

Six Months of Diazoxide Treatment at Bedtime in Newly Diagnosed Subjects With Type 1 Diabetes Does Not Influence Parameters of β -Cell Function and Autoimmunity but Improves Glycemic Control

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OBJECTIVE — Continuous β -cell rest with diazoxide preserves residual endogenous insulin production in type 1 diabetes. However, side effects have hampered therapeutic usefulness. In a double-blind study, we tested whether lower, intermittent dosing of diazoxide had beneficial effects on insulin production, metabolic control, and autoimmunity markers in the absence of side effects.

RESEARCH DESIGN AND METHODS — Forty-one newly diagnosed type 1 diabetic patients were randomized to 6 months of treatment with placebo or 100 mg diazoxide at bedtime. A1C, C-peptide (fasting and glucagon stimulated), and FoxP3⁺ regulatory T-cells (Tregs) were measured. Patients were followed for 6 months after intervention.

RESULTS — Of six dropouts, three were due to perceived side effects; one subject in the diazoxide group experienced rash, another dizziness, and one in the placebo group sleep disturbance. Adverse effects in others were absent. Diazoxide treatment reduced A1C from 8.6% at baseline to 6.0% at 6 months and 6.5% at 12 months. Corresponding A1C value in the placebo arm were 8.3, 7.3, and 7.5% ($P < 0.05$ for stronger reduction in the diazoxide group). Fasting and stimulated C-peptide decreased during 12 months similarly in both arms (mean -0.30 and -0.18 nmol/l in the diazoxide arm and -0.08 and -0.09 nmol/l in the placebo arm). The proportion of Tregs was similar in both arms and remained stable during intervention but was significantly lower compared with nondiabetic subjects.

CONCLUSIONS — Six months of low-dose diazoxide was without side effects and did not measurably affect insulin production but was associated with improved metabolic control.

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Preservation of residual insulin production in type 1 diabetic patients is accompanied by improved glycemic control, reduced microvascular complications, and reduced number of hypoglycemic events (1,2). To retain residual insulin secretion is thus highly desirable.

Autoimmune mechanisms are of main importance for β -cell destruction in type 1 diabetes. Accordingly, immunosuppressive treatment retards the destructive process (3–5) and thus has therapeutic potential. But also, the degree of metabolic control affects, whether by modulation of autoimmune activity or by other mechanisms, the rate of β -cell deterioration. Thus, in the Diabetes Control and Complications Trial (DCCT), intensive insulin treatment, which achieved lower A1C than conventional treatment, also markedly retarded deterioration in C-peptide levels (2). This favorable effect could be due to lesser hyperglycemia, per se, but also to a lesser degree of overstimulation of the β -cells (i.e., " β -cell rest").

Diazoxide provides β -cell rest by reversibly suppressing glucose-induced insulin secretion through opening ATP-sensitive K⁺ channels in the β -cell (6). A beneficial effect of 3 months treatment with diazoxide was documented in 20 newly diagnosed type 1 diabetic subjects. Diazoxide ($4\text{--}6\text{ kg} \cdot \text{kg}^{-1} \cdot 24\text{ h}^{-1}$, i.e., 280–420 mg for a 70-kg subject) or placebo was divided into capsules taken three times daily (7). After the intervention, C-peptide levels were better preserved in diazoxide- versus placebo-treated subjects for up to 18 months. Ortqvist et al. (8) obtained similar results with diazoxide $5\text{--}7.5\text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ given to pediatric patients for 3 months. However, disturbing side effects (lanugo hair growth, edema, and hypotension) were frequent and have hampered further studies with diazoxide (7,8).

No studies have tested whether a

lower dosage of diazoxide would eliminate side effects and still exert a beneficial effect on insulin production and metabolic control in type 1 diabetes. We recently treated type 2 diabetic subjects using a reduced, intermittent dosing of diazoxide (i.e., 100 mg at bedtime) (9,10). Side effects were then absent and insulin production improved provided that patients were simultaneously treated with bedtime insulin (9). These results encouraged us to perform a similar study in type 1 diabetes.

Beneficial effects of diazoxide in previous type 1 diabetes studies have been proposed to be due to β -cell rest and diminishing cellular autoimmune activity (11,12). However, studies on the effects on T-cell subpopulations are lacking. Among these, much recent evidence points to the importance of regulatory T-cells (Tregs) (13). Tregs were originally characterized by strong expression of interleukin (IL)-2R, CD25, and recently and more specifically by expression of the transcription factor forkhead box P3 (FoxP3) (14,15). It was therefore of interest in our trial to look for a relative change in Treg populations.

The aims of this study were thus to investigate in newly diagnosed subjects with type 1 diabetes whether a low-dose and intermittent treatment with diazoxide would 1) be devoid of side effects; 2) lead to better endogenous insulin secretion, measured by fasting and stimulated C-peptide; 3) have beneficial effects on metabolic control, measured by A1C and home glucose monitoring; and 4) affect autoimmune processes, measured by glutamic acid decarboxylase (GAD)-65 and islet antigen (IA)-2 antibody titers and by the FoxP3 marker of regulatory T-cells.

RESEARCH DESIGN AND METHODS

— Patients were recruited between February 2005 and June 2007 from the university hospitals of St. Olav, Akershus, Stavanger, Haukeland, and North Norway as well as Levanger Hospital. Inclusion criteria were age 18–40 years, insulin-dependent diabetes with positive test for GADA and/or IA-2A (antibodies against GAD65 and IA-2), fasting C-peptide level ≥ 0.2 nmol/l, and diabetes duration ≤ 12 weeks. Exclusion criteria were evidence of drug and alcohol abuse. Further, we did not include women who were pregnant or who did not use contraception. Patients were given oral and written information before consenting to participate. The study pro-

ocol was approved by the regional ethics committee and the Norwegian Drug Agency. Details of study design can be found in the online appendix (supplemental Table 1 in the online appendix [available at <http://care.diabetesjournals.org/cgi/content/full/dc09-1436/DC1>]).

Before inclusion, all patients underwent a clinical examination and blood sampling for assessment of blood count, lipid profile, and renal and liver function. All patients received a standard multi-injection regimen consisting of mealtime monomeric short-acting insulin and long-acting NPH insulin twice daily (except for two subjects in the diazoxide group and three in the placebo group who received NPH insulin only at night). After obtaining fasting blood glucose consistently between 4 and 6 mmol/l and postprandial levels between 5 and 7 mmol/l, the patients were double-blindly randomized to either 100 mg diazoxide at night or placebo. Randomization was stratified for age < 25 or ≥ 25 years. The intervention lasted 6 months and the follow-up period another 6 months. The primary end point was β -cell function, assessed by fasting and glucagon-stimulated C-peptide. Secondary end points were glycemic control (A1C and blood glucose), insulin dosage, and markers of autoimmunity (GADA, IA-2A, and Tregs).

The study visits took place at baseline and once every 3 months. Visits were focused on glycemic control and occurrence of side effects. Each visit included a clinical investigation and measurements of fasting blood glucose, blood pressure, and body weight. Patients were also examined for presence of edema and asked about the occurrence of hypoglycemia and adverse events. Hypoglycemic episodes were registered as minor or major events depending on coping or not. We registered current insulin dosage (fast- and long-acting) at each visit.

C-peptide glucagon stimulation tests were performed in duplicate at baseline and every 3 months thereafter. In total, 10 tests were performed in each participant. Blood was sampled in the overnight-fasted state and 6 min after the intravenous injection of 1 mg glucagon.

Patients were to perform seven-point home glucose monitoring (7pHGM) during 3 consecutive days at baseline, during intervention, and between 3 and 6 months after intervention. Blood glucose was measured fasting, 2 h after breakfast, before lunch, 2 h after lunch, before dinner, 2 h after dinner, and at bedtime us-

ing the patients' own blood glucose-measuring device.

Diazoxide was provided by TEVA Pharmaceuticals Europe (Utrecht, the Netherlands). Manufacture of drug and placebo capsules was performed by the Kragerø Tablet Production Unit (Kragerø, Norway). At baseline and at the end of intervention, we isolated peripheral blood mononuclear cells (PBMCs) from 11 subjects in the diazoxide-treated and 9 subjects in the placebo-treated group. Cells were isolated by separation on Lymphoprep (Axis-Shield, Oslo, Norway) and then stored below -140°C . We also isolated PBMCs from 20 nondiabetic, age- and sex-matched blood donors. After thawing, the cells were processed through a FACScan flow cytometer (BD Biosciences, San Jose, CA). For details see the online appendix.

Assays

A1C was assayed by a DCA 2000 (Bayer AS Diagnostics, Oslo, Norway). Reference levels defining normality were between 3.0 and 6.0%. C-peptide, insulin, glucagon, and proinsulin were assayed by radioimmunoassay (Linco Research, St. Louis, MO). GADAs and IA-2As were determined by enzyme-linked immunosorbent assay (Medizym; Medipan Diagnostica, Selchow, Germany). Titers of GADA > 5 units and of IA-2A > 10 units were regarded as positive.

Statistics

The coefficient of variation (CV) for fasting C-peptide is 13% (16). To detect a 20% difference with 80% certainty, one would only need eight patients in each group. Considering a spontaneous variation in C-peptide decline, we planned to include 50% more patients. Assuming a 10% dropout, 36 patients would then need to be included.

For C-peptide glucagon tests, we used the mean values of duplicates for analyses. For 7pHGM, we used the median because of high spread in some of the values. All other results are given as means \pm SE. Significance testing was performed using the Mann-Whitney test. Dichotomous variables were analyzed using the Pearson's χ^2 test. To assess insulin sensitivity, we used the homeostasis model assessment (HOMA) calculator from Oxford university (<http://www.dtu.ox.ac.uk>).

Table 1—Baseline characteristics

| | Diazoxide | Placebo | Dropouts |
|---|-------------|-------------|-------------|
| Age (years) | 27.5 ± 1.60 | 27.0 ± 1.76 | 30.8 ± 1.64 |
| Sex (male/female) | 13/6 | 12/4 | 4/2 |
| BMI (kg/m ²) | 24.8 ± 0.78 | 26.0 ± 1.38 | 27.2 ± 3.53 |
| Systolic blood pressure (mmHg) | 122 ± 2.7 | 116 ± 3.6 | 126 ± 5.1 |
| Diastolic blood pressure (mmHg) | 75 ± 2.1 | 72 ± 1.9 | 79 ± 3.8 |
| Time of inclusion after diagnosis of diabetes (weeks) | 5.0 ± 1 | 8.0 ± 2 | 7.6 ± 1 |
| Nicotine use (yes/no) | 6/13 | 6/10 | 2/4 |
| Fasting glucose (mmol/l) | 7.5 ± 0.59 | 7.9 ± 0.46 | 7.8 ± 1.50 |
| A1C (%) | 8.6 ± 0.38 | 8.3 ± 0.56 | 8.1 ± 0.90 |
| C-peptide (nmol/l) | 0.31 ± 0.03 | 0.34 ± 0.05 | 0.56 ± 0.08 |

Data are means ± SE.

RESULTS

Dropouts

Forty-one patients were randomized to either diazoxide (no. 22) or placebo (no. 19). Six patients were excluded during intervention, all of these during the first 3 months of intervention. Three patients dropped out for personal reasons (one from the diazoxide group and two from the placebo group). Three patients were excluded due to assumed side effects (from the diazoxide group, one due to a rash and another due to dizziness). In the placebo group, one patient experienced sleep disturbances, which disappeared after drug cessation. Compared with completers, the dropouts were slightly older, more overweight, had higher blood pressure, and had higher fasting C-peptide at baseline but comparable glycemic control (Table 1). The remaining 35 patients completed the study. A random third was asked to return the unused capsules after intervention. The amount of unused medication agreed with the prescribed doses of the study medication.

Baseline characteristics

Age and sex distribution did not differ between groups. Ketoacidosis at first referral was registered in three patients in each group. The diazoxide group had significantly shorter disease duration before inclusion versus the placebo group. There was a significant decrease in C-peptide levels of -0.17 ± 0.04 ($P = 0.003$) in subjects as a whole from the time of diagnosis to inclusion.

Clinical examination and routine blood testing (See RESEARCH DESIGN AND METHODS) were normal (data not shown). Blood pressure, BMI, glycemic control, and fasting C-peptide levels did not differ

between the groups. The use of nicotine was equally distributed. In the diazoxide group, two patients used oral contraceptives, one used a statin, one used a proton pump inhibitor, and one used gabapentin. In the placebo group, three patients used oral contraceptives and one was on asthma inhalation therapy containing glucocorticoids (Table 1).

Body weight

The diazoxide group gained 1.1 ± 0.75 kg in total during the study period. The placebo group gained 1.8 ± 1.0 kg during the first 3 months and had a total weight gain of 2.0 ± 1.5 kg. This was significantly higher than in the diazoxide group ($P = 0.040$).

Insulin dosage

There was no significant difference in insulin dosage (total and long acting) between groups (supplemental Table 2).

Glycemic control

A1C improved in the diazoxide-treated group compared with the placebo group both during intervention and during follow-up (Fig. 1). Neither fasting blood glucose at the study visits nor the 7pHGM registration showed significant changes during intervention. However, 3 months after intervention, the fasting blood glucose decreased significantly, by -1.4 mmol/l in the diazoxide-treated group, while increasing by 1.1 mmol/l in the placebo group ($P = 0.017$ for difference between groups). The same tendency was seen 6 months after intervention. Only 51% of the patients completed the 7pHGM registrations after the intervention. By these data, there was a tendency ($P = 0.158$) for a reduction in blood glucose before and after breakfast in the diazoxide-treated versus placebo group.

Hypoglycemic events

The self-reported frequency of hypoglycemic events before inclusion was comparable between the two groups. During intervention, five patients in the diazoxide group reported frequent minor hypoglycemic events (>10 events during 6 months). Five patients in the placebo group reported frequently minor hypoglycemia and two reported one major event each.

Parameters of β -cell function

Fasting and glucagon-stimulated C-peptide levels decreased similarly in both groups throughout the study. From comparable baseline values, the fasting C-peptide in the diazoxide group decreased by 0.05 ± 0.03 nmol/l after intervention and by 0.13 ± 0.02 nmol/l after 12 months. The decrement in the placebo group was 0.02 ± 0.05 and 0.08 ± 0.04 , respectively (supplemental Table 3). Stimulated C-peptide levels showed the same pattern; from a baseline value of 0.53 ± 0.07 nmol/l, the decrement was 0.1 ± 0.04 and 0.18 ± 0.04 in the diazoxide group. In the placebo group, the baseline value was 0.6 ± 0.1 nmol/l and decreased by 0.04 ± 0.07 and 0.09 ± 0.07 , respectively.

To adjust for different glucose concentrations, we calculated the ratio of C-peptide to glucose (Fig. 2). No significant difference between groups was observed. Also fasting levels of insulin, proinsulin, and glucagon did not change during the study (supplemental Table 3).

Insulin sensitivity

Insulin sensitivity as assessed by HOMA-S% was comparable between the groups at baseline. After 3, 9, and 12 months, the HOMA-S% increased by, respectively, 16, 14, and 32% in the diazoxide group. Sensitivity remained stable in the placebo group (-3 , 0 , and 4% for the same time periods, $P = 0.020$, 0.147 , and 0.072 , respectively, for differences between the groups).

Measurements of GADA and IA-2A

All patients were by selection criteria positive for GADA. Twenty-four subjects were additionally positive for IA-2A. Divided by group adherence, 74% in the diazoxide and 63% of subjects in the placebo group were positive for both antibodies. Titers of GADA and IA-2A before intervention were similar between groups. Participants taken together had a mean GADA titer at the start of 182 ± 15 and 168 ± 15 units after 12

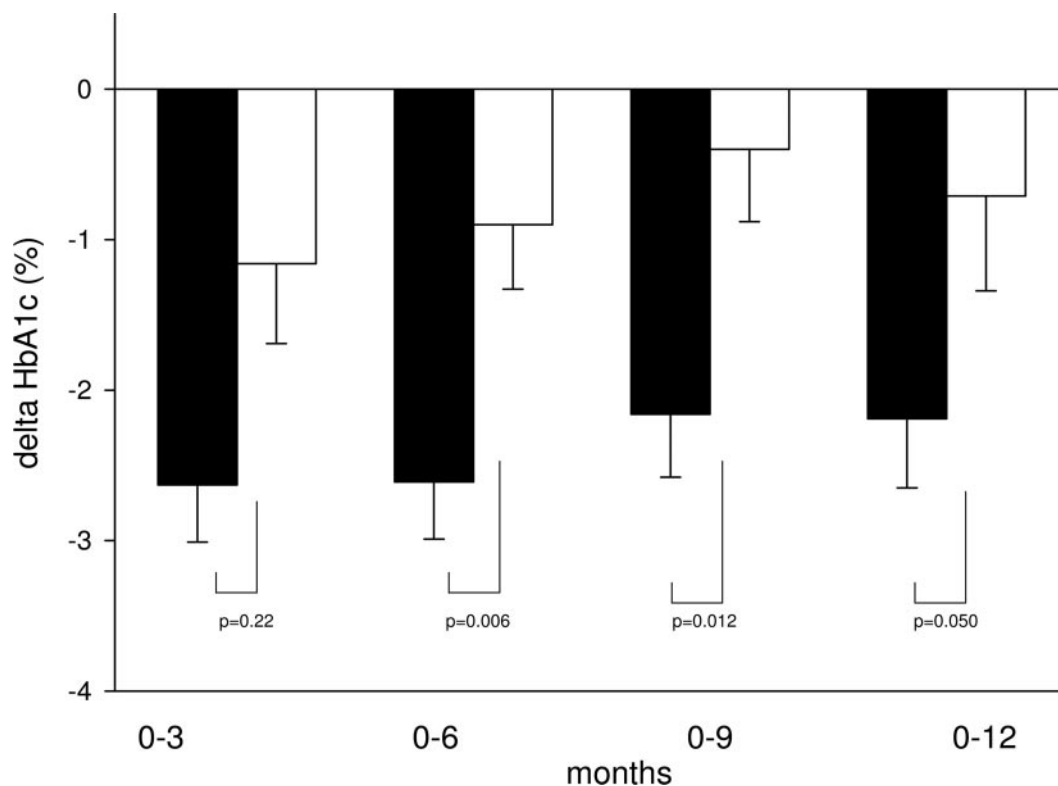


Figure 1—Changes in A1C from baseline, recorded after 3, 6, 9, and 12 months of the study (means \pm SE). Baseline mean values for diazoxide: 8.6%; for placebo: 8.3%. ■, diazoxide-treated subjects; □, placebo-treated subjects.

months. The decline in GADA was nonsignificantly smaller in the diazoxide-treated group ($P = 0.133$). The mean IA-2A (all participants) was 188 ± 30 units at baseline and 168 ± 31 units after 12 months.

Subjects who were positive for both GADA and IA-2A had a tendency for a

more pronounced decline in fasting C-peptide after 6 ($P = 0.092$) and 12 months ($P = 0.128$) compared with subjects who were only GADA positive, with a similar aggravating effect on stimulated C-peptide after 12 months ($P = 0.083$).

PMBCs and Tregs

The proportion of $CD4^+$ lymphocytes to the total number of lymphocytes did not differ between the diabetic groups and the healthy control group (supplemental Fig. 1). The proportion of rTregs and aTregs, expressed, respectively, as the proportion of $CD4^+/CD45RA^+/CD25^+/FoxP3^+$ cells to the total number of $CD4^+/CD45RA^+$ cells and the proportion of $CD4^+/CD45RA^-/CD25^+/FoxP3^+$ to the total number of $CD4^+/CD45RA^-$ cells, did not differ between the diazoxide- and placebo-treated groups (supplemental Figs. 2 and 3). The results were the same when the groups were split into C-peptide decline over or below the median (supplemental Fig. 4). Likewise, when Tregs were compared in high versus low titers of GADA and IA-2A or numbers of antibodies (i.e., one or two antibodies), there was no detectable correlation of the Treg ratio to disease activity (data not shown). However, we found evidence for a small overall downregulation of Tregs in the diabetic group versus the nondiabetic group. This significant difference was found both for the rTregs and the aTregs (supplemental Figs. 2–4).

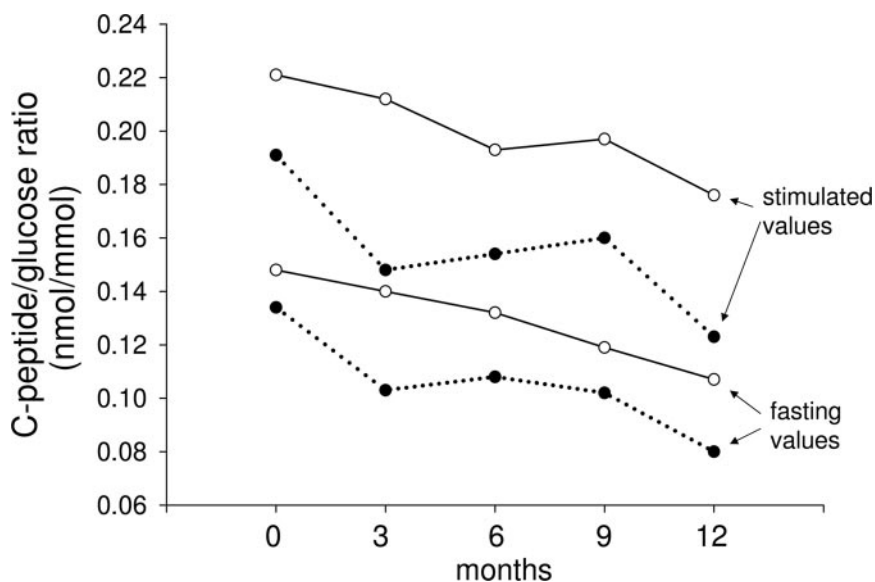


Figure 2—Fasting and stimulated values of C-peptide-to-glucose ratio at 0, 3, 6, 9, and 12 months. ●, diazoxide-treated subjects; ○, placebo-treated subjects.

CONCLUSIONS— This study is the longest intervention trial with diazoxide so far performed in subjects with diabetes. The main findings are that 6 months of low-dose diazoxide treatment was accompanied with few side effects and did not measurably affect residual insulin production but was associated with improved metabolic control.

The absence (or near absence) of well-known side effects (7,8) is in line with our previous studies in type 2 diabetic patients (9,10). This study extends these observations to a younger age-group and a longer treatment period. A low drop-out rate attests to acceptability of treatment. Compliance with the intervention was further corroborated by satisfactory results from capsule counting. One may conclude that further long-term studies with low-dosage diazoxide, if they are indeed indicated, is not hampered by concerns about safety or compliance.

In contrast to the previous studies (7,8), we did not observe any postintervention effect on fasting or glucagon-stimulated levels of C-peptide. The study of Björk et al. (7), which reported beneficial effects of diazoxide treatment, is the study most similar to ours; a discussion of differences of potential importance is thus in order. Age, C-peptide levels at baseline, and method of testing insulin production were similar. Participants in the Björk et al. study were included within a week after diagnosis, while intervention in our subjects started up to 12 weeks after initiation of insulin treatment (in line with current recommendations [17]). It seems possible that our subjects were in partial remission at baseline, whereas the participants in the Björk et al. study had at that time not entered remission. The implications of such a difference are however not clear.

A simple explanation for the lack of measurable effects on insulin production might be the lower dosage of diazoxide. We used a dose of diazoxide that was about one-third of the dose used in the Björk et al. study. We reasoned that a longer intervention period (i.e., 6 rather than 3 months in the previous studies) could compensate for a lower dose. Such a putative effect may, however, have been nullified by a blunting of the effects of diazoxide with time. Thus, there was at least a tendency for inhibition of fasting and stimulated C-peptide after 3 months of intervention but no such effect at the end of the 6-month intervention period. The notion of time-dependent blunting of

effects is compatible with the Björk et al. study, in which the suppression of C-peptide during intervention was blunted already at the end of the 3-month intervention period. Björk et al. assumed blunting to be due to a recovery of the secretory capacity of the β -cell. This explanation cannot be ruled out but is less likely in the light of the postintervention data in our study.

A1C improved significantly in the diazoxide group compared with placebo. This effect was seen already during intervention but persisted during the follow-up. The cause of improved glycemic control is not clear. One possibility is improved insulin production that was not picked up by the present methods of testing (i.e., fasting and glucagon-stimulated C-peptide). The fact that the beneficial effect lingered postintervention would be compatible with a hidden postintervention effect of diazoxide on β -cell function. One cannot rule out that other tests of β -cell function, such as meal-stimulated secretion, would have detected a difference. However, we have no evidence to support this notion. An alternative or additional possibility is that diazoxide favorably affects metabolic control by improving insulin sensitivity. Such a notion is compatible with the insulin requirement not being enhanced in the diazoxide-treated group despite the expected inhibitory effect of diazoxide on insulin secretion. Similar observations were made in the two previous clinical studies (7,8), and data from animal studies have indicated improved sensitivity (18). Further, when we calculated HOMA-5% (rarely done in type 1 diabetes but deemed admissible [19]), there was significant improvement in the diazoxide-treated group during the intervention.

The molecular mechanisms for an insulin-sensitizing effect of diazoxide remain to be clarified. Of possible significance is the fact that body weight increased more in the placebo group during the first 3 months. In animal studies, diazoxide decreases appetite (20), and a similar, albeit unproven, effect could be operative in humans (21).

To our knowledge, ours is the first study to prospectively analyze the relative Treg proportion in PBMCs during the early phase of type 1 diabetes. The FOXP3 marker was unaltered through the 6-month intervention period both in the diazoxide and the placebo group. On the other hand, the Treg ratio differed significantly between age-matched nondiabetic

subjects and the type 1 diabetic subjects. These observations are in contrast to a previous report (22), which, however, did not focus specifically on differences between newly diagnosed type 1 diabetes versus healthy subjects. In our study, the samples from the healthy control subjects were assembled in one center (Trondheim), thus avoiding the transportation necessary for many of the diabetes samples. However, we did not find any differences between samples from Trondheim and those from other centers (results not shown). Nevertheless, the observed differences between nondiabetic and diabetic subjects need to be confirmed by an independent study, ideally performed in freshly isolated cells. As to humoral autoimmunity, we, as others (7,8), did not detect any significant influence by diazoxide on GADA levels.

Are further studies with diazoxide in type 1 diabetes warranted? At first glance, the lack of effects on endogenous insulin production would speak against such endeavors. However, since diazoxide at higher dosage does exert beneficial effects, a beneficial effect on insulin production at the present dose in a combination therapy, for instance together with an immunosuppressant, does not seem unreasonable. In any case, the beneficial effects of diazoxide on metabolic control that we observe could be welcome in a combination therapy.

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