Magnesium Deficiency and Glucose Metabolism in Rat Adipocytes

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We examined the effect of reducing ambient and intracellular free Mg ion ([Mg]i) concentrations on insulin action in epididymal adipocytes from male Sprague-Dawley rats in terms of (1) cellular transport of nonmetabolizable 2-deoxyglucose, (2) [U-^{14}C]glucose oxidation to CO_2, and (3) D-[^{3}H]glucose incorporation into triglycerides. There were no significant differences in basal or insulin-stimulated transport of 2-deoxyglucose between adipocytes cultured in physiologic (1.24 mmol) or low (0.16 mmol) Mg for up to 24 hours. In contrast, insulin-stimulated but not basal [U-^{14}C]glucose oxidation to CO_2 was significantly reduced in adipocytes cultured in low versus physiologic Mg ($P < .05$ to .01). Similarly, there were no differences in basal glucose incorporation into triglycerides between cells cultured in low or physiologic Mg media for up to 24 hours. However, long-term (24-hour) but not short-term (2-hour) exposure of cells to low Mg was associated with a significant 30% reduction in insulin-stimulated D-[^{3}H]glucose incorporation into triglycerides. When adipocytes incubated in low Mg were reincubated in high Mg (1.24 or 5 mmol) for 30 minutes, normal insulin-stimulated D-[^{3}H]glucose incorporation into triglycerides was restored. Incubation of adipocytes in low Mg (0.16 mmol) for 24 hours resulted in a significant decrease in [Mg]i (264 ± 89 *v* 437 ± 125 μmol/cell [mean ± SEM]) as compared with cells incubated in physiologic Mg (1.24 mmol; $P < .01$). These data support a role for intracellular Mg deficiency in the development of insulin resistance and suggest that the effect occurs at a site(s) distal to glucose entry into the cell. The effect of Mg deficiency on insulin action appears to be reversible.

MAGNESIUM deficiency is a common problem in insulin-dependent and non–insulin-dependent diabetes mellitus.[1-3] Reduced intracellular Mg content in states of insulin resistance[4,5] has been attributed to a reduction in insulin-stimulated entry of Mg ions ([Mg]i) into the cell.[6-12] Conversely, hypomagnesemia has been implicated in the pathogenesis of ketoacidosis-associated insulin resistance.[13] Thus, hypomagnesemia and low intracellular magnesium can be both a consequence[1-3,6,14,15] and a cause[7,16] of insulin resistance. Further, in addition to the development of poor glycemic control, Mg deficiency is an important predisposing factor for the development of vascular changes implicated in diabetes, including increased vasomotor tone and platelet reactivity.[17] Furthermore, the deficiency of [Mg]i was proposed as the link between insulin resistance and hypertension.[18]

One of the major metabolic actions of insulin is to stimulate glucose transport into adipocytes and muscle cells.[19] Adipocytes from the epididymal fat pads of male Sprague-Dawley rats have been repeatedly shown to be a suitable in vitro model for studying insulin action.[19-22] We examined the effect of reducing ambient Mg and [Mg]i on the corresponding changes in insulin action in adipocytes, as reflected by changes in cellular transport of nonmetabolizable glucose (2-deoxyglucose), glucose ([U-^{14}C]glucose) oxidation to CO_2, and glucose incorporation into triglycerides.

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MATERIALS AND METHODS

Chemicals

Crystalline biosynthetic human insulin (lot #615-707-208) was obtained from Eli Lilly & Co (Indianapolis, IN), collagenase (lot # MOP123j-4197) from Worthington Biochemical (Freehold, NJ), Fraction V bovine serum albumin (BSA) from Boehringer Mannheim Biochemicals (Indianapolis, IN), ethanolamine from Sigma Biochemical, and silicon oil from Union Carbide (New York, NY).

Adipocytes

Male Sprague-Dawley rats (180 to 220 g) were used in the studies, and were fed lab chow ad libitum. All studies were approved by our animal safety review committee. Animals were killed with phenobarbital anesthesia (5 mg/100 g body weight intraperitoneally), and the epididymal fat pads were removed. Isolated fat cells were prepared by the method of Rodbell[23] as modified by Ciaraldi et al,[24] using HEPES/salt buffer (pH 7.4) consisting of 150 mmol NaCl, 5 mmol KCl, 1.2 mmol $MgSO_4$, 1.2 mmol $CaCl_2$, 2.5 mmol NaH_2PO_4, 3 mmol glucose, 10 mmol HEPES, collagenase (2 mg/mL) and 4% BSA. After digestion and filtration sequentially on 400- and 200-μm Nylon mesh, the cells were washed four times in a collagenase-free buffer.

Cell Cultures

Cells were resuspended in Dulbecco's modified Eagle's medium containing 2% fetal calf serum, 1% BSA, and physiologic (1.24 mmol) or low (0.16 mmol) Mg, and incubated at 37°C in a 5% CO_2 incubator for periods specified later. Cells were then washed in Krebs-Ringer bicarbonate buffer containing the respective Mg concentrations (1.24 or 0.16 mmol) and resuspended at a concentration of 2×10^5 cells/mL. Cell counts were performed by a modification of method III reported by Hirsch and Gallian,[25] in which cells were fixed in 2% osmium tetraoxide and counted in a model ZBI Coulter (Hialeah, FL) counter with a 100-μm aperture tube.

2-Deoxyglucose Transport Into Isolated Adipocytes

Adipocyte glucose transport was assessed by measuring baseline and insulin-stimulated uptake of 2-deoxyglucose, which is a nonmetabolizable analog of D-glucose. The method has previously been described by Ciaraldi and Olefsky[26] and Robertson et al.[27] At the conclusion of culturing periods in media containing physiologic or

low Mg, cells were washed twice in glucose-free HEPES/salt buffer containing 1% BSA and the respective concentration of Mg (1.24 or 0.16 mmol) and incubated in an air atmosphere with constant rotary shaking at 37°C for 60 minutes, in the absence and in the presence of insulin (final concentration, 0 to 25 ng/mL). The transport reaction was started by addition of 10 μL mixed unlabeled and tritium-labeled 2-deoxy-D-glucose (0.1 mmol 2-deoxy-D-glucose and 0.2 μCi [1,2^{3}H]-2-deoxy-D-glucose) or mixed unlabeled and tritium-labeled L-glucose (0.1 mmol L-glucose and 0.2 μCi [1-^{3}H]-L-glucose). The transport reaction was terminated by transferring 200 μL of the reaction mixture to microfuge tubes containing 150 μL silicone oil and centrifuging for 30 seconds. The microfuge tubes were then cut at the oil layer, and the upper portion containing the cell pellet was transferred to a miniscintillation vial with 4 mL liquid scintillation cocktail. Total counts were obtained by placing 100 μL pooled reaction mixture directly into the scintillation vials. Vials were shaken and counted in a beta-counter after dark adaptation. Radioactivity in vials containing L- or D-glucose–treated cells was expressed as percent of the respective total counts. Nonspecific radioactivity trapping was adjusted for by subtracting percent uptake of ^{3}H-L-glucose blank counts from percent uptake of 2-[1,2-^{3}H]-deoxy-D-glucose. Counts for each data point were normalized to 2×10^5 cells.

Glucose Oxidation in Isolated Adipocytes

Glucose oxidation by adipocytes was measured by the method of Rodbell[23] as adapted by Olefsky[28] and Robertson et al.[27] Cells were diluted to 2×10^5/mL in buffer gassed with 95% O_2/5% CO_2 for 15 minutes and containing 1% BSA (pH 7.4). Nine hundred fifty microliters of cell suspension were mixed with 50 μL insulin (final concentration, 0 to 50 ng/mL) and 0.2 μCi [U-^{14}C]-D-glucose (premixed with D-glucose) in polypropylene tubes. Final glucose concentration was 2 mmol to allow for glucose oxidation to CO_2 to be the rate-limiting factor in the reaction. The tubes were capped with stoppers (Kontes # 882310-0000, Vineland, NJ) attached to center wells (Kontes # 882320-0000); each well contained a 1.5 × 4.5-cm strip of Whatman # 1 filter paper (Hillsboro, OR). The tubes were placed in a rotary shaking water bath adjusted to 37°C and incubated for 60 minutes. Using a syringe, 0.2 mL ethanolamine was added to each center well to be absorbed by the filter paper, followed by addition of 0.2 mL 8N H_2SO_4 to the cell suspension (without touching the center well), and tubes were incubated with gentle shaking for an additional 60 minutes. The stoppers were removed, and the wells were cut and placed in scintillation vials containing 10 mL liquid scintillation cocktail. Blank counts were obtained in reactions performed with no added cells. Total counts were obtained by counting 10 μL labeled substrate. Percent glucose oxidation to CO_2 was derived from the ratio of counts obtained from cell-containing tubes to total counts added.

Glucose Incorporation Into Triglycerides in Isolated Adipocytes

Freshly prepared adipocytes cultured in media containing physiologic (1.24 mmol) or low (0.40 or 0.16 mmol) magnesium concentrations at 37°C for 2 or 24 hours were used for determination of insulin-stimulated glucose incorporation into triglycerides, as previously described by our laboratories.[29] Briefly, 400 μL adipocyte suspension in Krebs-Ringer-HEPES buffer containing 0.27 mmol glucose was mixed in triplicate with insulin (0.05 to 100 ng/mL) and 0.2 μCi [3-^{3}H]-D-glucose in a total volume of 0.5 mL. Tubes were incubated for 2 hours at 37°C under gentle shaking. The incubation was interrupted by adding 5 mL/toluene-based scintillation mixture per tube, followed by a rest of at least 1 hour to allow extraction of lipids into the toluene phase before counting.

Measurement of [Mg]i

[Mg]i was measured using a specific fluorescent probe (MAG-Fura2-AM; Molecular Probes, Eugene, OR) as described by Grynkiewicz et al[30] and modified by us.[31] HEPES buffer–washed adipocytes were incubated with 1 mmol fluorescent probe at 37°C in the presence of 5.5 mmol glucose for 30 minutes, followed by centrifugation at 50 × *g* for 10 minutes to remove excess dye. [Mg]i was then measured at excitation wavelength 335 to 370 nm and emission wavelength 510 nm on a Hitachi F-2000 fluorescence spectrophotometer (Hitachi Instruments, Tokyo, Japan). Intracellular ion concentration was calculated using the formula, $[ion]i = K_d \times [R - R_{min}/R_{max} - R] \times [S_{f2}/S_{b2}]$, where R is the ratio of intensities at the two wavelengths, R_{max} is the value when cells are lysed with Triton X-100 (0.1%), R_{min} is the ratio after addition of 75 mmol EDTA, and S_{f2} and S_{b2} are values for the second wavelength at minimum and maximum intensities. The concentration of magnesium used for R_{max} was 40 mmol and for R_{min} 0 mmol. All magnesium was chelated with EDTA. The K_d used in the equation was 1,500 μmol.

Statistical Analysis

Each experimental protocol was repeated six to nine times, unless stated otherwise. Collected data were analyzed with two-way ANOVA with replication, with treatment (low and normal Mg) and insulin (dose-response curve) as the two cross-classifying factors. When ANOVA was statistically significant, *t* test for paired samples was used to assess the significance of differences between low and physiologic Mg-treated cell responsiveness to insulin.

RESULTS

Effect of Mg Deficiency on Glucose Transport Into Isolated Adipocytes

Isolated adipocytes were cultured in media containing low (0.16 mmol) or physiologic (1.2 mmol) Mg concentrations for either 4 or 24 hours before determination of basal and insulin-stimulated cellular glucose uptake. There were no significant differences in basal or insulin-stimulated uptake of 2-deoxyglucose between cells cultured in media containing a low or physiologic Mg concentration for either 4 or 24 hours. However, basal glucose uptake was significantly higher in cells cultured for 24 hours than in cells cultured for 4 hours regardless of the ambient Mg concentration ($P < .001$ for cells cultured at physiologic Mg and $P < .02$ for cells cultured at low Mg). Insulin-stimulated glucose uptake after 4 and 24 hours was similar in cells cultured in low and physiologic Mg. Figure 1 depicts the mean results obtained in nine 4-hour (Fig 1A) and six 24-hour (Fig 1B) culturing experiments.

Effect of Mg Deficiency on Glucose Oxidation to CO_2 in Isolated Adipocytes

In contrast to the lack of changes in glucose uptake described above, insulin-stimulated glucose oxidation to CO_2 was significantly reduced in adipocytes cultured for 4 hours in low Mg versus cells cultured in physiologic Mg, with a more pronounced decrease in glucose oxidation at higher insulin concentrations (Fig 2A). Although mean basal glucose oxidation tended to be slightly lower in cells

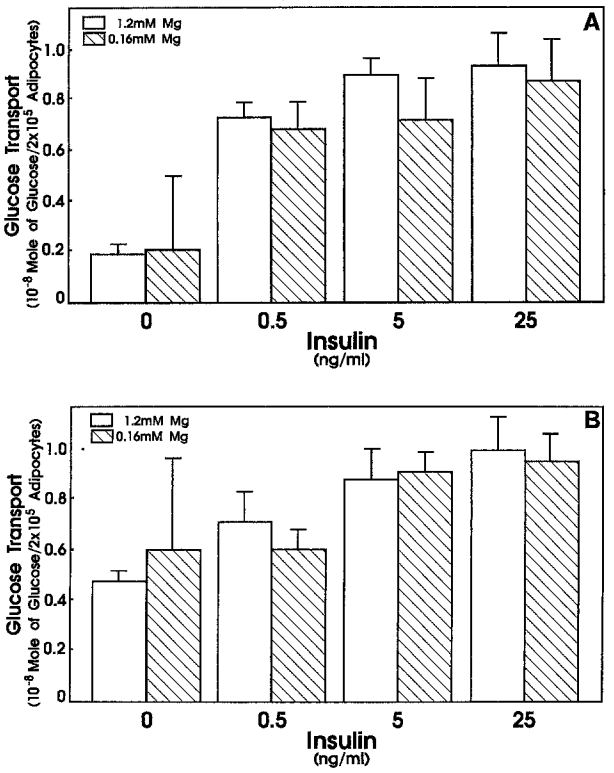

Fig 1. Glucose transport in isolated adipocytes cultured in media containing low (0.16 mmol) or physiologic (1.2 mmol) Mg concentrations for either 4 hours (A, n = 9) or 24 hours (B, n = 6) before determination of basal and insulin-stimulated glucose uptake.

cultured in low-Mg media, the differences were not statistically significant. Furthermore, insulin-stimulated glucose oxidation adjusted for basal glucose oxidation (by subtraction) remained significantly higher for cells cultured in physiologic Mg versus low Mg (Fig 2B).

Effect of Mg Deficiency on Glucose Incorporation Into Triglycerides in Isolated Adipocytes

Short-term exposure (2 hours) of adipocytes to low extracellular Mg did not influence basal or insulin-stimulated glucose incorporation into triglycerides (Fig 3A). In contrast, long-term exposure (24 hours) to low Mg was associated with a significant reduction in insulin-stimulated glucose uptake and incorporation into triglycerides over the entire range of the insulin-response curve (Fig 3B). Basal glucose incorporation into triglycerides was not influenced by low ambient Mg concentration. In another experimental protocol, adipocytes incubated in low Mg were divided into two portions: one portion continued to be incubated at the same low ambient Mg concentration, while the other portion was incubated in a physiologic-Mg media (1.24 mmol) for 30 minutes. Incubation of Mg-depleted adipocytes in media containing physiologic Mg resulted in restoration of insulin-stimulated glucose incorporation into triglycerides over the entire range of insulin concentrations used (Fig 3C). Similar data on restoration of insulin-stimulated glucose incorporation into triglycerides were obtained when Mg-depleted adipocytes were reincubated in high-Mg (5 mmol) media for 30 minutes (data not shown).

[Mg]i

Incubation of adipocytes in media containing low (0.16 mmol) Mg for 24 hours was associated with a significant decrease in [Mg]i (264 ± 89 μmol, mean ± SEM) as compared with culture in media containing physiologic (1.24 mmol) Mg concentrations (437 ± 125, $P < .01$). [Mg]i values in adipocytes cultured for only 4 hours in media containing low ambient Mg were, on average, lower than but not statistically different from [Mg]i values in cells cultured in physiologic-Mg media.

DISCUSSION

Diabetes mellitus is a complex metabolic disorder with many long-term complications, including retinopathy, neuropathy, nephropathy, and increased risks for hypertension, coronary artery disease, and peripheral vascular disease.[32,33] Insulin deficiency and insulin resistance could coexist, albeit at varying degrees, in both insulin-dependent and non–insulin-dependent diabetes. Mg deficiency has increasingly been recognized as a common problem in both types of diabetes, and its presence is inversely related to the level of glycemic control and to the development of complications, including hypertension.[1,18,34] It has also been dem-

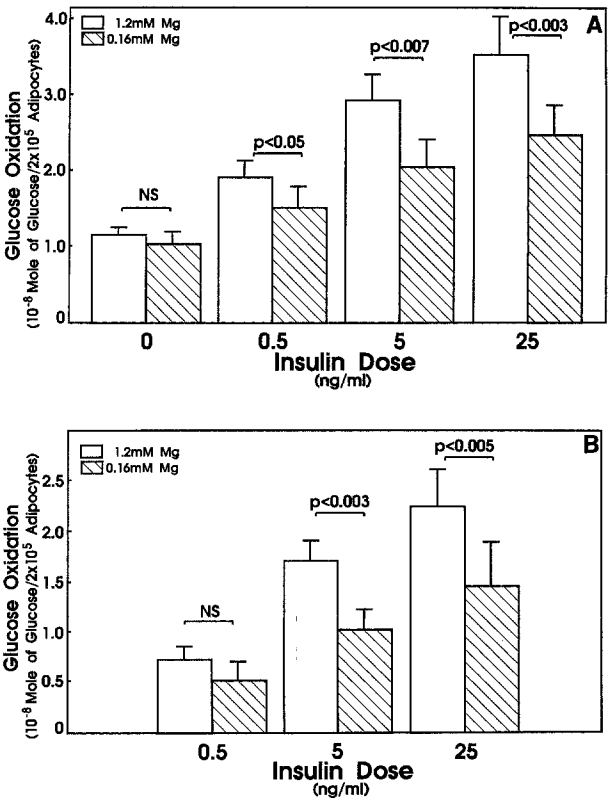

Fig 2. (A) Insulin-stimulated glucose oxidation to CO_2 in adipocytes cultured in low (0.16 mmol) versus physiologic (1.2 mmol) Mg concentration (n = 9). (B) Insulin-stimulated glucose oxidation adjusted for basal glucose oxidation (by subtraction; n = 6).

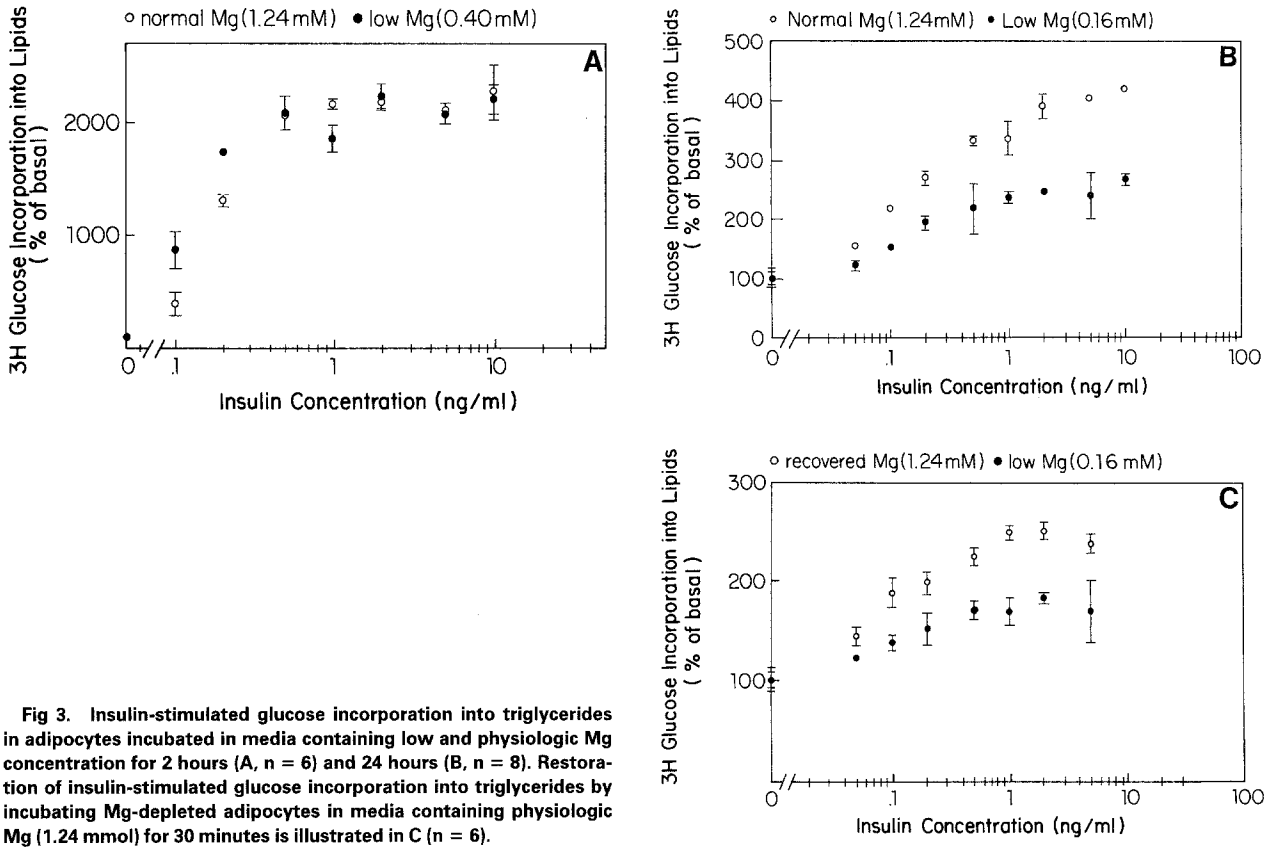

Fig 3. Insulin-stimulated glucose incorporation into triglycerides in adipocytes incubated in media containing low and physiologic Mg concentration for 2 hours (A, n = 6) and 24 hours (B, n = 8). Restoration of insulin-stimulated glucose incorporation into triglycerides by incubating Mg-depleted adipocytes in media containing physiologic Mg (1.24 mmol) for 30 minutes is illustrated in C (n = 6).

onstrated that Mg deficiency in normal human volunteers leads to insulin resistance.[35,36] Conversely, Mg supplementation improves glucose disposal in patients with diabetes.[6,17,37] In addition, our group has recently shown that Mg supplementation can prevent fructose-feeding–induced insulin resistance in rats,[38] and increased dietary Mg intake can maintain insulin responsiveness and reduce the development of diabetes in male Zucker Diabetic Fatty rats.[39]

The data presented in this communication showing reduced glucose metabolism (glucose oxidation and incorporation into triglycerides) in cells subjected to low ambient Mg concentrations provide further support for the evidence implicating magnesium deficiency as a factor in the development of insulin resistance.[7,15,16] In addition, the data presented extend the previous observations of hypomagnesemia-induced insulin resistance to indicate that such an effect in adipocytes occurs at a site(s) in glucose metabolism distal to glucose entry into the cell. This is illustrated by a lack of difference in the uptake of 2-deoxyglucose between cells cultured in low-Mg or physiologic-Mg media. Further, the effects of low ambient Mg on glucose incorporation into triglycerides are not immediate and require a period of 4 to 24 hours to be appreciable. A more pronounced effect on insulin-stimulated glucose oxidation to CO_2 appeared to occur at or before 4 hours and extended to 24 hours. The more prolonged latency for the manifestation of a reduction in insulin-stimulated glucose incorporation into triglycerides versus a reduction in glucose oxidation to CO_2 may be due to the fact that fatty acid synthesis is an extramitochondrial process dependent on the availability of substrates from the mitochondrial citric acid cycle, whereas CO_2 generation is a direct product of mitochondrial glucose oxidation through the citric acid cycle.

Magnesium is the second most abundant intracellular cation and is involved in many of the enzyme systems regulating intracellular glucose metabolism.[40] Although the normal range for [Mg]i in adipocytes has not been well established, measurement of [Mg]i in other cell types using newer methods[40,41] has revealed a range that is close to the K_m of many important enzyme systems using adenozine triphosphate or phosphate transfer. Thus, a reduction in [Mg]i could result in reduced activities of these enzyme systems.[40,42] Target enzyme systems include hexokinase and/or pyruvate dehydrogenase. Hexokinase is an extramitochondrial enzyme that phosphorylates glucose upon its entry into the cell, whereas pyruvate dehydrogenase is an important mitochondrial enzyme that regulates the synthesis of acetyl coenzyme A and consequently its availability for the citric acid cycle and citrate generation. Some of the citrate is transported outside the mitochondria to provide a substrate for fatty acid synthesis. Both the 2-deoxyglucose uptake and lipogenesis assays at low substrate concentration measure glucose entry and phosphorylation. Since it is generally believed that glucose entry but not phosphorylation is the rate-limiting step in the 2-deoxyglucose uptake assay, the different effects of low ambient Mg concentration on glucose metabolism measured by this and the lipogenesis assays would suggest phosphorylation (the site of hexoki-

nase enzyme activity) as a likely target site for regulation by [Mg]i. Future measurement of adipocyte uptake of a nonphosphorylated analog, such as 3-*O*-methylglucose, and time-course glucose-uptake studies are planned to clarify this possibility. Other studies evaluating the effects of Mg deficiency on the activity of pyruvate dehydrogenase and other target enzyme systems are also needed.

Besides a decrease in [Mg]i, low ambient Mg concentrations may lead to a reduction in total cellular Mg content. However, a change in total intracellular Mg content, even if it occurs, is less important than the associated change in [Mg]i in influencing glucose metabolism, since protein-bound Mg would not be available for regulation of metabolic enzyme activities. Another possible effect of magnesium deficiency is a change in cellular calcium levels.[43-46] Increased [Ca]i has been implicated in insulin resistance.[47,48] It is therefore possible that magnesium deficiency–induced insulin resistance may have been caused, in part, by an increase in [Ca]i. Preliminary studies assessing [Ca]i under similar experimental protocols did not show significant changes in [Ca]i (data not shown). If these results are confirmed, changes in insulin action in adipocytes incubated in low ambient Mg concentrations would most likely be related to a reduction in [Mg]i and not to an increase in [Ca]i. Lastly, the possibility that the present results of Mg deficiency–induced insulin resistance reflect nonspecific effects, not related to changes in any particular enzyme system, could not be ruled out.

The effect of Mg deficiency on insulin action is reversible. The restoration of insulin-stimulated glucose incorporation into triglycerides when Mg-deprived adipocytes were incubated in physiologic-Mg or high-Mg media supports this possibility. Further, culture of adipocytes in a reduced Mg concentration is not toxic, and the time required for restoration of insulin action appears to be much shorter than the time needed for development of insulin resistance.

In summary, the data presented herein indicate that low ambient Mg concentrations can lead to insulin resistance in cultured male rat adipocytes. In addition, the data suggest that low magnesium–induced insulin resistance occurs at a site(s) in glucose metabolism distal to glucose entry into the cell. Further, the effect of low ambient Mg concentration on glucose incorporation into triglycerides is not immediate and requires a period of between 4 and 24 hours to be appreciable. In contrast, the effect of low Mg on glucose oxidation to CO_2 occurs at or before 4 hours and extends to 24 hours. The effect of low ambient Mg concentration on insulin-mediated intracellular glucose metabolism could be the result of decreased [Mg]i. Finally, the effect of Mg deficiency on insulin action is reversible. These studies provide further support for the role of Mg deficiency in reduced insulin-mediated glucose metabolism.

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