Intracellular Magnesium of Platelets in Children With Diabetes and Obesity

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Magnesium (Mg^{2+}), the second most abundant intracellular cation, is a critical cofactor in numerous enzymatic reactions. By using a fluorescent probe, mag-fura-2, we examined the basal levels and changes in intracellular Mg^{2+}($[Mg^{2+}]_i$) of platelets in diabetic and obese children. Under the basal condition, the platelet $[Mg^{2+}]_i$ of both type 1 and type 2 diabetes mellitus (DM) and the obesity groups was significantly lower than the values in the nondiabetic control group (377 ± 62 μmol/L, 312 ± 72 μmol/L, 373 ± 35 μmol/L *v* 594 ± 62 μmol/L, respectively, *P* < .05). $[Mg^{2+}]_i$ was increased after the stimulation with 100 μU/mL of insulin. After 60 seconds of insulin stimulation, the value of $[Mg^{2+}]_i$ was lower in the type 1 DM group compared with the control group (729 ± 85 μmol/L *v* 1,078 ± 67 μmol/L, *P* < .005). The increase in percentage over the resting $[Mg^{2+}]_i$ was higher in the type 2 DM group than in the stimulated control group (222% ± 51% *v* 98% ± 18 %, *P* < .05), although the stimulated $[Mg^{2+}]_i$ did not reach the level of the control group. The diabetic patients and obese subjects have $[Mg^{2+}]_i$ deficiency. In the type 2 DM and obese groups, platelets responded well to insulin. In children under insulin-resistant states, $[Mg^{2+}]_i$ decreases before the poor reactivity to insulin occurs in platelets. Decreased $[Mg^{2+}]_i$ might underlie the initial pathophysiologic events leading to insulin resistance and abnormality of platelet coagulation.

MAGNESIUM (Mg^{2+}) deficiency occurs in patients with diabetes and vascular disease, in whom platelet hyperactivity is a common factor.[1,2] Diabetic subjects show an increase in platelet reactivity that can enhance the risk of vascular diseases.[2] Mg^{2+} modulates platelet-dependent thrombosis.[1] The intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) and its role in the platelet reactivity in the state of insulin resistance is not known.

Platelets are often used in the study of cellular cation metabolism in diseases,[3] because they are readily available for study and are thought to share a number of features with vascular smooth muscle cells. Human platelets have insulin receptors with similar characteristics as those in other cells.[4] We and other investigators reported that insulin could mediate $[Mg^{2+}]_i$ in platelets.[5,6] Due to changes in lifestyle around the world, the rate of type 2 diabetes mellitus (DM) in children has increased recently.[7,8]

Intracellular Mg^{2+} and its role in the pathogenesis of insulin resistance are not known. This study aims to evaluate the dysfunction of magnesium regulation in platelets of children with diabetes and obesity.

MATERIALS AND METHODS

Experimental Subjects

Fifteen patients with type 1 diabetes (9 boys and 6 girls, aged 14.5 ± 1.9 years [range, 3 to 18]); mean years after onset, 5.3 ± 1.1), 7 subjects with type 2 diabetes (6 boys and 1 girl, aged 15.0 ± 0.8 years [range, 14 to 17]; mean years after onset, 0.9 ± 0.1), and 7 obese subjects (4 boys and 3 girls, aged 11.2 ± 0.8 years [range, 8 to 15]) were investigated. Diabetes was defined using the criteria of the American Diabetes Association.[9] Obesity was expressed as a body mass index (BMI), ie, weight in kilograms divided by the square of the height in meters, greater than 25. Type 1 diabetes patients were on therapy with subcutaneous insulin, the last dose given 6 hours before sampling. The control group consisted of 14 subjects (10 boys and 4 girls, aged 12.7 ± 1.9 years [range, 6 to 17]) with normal blood pressure and a negative family history of DM.

Control and type 2 diabetes subjects were not treated with any medications, including insulin, and did not show any evidence of endocrine malfunction or recent use of drugs that might potentially alter electrolyte balance. Informed consent was obtained from the parents if the subjects were less than 16 years old and from the subjects themselves when they were older than 16 years.

Platelet Preparation

Platelets were isolated as previously described.[10] Approximately 10 mL of venous blood was drawn into 3.8% (wt/vol) acid citrate buffer (10:1, vol/vol) and was centrifuged at 200 × *g* for 10 minutes at room temperature. The platelet-rich plasma was decanted, further centrifuged at 1,000 × *g* for 10 minutes, and the cells were washed 3 times in Hepes buffer solution (HBS) containing (mmol/L) NaCl 140, KCl 5, glucose 25, $MgCl_2$ 1, NaH_2PO_4 1, Hepes 25 (pH 7.2), and EGTA 0.2. EGTA was omitted from the third washing, and 0.1% fatty-acid free bovine serum albumin (BSA) was added. Platelets were counted in a Celltac counter (Nihon Kohden, Tokyo, Japan). Unless otherwise indicated, platelets at a concentration of 2 to 3 × 10^7 platelets/mL were suspended in suspension buffer solution (SBS) containing (mmol/L) NaCl 20, KCl 115, Hepes 10 (pH 7.05). Platelets were studied within 4 hours after drawing blood.

Measurements of Intracellular Magnesium Concentrations

Intracellular ionic $[Mg^{2+}]_i$ concentrations were measured with a Hitachi F-2000 fluorescence spectrophotometer (Hitachi Instruments, Tokyo, Japan) by using mag-fura-2 probes as described by Raju et al.[11] A 2-μmol/L quantity of mag-fura-2/acetoxymethyl dye was added to the platelet suspension and incubated at 37°C for 30 minutes. After loading of the dyes, the platelets were washed twice with SBS, the fura dyes were removed by centrifugation, and the platelets were resuspended in SBS. The excitation wavelengths were set at 335/370 nm, and the emission wavelength was 510 nm. Each intracellular ionic concentration was calculated as described[11,12] by using dissociation constant (Kd) = 1,500 (μmol/L). The maximum intensities were determined by disrupting the cells with 0.1% Triton in the presence of 30

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Table 1. Clinical Characteristics

	Controls (n = 14)	Type 1 DM (n = 15)	Type 2 DM (n = 7)	Obesity (n = 7)
Age (yr)	12.7 ± 1.9	14.5 ± 1.9	15.0 ± 0.8	11.2 ± 0.8
Boys/girls	10/4	9/6	6/1	4/3
BMI (weight [kg]/height [m]^2)	19.0 ± 1.2	19.7 ± 1.6	26.1 ± 0.2*	30.4 ± 4.1†
Fasting plasma glucose (mg/dL)	88 ± 9	218 ± 30‡	177 ± 47	100 ± 8
Total cholesterol (mg/dL)	174 ± 7	174 ± 9	204 ± 21	200 ± 16
Hemoglobin A_{1C} (%)	ND	7.6 ± 0.3	6.9 ± 1.7	ND
Years from onset	—	5.3 ± 1.1	0.9 ± 0.1	—

Abbreviation: ND, not determined.
*$P < .001$, †$P < .01$, ‡$P < .05$ *v* control.

mmol/L $MgCl_2$. The minimum intensities were the values determined in the presence of 60 mmol/L EDTA. $MnCl_2$ (0.05 mmol/L) was used to quench the fluorescence from extracellular dye according to the methods of Ng et al.[13] Insulin and thrombin were dissolved in deionized water. Twenty-five microliters of insulin and thrombin were added to 2.5 mL of platelet suspension.

Chemicals

All chemicals were purchased from Sigma Chemical Co (St Louis, MO), unless stated otherwise. Mag-fura-2/acetoxymethyl was from Molecular Probes (Eugene, OR).

Statistical Analysis

Data were expressed as the mean ± SE. Statistical significance was assessed using analysis of variance (ANOVA). A value of $P < .05$ was considered significant.

RESULTS

Profile of Each Group

Clinical characteristics of the study subjects are listed in Table 1. No statistical differences among the groups were observed for age. Boys were dominant for sex distribution only in the type 2 DM group. BMI was significantly higher in the obesity group and the type 2 DM group compared with the control group. Overnight fasting glucose in the type 1 DM was significantly higher than in the controls.

Basal and Insulin-Stimulated Intracellular Magnesium

In both of the type 1 and type 2 diabetic patients, and the obesity group, basal $[Mg^{2+}]_i$ was significantly lower than in the control ($P < .05$; Fig 1).

To examine the response for insulin in platelets, we studied the $[Mg^{2+}]_i$ after insulin stimulation. In the type 1 DM group, at 60 seconds after the stimulation with 100 μU/mL of insulin, stimulated $[Mg^{2+}]_i$ was significantly lower than in the control group ($P < .005$; Fig 1). The actual increased value of $[Mg^{2+}]_i$ after insulin stimulation in the type 1 DM group was lower than in the type 2 DM group ($P < .05$; Table 2).

The increased percentage of insulin stimulation in type 2 DM group was significantly higher than in the control group ($P < .05$; Table 2).

There were no significant gender- nor age-related differences of $[Mg^{2+}]_i$ in the basal state and the reactivity to insulin. No significant percent increase after insulin stimulation was observed in the obesity group.

DISCUSSION

In our previous study, we demonstrated that insulin and insulin-like growth factor-1 (IGF-1) induced an immediate rise of $[Mg^{2+}]_i$ of platelets in a dose-dependent manner.[5] A similar phenomenon was observed in erythrocytes.[14,15] Although higher than the physiologic level, the dose of insulin concentrations that we used was 100 μU/mL, which was found to show the maximal effect according to a dose-response curve.[5] Barbagallo et al used the same level of insulin concentration in their study.[16,17] The basal level of $[Mg^{2+}]_i$ was reported between the range of 270 and 630 μmol/L.[18,19] Our results were consistent with the previous data.

The mean basal $[Mg^{2+}]_i$ was lower in both of the type 1 and type 2 DM as well as the obesity group. Hyperglycemia may also have an effect on Mg transport and induce a decline of $[Mg^{2+}]_i$. This hypothesis is supported by the report by Barbagallo et al.[20] They reported that responses to in vitro hyperglycemia were blunted in adult hypertensive subjects and that these responses were closely linked to basal $[Mg^{2+}]_i$ levels.[20]

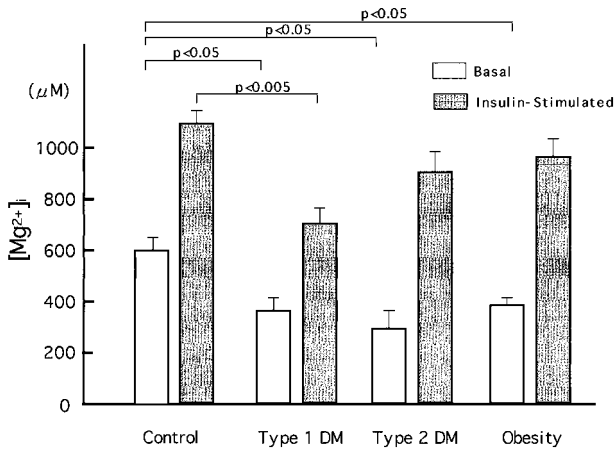

Fig 1. Intracellular magnesium of each group. The basal levels and $[Mg^{2+}]_i$ of platelets in diabetic, obese, and control subjects were measured by using a fluorescent probe, mag-fura-2 . Under the basal condition, the platelet $[Mg^{2+}]_i$ of both type 1 and type 2 DM and the obesity groups was significantly lower than the values in the nondiabetic control group. $[Mg^{2+}]_i$ was increased after the stimulation with 100 μU/mL of insulin. After 60 seconds of insulin stimulation, the value of $[Mg^{2+}]_i$ was lower in the type I DM group compared with the control group.

Table 2. Intracellular Magnesium of Each Group

	Controls (n = 14)	Type 1 DM (n = 15)	Type 2 DM (n = 7)	Obesity (n = 7)
Basal (μmol/L)	594 ± 62	377 ± 62*	312 ± 72*	373 ± 35*
Insulin stimulated (μmol/L)	1,078 ± 67	729 ± 85†	899 ± 153	933 ± 145
Percentage increased after insulin stimulation	98% ± 18%	137% ± 29%	222% ± 51%	144% ± 45%
Actually increased value after insulin stimulation (μmol/L)	475 ± 73	333 ± 48	567 ± 106	545 ± 149

*$P < .05$.
†$P < .005$.

We washed the platelets 3 times with the buffer and excluded the difference of glucose level of each subject. Barbagallo et al reported a role of basal ion levels in determining cellular responsiveness.[17] However, in our study, basal $[Mg^{2+}]_i$ was not correlated with ion responsiveness to insulin.

The actual increased value of $[Mg^{2+}]_i$ in the type 1 DM was less than any other group, which expressed the impairment in response to insulin. On the other hand, in the type 2 DM, the actual increased value after insulin stimulation was higher than the control. Reaven pointed out that hyperinsulinemia clusters with impaired glucose tolerance and obesity.[21] Under the hyperinsulinemia per se, ie insulin resistance, platelets are hyperreactive to insulin. Although both of the type 1 and type 2 diabetic patients have low $[Mg^{2+}]_i$, the platelets in only type 2 DM group have good potentiality to compensate for low $[Mg^{2+}]_i$. In our results, the percent increase over the resting level in the presence of insulin was 2 times higher in the type 2 DM compared with the control group. These results indicated that the platelets of the type 2 DM group has hyper-reactivity for insulin and stimulated $[Mg^{2+}]_i$ increased up to the control level. When the plasma insulin level decreases after the failure of β-cell function, dysregulation of $[Mg^{2+}]_i$ would be markedly enhanced in the type 2 DM.[22]

The etiology of insulin resistance is not completely understood. Mg^{2+} deficiency may predict the risk of insulin resistance. Mg^{2+} supplementation can improve glycemic control in type 2 diabetic patients. Some clinical evidence suggests that Mg^{2+} deficiency may play a role in insulin resistance.[23] It has been difficult to determine the role of the Mg^{2+} deficit in causing insulin resistance, as this deficit might also be the secondary result of resistance to the direct $[Mg^{2+}]_i$ -elevating action of insulin.[6,16] Lower $[Mg^{2+}]_i$ may contribute to insulin resistance, as Mg is important in the regulation of glucose utilization.[24]

The previously described insulin mediated accumulation in red blood cells is a slow process, peaking after 30 to 60 minutes.[16] However, our results showed a rapid increase in $[Mg^{2+}]_i$. We followed the effect of insulin over 30 minutes and found that $[Mg^{2+}]_i$ reached plateau at 5 minutes. Any increase of $[Mg^{2+}]_i$ afterwards was not observed except for the influence of fluorescent dye leak from the intracellular compartment. The discrepancies may come from the difference of materials and procedures, such as nuclear magnetic resonance and fluorescent-dye methods.

There are many metabolic abnormalities in diabetic or obese patients that may alter platelet function. We previously reported the intracellular calcium ($[Ca^{2+}]_i$) was higher both in the basal state and after stimulation with thrombin in the type 1 diabetic patients.[10] Mg^{2+} influences the regulation of $[Ca^{2+}]_i$ by inhibiting the influx of Ca^{2+} into the cytoplasm via Ca^{2+} channels and by facilitating the uptake of Ca^{2+}-adenosine triphosphatase (ATPase)–driven Ca^{2+} into different intracellular stores.[25] The mechanism of the Mg^{2+} accumulation following insulin stimulation is unclear. Insulin-induced elevations of $[Mg^{2+}]_i$ may reflect cellular uptake from intracellular storage sites for Mg^{2+} or from the extracellular space. Ishijima and Tatibana reported that bombesin rapidly induces magnesium mobilization from the intracellular pool through external Ca^{2+}- and tyrosine kinase–dependent mechanisms.[26]

The different reactivity between type 1 and type 2 DM groups in our study may also be due to the severity of metabolic dysregulation. The duration of the disease after the onset of type 1 DM was longer than that of type 2 DM. Therefore, the disease process might have been more advanced.

In summary, children with diabetes and obesity have $[Mg^{2+}]_i$ deficiency. In the type 2 DM and obese groups, platelets responded well to insulin. Under insulin-resistant states, $[Mg^{2+}]_i$ decreases before the poor reactivity to insulin occurs in platelets. Decreased $[Mg^{2+}]_i$ might underlie the initial pathophysiologic events leading to insulin resistance and abnormality of platelet coagulation.

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