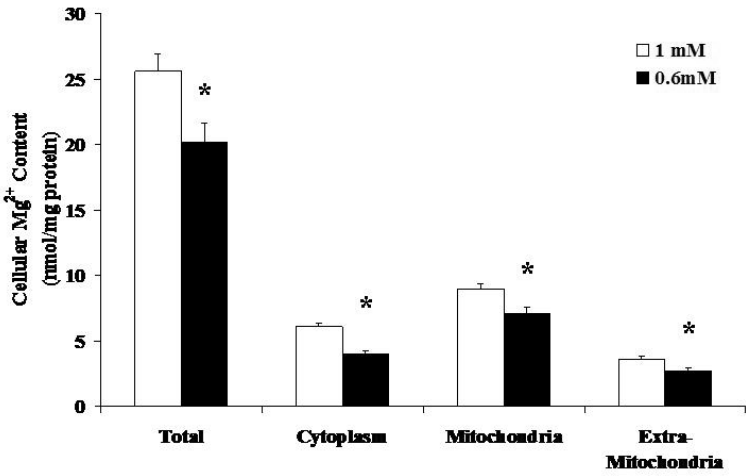

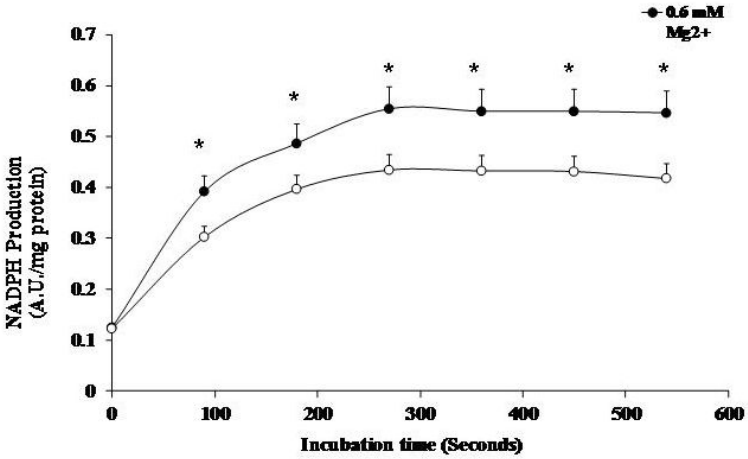

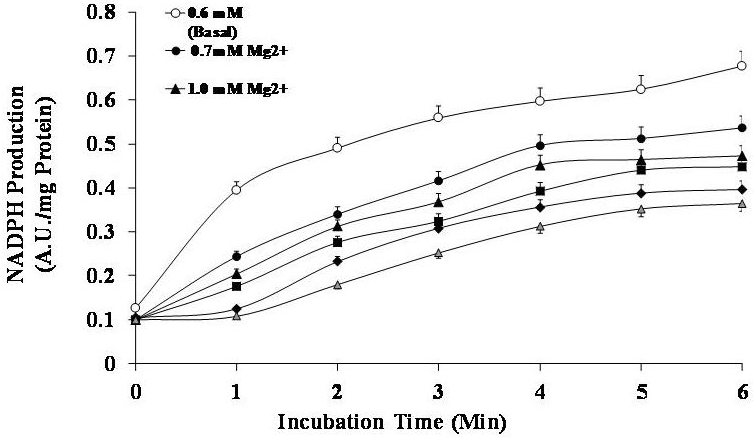

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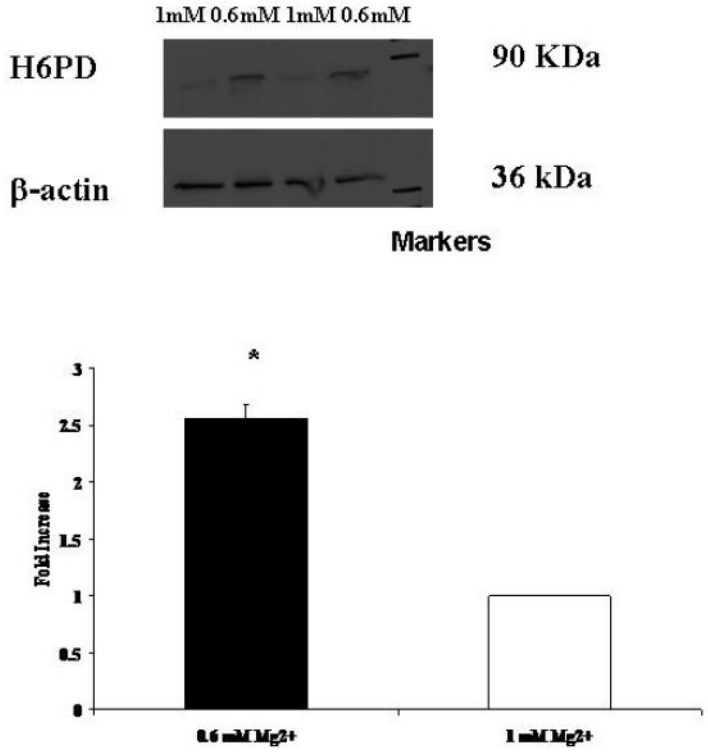

Figure 6. Hexose 6-phosphate dehydrogenase activity and expression in HL-60 cells

HL-60 cells were maintained in culture in the presence of 0.6, or 1 mmol/L $[Mg^{2+}]o$ for about 1 week. At 85% confluence, cells were harvested and assessed for total and compartmentalized Mg^{2+} content (Fig. 6A) as indicated under Materials and Methods. Digitonin-permeabilized cells (50 μg digitonin/ml) were washed twice and incubated in a cytosol-like medium in the presence of 1 mmol/L G6P, at 37°C. H6PD-coupled NADPH production was measured fluorimetrically as described under Materials and Methods (Fig. 6B). The inhibitory effect of increasing concentrations of exogenous Mg^{2+} on NADPH-production in HL-60 cells grown in the presence of 0.6 mmol/L $[Mg^{2+}]o$ is reported in Fig. 6C. H6PD expression in HL-60 cells grown in 0.6 or 1 mmol/L $[Mg^{2+}]o$ is reported in Fig. 6D. Western Blot analysis was carried out and normalized to β-actin as previously indicated for HepG2 cells (see legend to Figure 4). For each panel of Fig. 6, data are means ± S.E. of 4 different cell preparations for each experimental condition, each carried out in duplicate. Figure 6A: *Statistical significant versus the corresponding value in HL-60 cells grown in 1 mmol/L $[Mg^{2+}]o$. Figure 6B: *Statistical significant versus the corresponding value in HL-60 cells grown in 1 mmol/L $[Mg^{2+}]o$. Figure 6C: all the data points for the progressively increasing Mg^{2+} concentrations are statistical significant (ANOVA) vs. the corresponding values for basal 0.6 mmol/L Mg^{2+}. Labeling is omitted for simplicity. Figure 6D: *Statistical significant versus the corresponding value in HL-60 cells grown in the presence of 1 mmol/L $[Mg^{2+}]o$.

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Table 1

Primers used to assess G6PD expression by qRT-PCR

Protein		Primer sequence	Ref.
H6PD #1	forward primer	5′ ATGAAAGAGACCGTGGATGCTGAA 3′	22
	reverse primer	5′ CTCCATGGCCACGAGGGTGAG 3′	
H6PD #2	forward primer	5′ CAACTGGGGACCTGGCTAAGAAGT 3′	23
	Reverse primer	5′ GTTGATGAGAGGCAGGCTAAGGCT 3′	
β-actin	forward primer	5′ AGAAAATCTGGCACCACACC 3′	36
	reverse primer	5′ AGAGGCGTACAGGGATAGCA 3′	
GAPDH	forward primer	5′ ACCATCTTCCAGGAGCGAGATC 3′	22
	reverse primer	5′ GAGCCCCAGCCTTCTCCATGGT 3′	

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Table 2

Inhibitory effect of exogenous Mg^{2+} addition on NADPH production

	t = 0 Min	t = 2 min	t= 5 min[#]	t=10 min
1 mM HepG2 Cells				
No Mg^{2+}	0.126±0.014	0.874±0.061	1.560±0.187	2.666±0.098
1mM Mg^{2+} together with G6P	0.131±0.021	0.582±0.043[*]	0.954±0.105[*]	1.753±0.151[*]
1mM Mg^{2+} 5 min after G6P	0.130±0.009	1.034±0.077	1.579±0.081	2.769±0.112
0.6 mM HepG2 Cells				
No Mg^{2+}	0.189±0.028	1.783±0.080	2.912±0.106	3.569±0.073
1 mM Mg^{2+} together with G6P	0.179±0.036	1.017±0.112[*]	2.361±0.081[*]	2.871±0.113[*]
1mM Mg^{2+} 5 min after G6P	0.195±0.026	1.791±0.113	2.871±0.076	3.803±0.088
0.4 mM HepG2 Cells				
No Mg^{2+}	0.145±0.017	2.055±0.056	3.177±0.064	4.524±0.104
1 mM Mg^{2+} together with G6P	0.151±0.019	1.369±0.097[*]	2.835±0.076[*]	3.728±0.102[*]
1mM Mg^{2+} 5 min[#] after G6P	0.158±0.029	2.067±0.088	3.262±0.091	4.482±0.083

Data are expressed as A.U. NADPH production/mg protein, and are mean ± S.E. of 3 different cell preparations.

[*]Statistical significant vs. the No Mg^{2+} and the 1 mmol/L Mg^{2+} added 5 min after G6P

[#]Time of Mg^{2+} addition.

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Table 3

Body and liver weight, protein and Mg^{2+} content in total liver homogenate and purified liver microsomes from rats maintained for 2 weeks on normal Mg^{2+} or Mg^{2+} deficient diet.

	Normal Mg^{2+} diet	**Mg^{2+}-deficient diet**
Body weight (g)	287.1 ± 4.2	284.7 ± 5.4
Liver weight (g)	12.1 ± 1.2	11.5 ± 0.9
Hepatic protein (mg/g liver)	97.4 ± 3.2	95.9 ± 4.3
Hepatic Mg^{2+} (nmol/mg prot)	80.6 ± 4.6	62.3 ± 3.5*
Microsomes yield (mg prot/g Eq L)	15.4 ± 0.4	14.6 ± 0.3
Microsomal RNA[a] (mg/g Eq L)	280.3 ± 1.9	281.5 ± 2.2
Microsomal Mg^{2+} (nmol/mg prot)	1.98 ± 0.03	1.43 ± 0.02*

Data are means ± SE of 8 different preparations for each experimental group.

*Statistically significant vs. normal Mg^{2+} diet ($p < 0.05$).

[a]RNA was assessed according to Ragnotti [37]