Change in Glucose Homeostasis in Rats by Long-Term Magnesium-Deficient Diet

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Summary It is widely known that hypomagnesemia is one of the symptoms observed in diabetic patients. This study was performed to assess the effect of chronic magnesium (Mg) deficiency on glucose metabolism in rats. Male Sprague-Dawley rats (at the age of four weeks) were given a Mg-deficient diet or a control diet for two to eight weeks. The rats were orally administered sucrose solution (2 g/kg BW) every two weeks, and blood was drawn from a tail vein before and 15 min after sucrose loading to determine the concentrations of blood glucose and plasma insulin. At the same time, other rats in a non-fasted condition were sacrificed by decapitation (rats sacrificed at eight weeks were rats used for sucrose loading). The epididymal fat pads were immediately removed and adipocytes were isolated. The amount of glucose transporter 4 (GLUT4) in the plasma membranes and low-density microsomal membranes prepared from the adipocytes was measured by immunoblotting to estimate the influence of chronic Mg deficiency on glucose metabolism at the cellular level. In addition, plasma biochemical parameters and muscle mineral contents were also evaluated. The glucose concentration in fasted blood was significantly lower in Mg-deficient rats than in control rats throughout the experiment period. The feeding of a Mg-deficient diet also attenuated the response of blood glucose and plasma insulin: the glucose level in blood tended to be lower in Mg-deficient rats at 15 min after oral sucrose administration, and the difference was significant at two and eight weeks. The plasma insulin level in Mg-deficient rats was also lower, reaching a significant difference at two weeks. When animals were sacrificed in a non-fasted condition at 2-week intervals, the plasma glucose level was also significantly decreased in Mg-deficient rats as compared to control rats throughout the experiment period. The plasma insulin level in non-fasted Mg-deficient rats was also significantly decreased at two and six weeks. The Mg-deficient diet increased plasma triglyceride, but the difference was significant only at four weeks, and plasma cholesterol remained unchanged. The plasma Mg level was markedly lower in Mg-deficient rats throughout the experiment period. In Mg-deficient rats,
the Mg content in muscle was significantly reduced at two and eight weeks, whereas the calcium and sodium contents were significantly increased throughout the experiment period. In Mg-deficient rats, the degree of translocation of GLUT4 to plasma membranes in the adipocytes stimulated by insulin was reduced only at eight weeks. In conclusion, since fasted and non-fasted blood glucose levels and the response of blood glucose to sucrose loading were decreased in Mg-deficient rats, it is suggested that Mg deficiency induces changes in the glucose metabolism via impaired glucose absorption in the intestine or an altered glucose uptake in the liver and/or peripheral tissues.

**Key Words** Mg deficiency, blood glucose, plasma insulin, plasma triglyceride, plasma minerals, muscle minerals, adipocyte, GLUT4, rat

Hypomagnesemia has been reported in both insulin-dependent (IDDM) and non-insulin-dependent diabetic (NIDDM) patients (1–6). When compared with healthy subjects, diabetic patients have a decreased serum (or plasma) Mg level (1–6) and a decreased Mg content in erythrocyte (6) and muscular tissues (4), accompanied by increased urinary excretion of Mg (2, 3). The cause for the increased urinary Mg loss has been attributed to hyperosmotic diuresis by hyperglycemia (2), metabolic acidosis (7) and decreased Mg reabsorption in the kidney (8). Furthermore, decreased Mg consumption as a result of dietary restrictions for diabetic patients may also be responsible for Mg deficiency.

Mg plays an important role as a cofactor of many enzymatic reactions, including glucose metabolism. Mg promotes insulin-mediated glucose uptake into cells (9) and activates glycolysis (10). Mg also participates in phosphate-transfer reactions involving ATP and nucleotide triphosphatase (11), giving it an essential role in energy metabolism. Furthermore, Mg deficiency is known to be associated with decreased insulin sensitivity (7, 12–14) and insulin secretion from the pancreas (15–17). Reciprocally, insulin itself induces the transport of Mg into cells (18–20).

Although the biochemical role of Mg in glucose metabolism has been suggested as mentioned above, there is a limited amount of evidence regarding the implication of Mg in the etiology of diabetes. It has been reported that the oral administration of Mg improves insulin sensitivity in NIDDM patients (21) and decreases the insulin requirement of IDDM patients (22), and that depletion of the Mg levels in plasma and erythrocytes is reflected by poor metabolic control in diabetic patients (23). An animal study by Legrand et al. showed that impaired glucose tolerance and decreased insulin secretion occurred after oral glucose administration in Mg-deficient rats (17). However, in regard to the implication of Mg to diabetes mellitus, whether Mg deficiency is one of the factors inducing the onset of diabetes mellitus or whether Mg depletion occurs as the result of the onset of diabetes mellitus remains unclear.

In this study, to gain information on the cause-effect relationship between
diabetes and Mg deficiency, we determined the response to sucrose loading of glucose and insulin in blood, and mineral content in plasma and muscle in chronic Mg-deficient rats at 2-week intervals up to eight weeks. In addition, the effect of Mg on glucose transporter 4 (GLUT4) in adipocytes, as an indicator of insulin sensitivity at the cellular level, was also measured.

MATERIALS AND METHODS

Animals and experimental design. Three-week-old male Sprague-Dawley rats (Charles River Co., Shiga, Japan) were given a commercial diet (MF, Oriental Yeast Co., Tokyo) for one week. The rats were individually housed in a controlled environment (temperature 24±0.5°C, relative humidity 50±5%, lighting from 1900 to 0700). After acclimation for one week, the 4-week-old rats were divided into two groups and given either a Mg-deficient diet or a control diet for two to eight weeks. The composition of the experimental diet is shown in Table 1. The Mg-deficient diet was the same composition as the control diet (20% casein diet), except for the use of an MgO-free AIN-76 mineral mixture. The rats were allowed free access to food and deionized water.

At two-week intervals for eight weeks, rats fasted overnight were orally administered sucrose solution (2g/kg of body weight), and blood was obtained from tail veins before and 15 min after sucrose loading to determine the levels of blood glucose and plasma insulin. At the same time, non-fasted rats were given carbon dioxide to minimize lipolysis in adipose tissue and then sacrificed by decapitation. (Note: Because some rats died due to Mg deficiency during the period three to eight weeks after the start of the experiment, the number of animals used for sucrose loading and various parameters varied week to week. At eight weeks, the sacrificed rats were the same animals used for sucrose loading. Therefore, these rats were refed after sucrose loading and then sacrificed three days later in a

Table 1. Composition of the experimental diets.

| Component                | 20% casein diet (%) |
|--------------------------|---------------------|
| Casein                   | 20.0                |
| DL-Methionine            | 0.3                 |
| Corn oil                 | 5.0                 |
| Corn starch              | 15.0                |
| Sucrose                  | 50.2                |
| Mineral mixture<sup>1</sup> | 3.5               |
| Vitamin mixture<sup>2</sup> | 1.0               |
| Cellulose                | 5.0                 |

<sup>1</sup>AIN-76 mineral mixture.

<sup>2</sup>AIN-76 vitamin mixture.

The Mg-deficient diet was the same composition as the control diet (20% casein diet), except for the use of MgO-free AIN-76 mineral mixture.
The epididymal fat pads were immediately excised and adipocytes were isolated. The amount of glucose transporter 4 (GLUT4) in the plasma membranes and low-density microsomal membranes prepared from adipocytes were measured by immunoblotting. Additionally, biochemical parameters in plasma obtained after sacrificing were determined. The gastrocnemius of both hind legs were also removed to analyze mineral content.

**Blood biochemical parameters.** About 20 µl of blood obtained from tail veins before and 15 min after sucrose loading was immediately used to determine blood glucose using an autoanalyzer and an enzyme-electrode method (ANTSENSE, Sankyo-Miles Co., Tokyo). Further, about 350 µl of blood was drawn 15 min after sucrose loading, centrifuged at 11,900 g for 2 min at 4°C using a microcentrifuge (Himac CT15D, Hitachi Koki Co., Tokyo), and the plasma concentration of insulin was measured by RIA kit and antibody raised against rat insulin (Novo Nordisk, Denmark).

Non-fasted blood obtained after decapitation was immediately centrifuged at 1,900 g for 10 min at 4°C (Himac SCR20BA, Hitachi Koki Co., Tokyo). The plasma concentrations of glucose, triglyceride and total cholesterol were analyzed by enzymatic assay (Glucose CII-Test Wako, Wako Pure Chemical Industries Co., Osaka, Determiner TG-555 and Determiner TC-555, Kyowa Medex Co., Tokyo). The plasma concentrations of Mg, calcium, and potassium were measured by methods of xylidyl blue, OCPC and electrode, respectively.

**Mineral contents in diet and muscle.** The Mg content of the experimental diets and the mineral (Mg, calcium, potassium and sodium) content in muscle were determined by atomic absorption spectrophotometry.

**Adipocyte isolation and preparation of membrane fractions from adipocytes.** Adipocytes from the epididymal fat pads of 5–7 non-fasted rats were isolated by the method of Rodbell (24) using collagenase (Type II, Sigma Chemical Co., St. Louis, MO). The isolated adipocytes were incubated at 37°C for 30 min in the presence of insulin at a final concentration of 10⁻⁷ M (Sigma Chemical Co.). Subcellular membrane fractions from the isolated adipocytes were prepared according to the method of Simpson et al. (25). After incubation, adipose cells were homogenized in buffer containing 20 mM Tris-HCl, 1 mM EDTA and 250 mM sucrose with a pH of 7.4. Plasma membranes and low-density microsomal fractions were obtained using differential ultracentrifugation. Both pelleted fractions were appropriately resuspended in homogenate buffer, and the suspensions were immediately frozen in liquid nitrogen. The protein content in each fraction was measured using a protein assay kit (BCA Protein Assay, PIERCE, Rockford, IL). The samples were stored at −80°C until analysis.

**Determination of GLUT4.** Five microgram of membrane protein was subjected to SDS-PAGE by the method of Laemmli (26), followed by transfer to polyvinylidene difluoride membrane (GVHP304F0, Japan Millipore Limited Co., Tokyo). The transferred protein was incubated in the presence of rabbit anti-rat GLUT4 antibody (kindly provided by Dr. Y. Oka, Yamaguchi University, Yama-
guchi). GLUT4 signal was detected by ECL kit using a light-emitting non-radioactive method according to the manufacture's instruction (ECL Western Blotting Analysis System, Amersham International plc, Buckinghamshire, England).

Statistical analysis. Values are indicated as the M±SD, and statistical differences between the two groups were assessed by Student's t-test.

RESULTS

Physical condition and number of deaths
From day 3 after the beginning of the experiment, hyperemia of the ears, a typical symptom of Mg deficiency in rats, was observed in a few rats receiving the Mg-deficient diet. This symptom appeared in all Mg-deficient rats during the first week and disappeared by the third week. At the same time, generalized alopecia and ulcerative lesions were also observed in most Mg-deficient rats, with the latter disappearing by the sixth week. Subsequently, systemic convulsion occurred in most Mg-deficient rats from day 19 and lasted until the end of experiment.

Seven of 27 Mg-deficient rats died during the experimental period with events occurring on days 22, 31, 34, 43, 52 and 55.

Mg content in the experimental diets
The Mg content in the experimental diets given to rats during the study was 44±3 mg/kg in the Mg-deficient diet (three batches) and 466±23 mg/kg in the control diet (four batches).

Food consumption and body weight
Food consumption was reduced in the Mg-deficient rats from day 5. Food consumption of the Mg-deficient rats ranged from 60 to 80% that of the control rats by the end of experiment. Cumulative food consumption was significantly decreased in the Mg-deficient rats from the second to eighth week as shown in Table 2. As a result, food efficiency (body weight gain/food consumption) at eight weeks was significantly lower in the Mg-deficient rats (0.17±0.02, n=5) than in the control rats (0.28±0.02, n=7) (p<0.001).

Responses of blood glucose and plasma insulin to oral sucrose loading
The glucose concentration in blood obtained from tail veins before and 15 min after oral administration of sucrose is shown in Fig. 1. The glucose level in fasted blood decreased significantly in the Mg-deficient rats throughout the experiment period. At 15 min after sucrose loading, the Mg-deficient rats showed lower blood glucose than the control rats, and a significant difference was observed at two and eight weeks. The ratio of Δ blood glucose (Δ blood glucose was defined as the concentration of blood glucose increased for 15 min after sucrose loading) to blood glucose concentration at 15 min after sucrose loading is shown in Table 3. No significant difference was observed between the two groups throughout the experi-
Table 2. Cumulative food consumption and body weight.

| Duration | Control (n = 5) | Mg deficiency (n = 5) | Body weight (g) |
|----------|----------------|----------------------|-----------------|
| 0 weeks  |                |                      | 97.8 ± 3.2      |
| 2 weeks  | 302 ± 19       | 248 ± 10*            | 200 ± 11        |
| 4 weeks  | 543 ± 22       | 401 ± 22*            | 301 ± 15        |
| 6 weeks  | 872 ± 49       | 645 ± 28*            | 392 ± 20        |
| 8 weeks  | 1312 ± 83      | 890 ± 105*           | 459 ± 30        |

Values are M±SD; n = number of animals, see "Materials and Methods" in the text. *p < 0.01 vs. control, assessed by Student's t-test.

Fig. 1. Blood glucose concentrations before and 15 min after sucrose loading. Bars represent M±SD. Rats were orally administered sucrose solution (2 g/kg BW). The number of animals used is indicated in parentheses. Statistical significance was evaluated by Student’s t-test.

Fig. 2. The plasma concentration of insulin after sucrose administration is illustrated. Plasma insulin levels showed a tendency to be lower in the Mg-deficient rats except at four weeks, and they were significantly reduced at two weeks.

Analysis of plasma biochemical parameters after sacrificing

Figure 3 shows the plasma glucose, triglyceride and total cholesterol levels of
Table 3. The ratio of $\mathcal{A}$ blood glucose to blood glucose concentration 15 min after sucrose loading.

|       | $\mathcal{A}$ blood glucose/blood glucose |
|-------|------------------------------------------|
| 2 weeks | Control ($n = 7$) 0.44±0.05 |
|       | Mg deficiency ($n = 7$) 0.30±0.19 |
| 4 weeks | Control ($n = 7$) 0.37±0.05 |
|       | Mg deficiency ($n = 6$) 0.38±0.14 |
| 6 weeks | Control ($n = 7$) 0.32±0.11 |
|       | Mg deficiency ($n = 5$) 0.35±0.13 |
| 8 weeks | Control ($n = 7$) 0.38±0.12 |
|       | Mg deficiency ($n = 4$) 0.34±0.11 |

Values are M±SD; $n =$ number of animals, see “Materials and Methods” in the text. No significant difference was observed between the two groups throughout the experiment period.

Fig. 2. Plasma insulin concentrations at 15 min after sucrose loading. Bars represent M±SD. Rats were orally administered sucrose solution (2 g/kg BW). The number of animals used is indicated in parentheses. The numbers of animals used: See “Materials and Methods” in the text. Statistical significance was evaluated by Student’s t-test.

the animals sacrificed in a non-fasted state. As observed in fasted blood, the plasma glucose level was significantly lower in the Mg-deficient rats as compared to the control rats throughout the experiment period. In contrast to the plasma glucose, the plasma triglyceride level in the Mg-deficient rats tended to increased from the second to eighth week, with a significant difference at four weeks. Plasma total cholesterol levels were not different between the two groups. As shown in Fig. 4, in a non-fasted condition, Mg-deficient rats showed a lower concentration of plasma insulin throughout the experiment period, with a significant difference observed at two and six weeks. Figure 5 illustrates the plasma concentrations of Mg, calcium and potassium. The Mg-deficient diet markedly decreased the plasma Mg level throughout the experiment period. The plasma calcium and potassium levels were increased significantly in the Mg-deficient rats at four and eight weeks,
Fig. 3. Concentrations of plasma glucose, triglyceride and total cholesterol in non-fasted rats. Bars represent M±SD. The number of animals used is indicated in parentheses. The numbers of animals used: See “Materials and Methods” in the text. Statistical significance was evaluated by Student’s t-test.

Fig. 4. Plasma insulin concentrations in non-fasted rats. Bars represent M±SD. The number of animals used is indicated in parentheses. The numbers of animals used: See “Materials and Methods” in the text. Statistical significance was evaluated by Student’s t-test.

respectively.

Mineral content in gastrocnemius

The mineral content in muscle is shown in Fig. 6. The Mg-deficient diet
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Fig. 5. Concentrations of plasma minerals in non-fasted rats. Bars represent M ± SD. The number of animals used is indicated in parentheses. The numbers of animals used: See "Materials and Methods" in the text. Statistical significance was evaluated by Student’s t-test.

significantly lowered the Mg content in muscle at two and eight weeks. A similar tendency was observed for the potassium levels in muscle. In contrast, muscular calcium and sodium contents in the Mg-deficient rats were significantly higher than those in the control animals throughout the experiment period.

**GLUT4 in adipocytes**

Figure 7 shows the amount of GLUT4 in adipocytes incubated in the presence of insulin. Although Mg deficiency altered the mineral content in plasma and muscle, the effect of Mg on insulin sensitivity slowly appeared. Concerning the Mg-deficient rats, the degree of translocation of GLUT4 to plasma membrane in the presence of insulin was reduced only at eight weeks. In accordance with the translocation of GLUT4 to plasma membranes, the amount of GLUT4 in the intracellular pool (low-density microsomal membranes) was not different between the two groups until the sixth week. The amount of intracellular GLUT4 decreased in the Mg-deficient rats at eight weeks.
DISCUSSION

Although hypomagnesemia is widely known to be one of the symptoms in diabetic patients, the exact cause-effect relationship between Mg deficiency and abnormal glucose metabolism remains to be elucidated. The purpose of this study was to obtain information regarding the effect of chronic Mg deficiency on the glucose metabolism. At 2-week intervals for eight weeks, we observed changes in glucose tolerance and in the amount of GLUT4 in adipocytes in rats fed a Mg-deficient diet, in which the Mg content was approximately one-tenth that of the control diet.

Under the condition of a dramatically decreased plasma Mg level, our results clearly showed that Mg deficiency altered the glucose metabolism: glucose levels in fasted and non-fasted bloods were reduced unexpectedly, and the response to oral sucrose loading was attenuated. The observation by Legrand et al. differed in that the oral administration of glucose increased plasma glucose, whereas the intravenous injection of glucose decreased the plasma glucose level in Mg-deficient rats (17). On the contrary, Whang et al. found no impaired glucose tolerance in Mg-deficient rats after oral glucose loading (27). Since sucrose was used as a carbohydrate source in our diet, we administered a sucrose solution to the rats,
Fig. 7. Immunoblot analysis of GLUT4 in adipocytes in Mg-deficient rats. Isolated adipocytes from epidydimal fat pads incubated in the presence of insulin (10^{-7} M). Subcellular membrane fractions from isolated adipocytes were prepared by differential ultracentrifugation. Five ug of membrane protein was subjected to SDS-PAGE followed by transfer to polyvinylidene difluoride membrane. After the transferred protein was incubated in the presence of rabbit anti-rat GLUT4 antibody, GLUT4 signal was detected by a light-emitting non-radioactive method.

while Legrand et al. administered glucose as a tolerance test. The different source of carbohydrates may be partly responsible for the discrepancy. Furthermore, the fact that oral administration of sucrose in Mg-deficient rats did not increase plasma glucose to the level found in the control animals also suggests that Mg deficiency alters the handling of carbohydrates in the intestine, such as enzymatic digestion. Since Mg is essential for the maintenance of cellular function (10,11) and the physical state of plasma membrane (10,20), functional and/or morphological changes in epithelial cells in intestinal mucosa may be caused by Mg deficiency, leading to alterations in the digestion and absorption of nutrients.

It has been reported that Mg promotes insulin-mediated glucose uptake in the diaphragm obtained from normal rats (9). On the other hand, irrespective of the
presence of insulin, glucose uptake by the diaphragm obtained from Mg-deficient rats was enhanced in Mg-free medium when compared to the uptake by the control diaphragm. The addition of Mg to the Mg-free medium reduced glucose uptake by the diaphragm of Mg-deficient rats, while uptake by control diaphragm was not affected by the presence of Mg in medium (28). These results suggest that the decrease of extracellular Mg stimulates the uptake of glucose by tissue in a Mg-deficient state, which may explain the lower blood glucose level. Although the exact mechanism underlying the increased tissue uptake of glucose in Mg-deficient rats still remains to be established, Kahil et al. suggested that glucose uptake in the diaphragm of Mg-deficient rats is accomplished by both insulin-dependent and -independent mechanisms (29). Interestingly, Altura et al. found, in Mg-deficient rabbits, that cholesterol uptake in macrophages in the reticuloendothelial system was increased by a non-specific system independent from the receptor-mediated system (30). This may also be the case concerning the glucose metabolism in Mg-deficient rats: an insulin-independent pathway may play a role in glucose uptake by the tissues. Irrespective of fasted or non-fasted conditions, we found that Mg deficiency caused a reduction in the plasma insulin level, which was in agreement with the results of Gueux and Rayssiguier (16) and Legrand et al. (17). Furthermore, it has been shown that the removal of extracellular Mg inhibits insulin biosynthesis in rat islets (31). Taken together, these results indicate that the decreased level of blood glucose in Mg-deficient rats in this study might be in part due to enhanced glucose uptake in the liver and/or peripheral tissues by both insulin-independent and -dependent pathways.

Mg also influences blood lipids, especially the plasma triglyceride level (32). In this study, the plasma triglyceride level in non-fasted Mg-deficient rats tended to increase, although there was no significant difference except at four weeks. Rayssiguier et al. reported that the increase in plasma triglyceride resulting from Mg deficiency was due to a drop in lipoprotein lipase activity (33). Since insulin affects lipoprotein lipase activity (34, 35), and since diabetic patients with poor metabolic control often show hypertriglyceridemia (36), it would be of interest to study further the relationship between Mg deficiency and hyperlipidemia through the action of insulin.

It is known that hypomagnesemia is accompanied by hypocalcemia and hypopotassemia in humans (37), and hypercalcemia in Mg-deficient rats (38). However, in this study, plasma calcium and potassium levels in the Mg-deficient rats increased significantly only at four and eight weeks, respectively, and marked changes in these plasma minerals were not always observed throughout the experiment period. On the other hand, there was a tendency for reduced levels of Mg and potassium in the muscle of Mg-deficient rats, but significant differences were observed only at two and eight weeks. In contrast to these minerals, calcium and sodium contents were significantly increased in Mg-deficient rats throughout the experimental period, supporting previous findings that Mg has a role in regulating cellular ion channels and pumps, such as Na/K-ATPase (39) and the Ca^{2+}-

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activated K⁺ channel (40), to transport calcium and sodium out and potassium in. Glucose transport in adipose and muscle tissues is mainly carried out by cell surface GLUT4, which is an insulin-responsive glucose transporter (41,42). GLUT4 is predominantly located in an intracellular Golgi reticulum in the basal state, and is rapidly translocated to the cell surface in response to insulin (43,44). Accordingly, GLUT4 can be used as an indicator of insulin sensitivity at the cellular level. Streptozotocin treatment (45-47) and fasting (46,48) decreased both the intracellular pool of GLUT4 and its translocation to the cell surface in adipocytes, and also diminished GLUT4 mRNA in adipose tissue (47,49). However, the reduced protein and mRNA levels of GLUT4 were restored by subsequent insulin administration (45-47,49) and refeeding (46,48,49). In our study, despite the fact that the plasma insulin levels in Mg-deficient rats decreased both after sucrose loading and in the non-fasted condition throughout the experiment period, the degree of translocation of GLUT4 to the plasma membrane in adipose tissue by insulin stimulation in the Mg-deficient rats was different from the control rats only at eight weeks. In accordance with the decreased translocation of GLUT4 to the cell surface, the same effect of Mg deficiency was recognized in the amount of GLUT4 in the intracellular pool at eight weeks. Despite the decreased amount of insulin-responsive GLUT4, the Mg-deficient rats did not show hyperglycemia. Again, this may be the same as the case shown using Mg-deficient and cholesterol-fed rabbits (29): glucose uptake by the reticuloendothelium may be enhanced by Mg deficiency. The overall hypoglycemia observed in Mg-deficient rats in this study may have been due mainly to a non-insulin-responsive pathway, since Mg deficiency decreased insulin secretion (15−17).

In conclusion, the feeding of a Mg-deficient diet affects glucose metabolism as well as the levels of Mg in the plasma and muscle. The expression of GLUT4 to the cell surface in adipocytes, which was used as an indicator of insulin sensitivity, was slowly, but not acutely, affected by Mg deficiency. Since the blood glucose levels in both fasted and non-fasted conditions as well as the response of blood glucose to sucrose loading were decreased in Mg-deficient rats, these results suggest that Mg deficiency is related to either a reduction of glucose absorption in the small intestine or to an increase in glucose uptake by the liver and/or peripheral tissues.

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