Molecular effects of sulphonylurea agents in circulating lymphocytes of patients with non-insulin-dependent diabetes mellitus

I. Rabbone,¹ M. Piccinini,² M. Curto,¹ M. Mostert,³ S. Gamba,⁴ S. Mioletti,¹ R. Bruno² & M. T. Rinaudo¹

Departments of ¹Medicina e Oncologia Sperimentale -Sezione Biochimica-, ²Morfofisiologia Veterinaria, ³Scienze Pediatriche e dell'Adolescenza, Università di Torino, and ⁴Centro di Diabetologia 'Karen Bruni', Ospedale Maria Vittoria, Torino, Italy

Aims In circulating lymphocytes of NIDDM patients pyruvate dehydrogenase (PDH), the major determinant in glucose consumption through oxidative pathways, is poorly active. The aim of this study is to examine whether sulphonylurea drug treatment revives PDH activity in circulating lymphocytes from NIDDM patients.

Methods Twenty normal-weight individuals with NIDDM were enrolled in this study. They had maintained their glycaemic levels close to normal by means of a restricted diet that had no longer been successful in the proceeding 2 months. The treatment protocol consisted in 160 mg gliclazide daily for 5 weeks. Twenty healthy subjects, matched for age, body mass index and gender, were enrolled as a control group. Patients, before and after treatment, as well as controls were tested for PDH activity in their circulating lymphocytes. Nine other untreated patients and nine healthy subjects, with the above mentioned characteristics, were recruited for the assay of PDH activity in their circulating lymphocytes before and after exposure, *in vitro*, to gliclazide, to insulin, and to gliclazide and insulin in combination.

Results In gliclazide-treated NIDDM patients, PDH activity in circulating lymphocytes recovered. *In vitro*, in circulating lymphocytes of untreated patients and controls insulin at 5 $\mu\text{U ml}^{-1}$ was ineffective and highly effective, respectively, in raising enzyme activity; gliclazide at 10 ng ml^{-1} was ineffective on PDH in both groups, but in combination with insulin at 5 $\mu\text{U ml}^{-1}$ in both groups PDH was as active as in cells of controls exposed to insulin only. In cells of controls, gliclazide alone at 25–50 ng ml^{-1} caused enzyme activation, whereas above 50 ng ml^{-1} it caused inhibition; in cells of patients below 50 ng ml^{-1} it had no effects, but at 50 ng ml^{-1} and above raised enzyme activity to the basal level of controls.

Conclusions This study suggests that free gliclazide concentrations determine recovery of PDH activity in circulating lymphocytes of treated patients through drug-mediated enhanced insulin control over PDH or through the drug alone.

Keywords: NIDDM, sulphonylureas, pyruvate dehydrogenase, circulating lymphocytes

Introduction

Sulphonylurea drugs are largely employed to lower hyperglycaemia in patients with NIDDM. The mechanism underlying this effect is still debated. Evidence indicates these drugs improve cell responsiveness to insulin or even mimic some of the effects of insulin [1–7]. In a previous study it was observed that in circulating lymphocytes of NIDDM patients the activity of pyruvate dehydrogenase [1.2.4.1] (PDH), the major determinant of glucose consumption rate along oxidative pathways, is below normal and *in vitro* the enzyme is unresponsive to insulin at physiological concentrations [8]. In this study, in circulating lymphocytes of NIDDM patients, in whom glycaemic values had returned to normal following treatment with gliclazide (a second generation sulphonylurea agent), PDH activity recovered.

Gliclazide-assisted recovery of PDH activity in circulating lymphocytes may ameliorate glucose consumption in these

cells. An understanding of the mechanisms underlying this benefit might throw new light on the mechanisms through which sulphonylureas behave as hypoglycaemic agents.

In NIDDM patients systemic glucose consumption through oxidative pathways is abnormal and in tissues actively involved in preserving glucose homeostasis, such as skeletal muscle [9–11], this alteration is accompanied by poorly active PDH [12, 13]. Further, in adipocytes from these patients [14] enzyme response to insulin is impaired. Low enzyme activity is also a feature of cells and tissues of animals with experimental diabetes [15–21] and of circulating lymphocytes of newly diagnosed type I diabetic children [22].

Circulating lymphocytes are not elective targets for insulin and the role of PDH in these cells is not known to reflect that in cells and tissues actively involved in the preservation of glucose homeostasis. On the other hand, in individuals with impaired glucose tolerance low PDH activity and altered enzyme response *in vitro* to insulin at physiological concentrations in circulating lymphocytes correlate with increased area under the blood sugar and insulin curves obtained during an oral glucose tolerance test (OGT) [23].

Correspondence: Dr Maria Teresa Rinaudo, Dip. di Medicina e Oncologia Sperimentale, -Sezione Biochimica, Via Michelangelo, 27, 10126 Torino, Italy.

Therefore, the enzyme alterations in circulating lymphocytes might parallel those in skeletal muscle of NIDDM patients.

Some previous observations essential for the planning of this study, have been reported [8, 22, 24, 25]. Exposure of circulating lymphocytes from healthy subjects to insulin below $2.5 \mu\text{U ml}^{-1}$ leaves PDH activity unaffected; at $2.5 \mu\text{U ml}^{-1}$ the enzyme is activated and maximally so at $5 \mu\text{U ml}^{-1}$. With insulin at $10 \mu\text{U ml}^{-1}$ enzyme activity is again at basal levels and far below at $50 \mu\text{U ml}^{-1}$. In circulating lymphocytes of NIDDM patients PDH activity is below normal but rises to around the basal value of controls when these cells are exposed *in vitro* to insulin at $50 \mu\text{U ml}^{-1}$. The above described insulin effects are observed only when fresh intact cells are used and the contact medium is supplemented with Ca^{++} and Mg^{++} .

Some characteristic features of PDH are summarized below. In mammalian cells PDH is present in mitochondria in a polymeric structure in which one of the subunits behaves as a regulatory element in that it may exist in two forms, active dephosphorylated (PDHa) and inactive phosphorylated (PDHb). The balance between the two forms is controlled by PDH kinase and PDHP phosphatase. The latter is activated by $\mu\text{M Ca}^{++}$ and mM Mg^{++} and inhibited by mM fluoride [26] and there is evidence that it constitutes the actual insulin target [15–17, 27–30].

Methods

Subjects

Twenty normal-weight individuals (10 men and 10 women, mean age 53 ± 9 years, median 51 years, 95% CI 47.8–59.1, body mass index (BMI) $23 \pm 4 \text{ kg m}^{-2}$, median 23 kg m^{-2} , 95% CI 18.9–27.4), affected by NIDDM, as defined by the National Diabetes Data Group [31] were enrolled in this study. These individuals had been diabetic for 2–5 years, had never taken drugs known to affect glycaemia and had maintained their glycaemic levels close to normal by means of a restricted diet, that had no longer been successful in the previous 2 months. The treatment protocol consisted of 80 mg gliclazide twice a day before meals for 5 weeks. Routine laboratory tests performed before treatment were normal, apart from blood glucose levels, in all subjects. Blood samples for assays of blood sugar and insulin and for lymphocyte preparation were drawn from the antecubital vein at 08.00 h, after an overnight fast, at the start and end of the trial. The last gliclazide intake was at 19.00 h the day before the beginning of experiments.

Twenty healthy subjects, with no family history for diabetes, matched for age (53 ± 9 years, median 50, 95% CI 47.8–59.1), BMI ($23 \pm 4 \text{ kg m}^{-2}$, median 24 kg m^{-2} , 95% CI 18.9–27.0), and gender, were enrolled as a control group.

Patients before and after treatment as well as controls were tested for PDH activity in their circulating lymphocytes.

Another nine untreated patients and nine healthy subjects, with the above mentioned characteristics, were recruited for *in vitro* experiments, including the assay of PDH activity before and after exposure of circulating lymphocytes to the drug alone, to insulin alone and to the drug and insulin in combination.

The study was performed in accordance with the

principles of the Helsinki declaration and informed written consent was given by all subjects.

Materials

Gliclazide used in *in vitro* experiments was donated by Laboratoires Servier, Paris Cedex, France.

Enzymes, co-enzymes and substrates were supplied by Sigma Chemical Company (St Louis, Mo, USA) and Biochemia Boehringer (Mannheim, Germany). Lymphoprep (density, 1.077) was provided by Nyegaard & Co. (Oslo, Norway) and porcine insulin (40 U ml^{-1}) by Lilly & Co. (Indianapolis, Ind., USA). All the other products were analytical grade and were obtained from Merck (Darmstadt, Germany) and Carlo Erba (Milano, Italy).

Lymphocyte preparation

Lymphocyte preparation was performed according to Boyüm as already described [32]. At the end of preparation, cells were suspended in 110 mM NaCl buffered in 40 mM phosphate buffer pH 7.4 (PBS A) and identified by morphological, biochemical and immunological techniques [32]; in all preparations, the cell suspension consisted of 89–93% lymphocytes; monocytes were no more than 0.2–2.5%.

One part of the cell preparation was then used immediately for gliclazide, insulin and insulin + gliclazide contact procedures, another part for lymphocyte counts performed manually by means of a haemocytometer; the remaining cells, pelleted and then suspended in PBS A enriched with $2 \mu\text{g ml}^{-1}$ leupeptin, 20 μM EDTA (PBS B), were stored at -70°C (ideal storage conditions), and used for PDH activity assay within 4 days.

PDH activity assay

Frozen lymphocytes were lysed by thawing and freezing, followed by 10 s sonication at 0°C three times at 30 s intervals; a MSE soniprep 150 ultrasonic disintegrator was used, equipped with a 9.5 mm probe set at a 30 μm amplitude. Lysed cells were enriched with 1 mM DDT (dithiothreitol), 200 $\mu\text{g ml}^{-1}$ bovine serum albumin (BSA), both in 0.1 M Tris (tris[hydroxymethyl] aminomethane)-HCl pH 8, and then incubated for 10 min at 37°C in a medium containing: a) 50 mM NaF without supplementation with Ca^{++} and Mg^{++} , a condition strongly inhibiting PDHP phosphatase [26], thus allowing evaluation of the activity of PDH already in the active form (PDHa); b) 50 $\mu\text{M CaCl}_2$ and 10 mM MgCl_2 , a condition determining activation of PDHP phosphatase [26]; the ensuing increase of PDH activity, with respect to the activity determined according to a) [above] is a measure of the efficiency of the endogenous PDHP phosphatase in converting phosphorylated inactive into dephosphorylated active PDH; in this case PDH activity is referred to as PDH_{CaMg} ; c) in conditions of b) [above] with supplementation of exogenous PDHP phosphatase purified from bovine heart mitochondria, according to Teague *et al.* up to step H [33], so giving rise to full conversion of phosphorylated into dephosphorylated PDH and thus full PDH activation; in this case enzyme

activity is referred to as PDHt. Samples added with 50 mM NaF were then supplemented with MgCl₂ to a final concentration of 3 mM. PDH activity was evaluated on all preincubated samples on the basis of acetyl CoA formed in the presence of 1 mM CoASH, 20 mM pyruvate, 1 mM NAD⁺, 20 mM cysteine, 80 mM phosphate buffer pH 8, 0.1 U lactate dehydrogenase in a final volume of 250 µl and simultaneously transformed by excess of phosphotransacetylase into acetylphosphate [32]; the latter was tested according to Allred & Guy as previously described [32].

The availability of blood samples from controls was greater than from NIDDM patients. For this reason the experiments concerning NIDDM patients were performed on a number of blood samples from the same patient, whereas those concerning controls on a single sample. Consequently enzyme activity levels referred to NIDDM patients vary slightly more than those of controls.

Cell incubation experiments

Exposure of lymphocytes to gliclazide and insulin alone or in combination was performed using intact fresh cells, suspended in PBS A (2–3 × 10⁶ cells ml⁻¹) and incubated in a final volume of 200 µl in a medium containing 10 mM phosphate buffer pH 7.4, 200 µg BSA and 50 µM CaCl₂ and MgCl₂. After 15 min at 37° C, cells were added with 1 ml PBS A, quickly centrifuged, washed twice with PBS A, suspended in PBS B, frozen at -70° C and finally used for PDH activity assay.

Since gliclazide was stored as 1 mg ml⁻¹ stock solution in dimethylsulphoxide (DMSO) and diluted from the stock solution with 0.1% DMSO, DMSO was added to all samples to give the same final concentration; control samples indicated that the final DMSO concentration alone had no effect on PDH activity.

Cell viability at the end of incubation procedures was close to 98%.

Kinetic measurements

Drug effect, in terms of PDHa activity, was plotted *vs* drug concentration and the experimental data were fitted by non-linear regression analysis with the general equations for parameter estimation from allosteric-kinetic data and binding measurements in the presence (1) or absence (2) of inhibition [34]:

$$B = B_0 + \frac{(B_{\max} - B_0)K_i^m L^n + (B_{\infty} - B_0)L^{n+m}}{(K_d^n + L^n)(K_i^m + L^m)} \quad (1)$$

$$B = B_0 + \frac{(B_{\max} - B_0)L^n}{(K_d^n + L^n)} \quad (2)$$

B = biological effect, i.e. drug effect (E) in terms of PDHa activity.

B₀ = basal biological effect, E₀.

B_{max} = maximal biological effect or maximal efficacy, E_{max}.

B_∞ = biological effect at L = ∞

L = ligand concentration (nM gliclazide)

K_d = dissociation constant for L (E₅₀)

K_i = non-competitive inhibitory constant

n, m = Hill coefficients

Statistical analysis

Statistical analysis was performed using ANOVA and Student's *t*-test for paired and unpaired data as appropriate for each case and ANOVA and Newman Keuls for multiple comparison post-test. The level of significance was set at *P* < 0.05.

Results

In vivo experiments

In NIDDM patients after gliclazide treatment glycaemia in fasting conditions was close to that of controls and insulinaemia remained unvaried (Table 1). In circulating lymphocytes of patients before treatment PDH activity, in terms of PDHa and PDH_{CaMg}, was below normal, whereas PDHt was in the normal range of values. Following treatment PDHa and PDH_{CaMg} rose to about the levels of controls (Table 2).

In vitro experiments

The effects on PDH of insulin at 2.5 and 5 µU ml⁻¹ in cells of controls and at 50 µU ml⁻¹ in cells of patients were confirmed in this study. In addition it was observed that:

a) in cells of controls, gliclazide causes significant enzyme activation at 25 ng ml⁻¹ (*P* < 0.01) with a peak at 50 ng ml⁻¹ (*P* < 0.001); at 100 ng ml⁻¹ enzyme activity is at basal values and significantly below this at 500 ng ml⁻¹ (*P* < 0.001 with respect to basal) (Figure 1). In cells of patients, gliclazide at 50 ng ml⁻¹ affects PDH (enzyme activity is significantly above basal levels, *P* < 0.001) and in the range 50–500 ng ml⁻¹ it raises enzyme activity to levels close to those in cells of controls in basal conditions (Figure 1).

Gliclazide effects are detectable only if the contact medium is supplemented with 50 µM Ca⁺⁺ and Mg⁺⁺ and intact fresh cells are used; further, incubation of gliclazide with lysed cells leaves PDH activity unaffected (results not reported);

b) drug effect (expressed as PDHa activity) plotted versus drug concentration gives a bell-shaped sigmoidal curve in cells of controls and a monophasic curve in patients, more sigmoidal than in controls (Figure 2). Table 3 summarizes the values obtained by fitting the experimental data shown in Figure 2 with equations 1 and 2 (see Methods). It emerges that in line with the sigmoidal profile of the curves, the Hill

Table 1 Clinical data of NIDDM patients before and after gliclazide therapy (80 mg twice daily/5 weeks).

Clinical data	Gliclazide therapy	
	Before (20)	After (20)
BMI (kg m ⁻²)	23 ± 5	23 ± 4
Fasting blood glucose (mmol l ⁻¹)	10 ± 2 a	6 ± 2 b
Fasting blood insulin (µU ml ⁻¹)*	10 ± 5 c	12 ± 6 d

a → b *P* < 0.01; c → d *P*: NS.

*normal range: 5–12 µU ml⁻¹.

Data are the mean value ± s.d. In brackets number of cases.

Individuals	Gliclazide therapy	PDHa	PDH _{CaMg}	PDHt	PDHa/PDH _{CaMg} %	PDHa/PDHt %
NIDDM (20)	Before	17 ± 2.8*	25 ± 4.3*	52 ± 4	69 ± 0.8	34 ± 6
NIDDM (20)	After	28 ± 2.4	36 ± 2.8	53 ± 5	78 ± 0.7	53 ± 5
Control (20)	—	28 ± 2.0	38 ± 3.0	55 ± 3	74 ± 0.5	51 ± 4

PDH activity is expressed as nmoles acetylCoA/10⁶ cells h⁻¹. Values are the mean ± s.d. In brackets number of cases.

PDHa: activity of active PDH. PDH_{CaMg}: activity resulting from PDHa + newly activated PDH by means of endogenous PDHP phosphatase. PDHt: total PDH activity.

**P* < 0.001 *vs* controls and NIDDM patients after treatment.

Table 2 PDH activity in circulating lymphocytes from NIDDM patients before and after gliclazide therapy (80 mg twice daily/ 5 weeks) and controls.

Figure 1 PDH activity in circulating lymphocytes before and after exposure to gliclazide at different concentrations.

a) Activity of PDHa.

b) PDH_{CaMg} activity resulting from PDHa + newly activated PDH by means of endogenous PDHP phosphatase.

(—▲) controls and (---△) NIDDM patients.

Each point on the curves is the mean value from six independent duplicate experiments on circulating lymphocytes from six controls and six patients. Bars represent s.d.

coefficients are not equal to 1. In particular *n* is above 1 and more so in NIDDM patients, suggesting a homotropic cooperative effect of the drug in both cell groups, less well expressed in patients than in controls. Further, in NIDDM patients, along with almost comparable drug potency with

Figure 2 Dose dependent gliclazide effects.

Effects are expressed as PDHa activity (nmol acetylCoA/10⁶ cells h⁻¹). Results are the mean ± s.d. of four duplicate experiments performed on circulating lymphocytes from a single blood sample from one donor and one NIDDM patient. Comparable results were obtained in parallel experiments on blood samples from two other donors and NIDDM patients. Data from experiments on donors (▲) and NIDDM patients (△) were fitted to equation 1 and equation 2, respectively. Curves were computed with the parameters shown in Table 3.

Table 3 Kinetic parameters of dose dependent gliclazide effects.

Kinetic parameters	Controls	NIDDM patients
<i>E</i> _{max} (nmol/10 ⁶ cells h ⁻¹)(*)	40.30 (7.6)	31.50 (4.0%)
<i>E</i> ₅₀ (nM)	102.20 (16.0)	121.50 (4.1)
<i>n</i>	2.99 (25.0)	3.22 (5.2)
<i>K</i> _i (nM)	242.10 (4.2)	∞
<i>m</i>	8.22 (18.5)	∞

(*) refers to the activity of PDH in the active form, PDHa. Gliclazide concentrations ranged from 10 ng ml⁻¹ (33 nM) to 250 ng ml⁻¹ (825 nM). Kinetic parameters were evaluated according to equation 1 in circulating lymphocytes of controls and to equation 2 in those of NIDDM patients. Percentage ranges of variations are given in brackets.

respect to controls (*E*₅₀ is only slightly higher in the former than in the latter), *E*_{max} is 20% lower;

c) exposure of circulating lymphocytes from controls to gliclazide at 50 ng ml⁻¹ and insulin at 5 μU ml⁻¹, and those from patients to gliclazide at 100 ng ml⁻¹ and insulin at 50 μU ml⁻¹ (concentrations at which both compounds are equally effective in raising PDH activity in the respective

Table 4 PDH activity in circulating lymphocytes from controls and NIDDM patients following incubation with gliclazide (G) and insulin (I) in combination both at concentrations that alone are highly effective in raising enzyme activity.

Additive	Controls		Additive	Patients	
	PDHa	PDH _{CaMg}		PDHa	PDH _{CaMg}
—	26 ± 1	36 ± 3	—	15 ± 1	24 ± 3
I 5 μ U ml ⁻¹	36 ± 2	45 ± 4	I 50 μ U ml ⁻¹	26 ± 4	35 ± 4
G 50 ng ml ⁻¹	37 ± 2	46 ± 4	G 100 ng ml ⁻¹	29 ± 3	37 ± 2
I 5 μ U ml ⁻¹ + G 50 ng ml ⁻¹	35 ± 4 (NS)	45 ± 4 (NS)	I 50 μ U ml ⁻¹ + G 100 ng ml ⁻¹	27 ± 3 (NS)	35 ± 4 (NS)

PDH activity is expressed as nmol acetylCoA/10⁶ cells h⁻¹. Values are the mean ± s.d. of six separate duplicate experiments on lymphocyte preparations from six controls and six patients. PDHa: activity of PDH; PDH_{CaMg}: activity resulting from PDHa + newly activated PDH by means of endogenous PDHP phosphatase.

NS: *P* not significant *vs* G and I alone.

cells) causes the same enzyme activity levels observed in the respective cells when exposed to each compound alone, i.e. effects are not additive (Table 4);

d) in circulating lymphocytes from controls exposed to gliclazide at 10 ng ml⁻¹ (at this concentration and at 15 ng ml⁻¹ the drug has no effect on PDH, Figures 1 and 2) in combination with insulin at 1.25, 2.5 and 5 μ U ml⁻¹, enzyme activity is the same as in these cells when exposed to insulin at 5 μ U ml⁻¹ alone (at 1.25 μ U ml⁻¹, insulin has no effects over PDH; at 2.5 μ U ml⁻¹ it is effective but less than at 5 μ U ml⁻¹) (Figure 3 panels A and B). In the above circumstances, PDHt remains unaffected (data not reported);

e) exposure of circulating lymphocytes from diabetic patients to gliclazide alone at 10 (or 15) ng ml⁻¹ leaves PDH activity unaltered (Figures 1 and 2); however when the drug is in combination with insulin at 1.25 or 5 μ U ml⁻¹ (at these concentrations insulin alone is ineffective on PDH in these cells) PDH is sharply activated (*P* < 0.001 with respect to basal levels) and is as active as in cells of controls exposed to insulin alone at 5 μ U ml⁻¹ (Figure 3, panels A and B). In this context PDHt remains unaltered and PDHa/PDH_{CaMg} and PDHa/PDHt values are comparable to those in cells of controls exposed to insulin at 5 μ U ml⁻¹ alone (79.3 ± 1.3 and 65.4 ± 2.5 respectively, *vs* 80 ± 2.3 and 65.5 ± 0.5, respectively);

f) with reference to insulin at 1.25 μ U ml⁻¹, plotting drug effects (expressed in terms of PDHa activity) versus drug concentration in the range 2.5–15 ng ml⁻¹ (7.7–46.3 nM) gives a sigmoidal curve in cells of controls and patients, that is sharper in the latter (Figure 4). Data analysed according to equation 2 lead to define the kinetic parameters reported in Table 5. They suggest a homotropic cooperative effect of the drug (Hill coefficient above 1) with *E*_{max} comparable in the two cell groups but with decreased potency in cells of NIDDM patients (in the latter *E*₅₀ is twice that of controls);

g) in circulating lymphocytes from patients incubated with gliclazide at 10 ng ml⁻¹ and insulin at 50 μ U ml⁻¹ enzyme activity does not differ with respect to that evoked by insulin alone at 50 μ U ml⁻¹ (Table 6).

Discussion

In NIDDM patients, after 5 weeks of gliclazide treatment, blood glucose levels return to normal and in circulating lymphocytes PDH activity (in terms of PDHa and

Figure 3 PDH activity in circulating lymphocytes exposed to insulin at 1.25, 2.5 and 5 μ U ml⁻¹ alone or in combination with gliclazide at 10 ng ml⁻¹.

a) Activity of PDHa.

b) PDH_{CaMg} activity resulting from PDHa + newly activated PDH by means of endogenous PDHP phosphatase.

(—Δ—Δ) and (—▲—▲) controls and NIDDM patients respectively in the presence of insulin alone and (—▲) (—Δ) controls and NIDDM patients respectively in the presence of insulin and gliclazide.

Each point on the curves is the mean value from six independent duplicate experiments on circulating lymphocytes from six controls and six patients. Bars represent s.d.

PDH_{CaMg}) recovers. Before and after treatment PDHt is normal and thus low PDH activity in untreated patients does not appear to depend on a quantitative enzyme defect. Unaltered PDHt and low PDHa activity also characterize various tissues in animals with experimental diabetes [15–17].

Figure 4 Dose dependent gliclazide effects in the presence of insulin at $1.25 \mu\text{U ml}^{-1}$. Effects are expressed as PDHa activity (nmol acetylCoA/ 10^6 cells h^{-1}). The results are the mean $\pm$ s.d. of four duplicate experiments performed on circulating lymphocytes of a single blood sample from one donor and one NIDDM patient. Comparable results were obtained in parallel experiments on blood samples from two other donors and NIDDM patients. Data of experiments from donors ($\blacktriangle$) and NIDDM patients ($\triangle$) were fitted to equation 2 and the curves were computed with the parameters shown in Table 5.

Table 5 Kinetic parameters of dose dependent gliclazide effects in circulating lymphocytes exposed to insulin at $1.25 \mu\text{U ml}^{-1}$.

Kinetic parameters	Controls	NIDDM patients
E_{max} (nmol/ 10^6 cells h^{-1})(*)	41.27 (2.5)	39.70 (1.1)
E_{50} (nM)	10.56 (9.5)	20.41 (1.4)
n	1.99 (19.1)	5.56 (5.9)

(*) refers to the activity of PDH in the active form, PDHa. Drug concentrations ranged from 2.5 ng ml^{-1} (7.7 nM) to 15 ng ml^{-1} (46.3 nM). Kinetic parameters were evaluated according to equation 2. Percentage ranges of variations are given in brackets.

Table 6 PDH activity in circulating lymphocytes of NIDDM following incubation with insulin (I) at $50 \mu\text{U ml}^{-1}$ alone or in combination with gliclazide (G) at 10 ng ml^{-1} .

Additive	PDHa	PDH _{CaMg}
—	16 ± 0.5	25 ± 4.0
I $50 \mu\text{U ml}^{-1}$	26 ± 3.0	34 ± 3.0
G 10 ng ml^{-1}	15 ± 1.0	26 ± 3.0
I $50 \mu\text{U ml}^{-1}$ + G 10 ng ml^{-1}	24 ± 4.0 (NS)	33 ± 4.0 (NS)

PDH activity is expressed in nmol acetylCoA/ 10^6 cells h^{-1} . Values are the mean $\pm$ s.d. of six separate duplicate experiments performed on lymphocyte preparations from six controls and six patients. PDHa: activity of PDHa; PDH_{CaMg}: activity resulting from PDHa + newly activated PDH by means of endogenous PDHP phosphatase. NS: P not significant vs I $50 \mu\text{U ml}^{-1}$.

PDH_{CaMg} activity is a measure of PDHP phosphatase efficiency in converting PDHb into PDHa [26]. Low PDH_{CaMg} activity in untreated and its recovery in treated patients suggest the phosphatase encounters difficulties in converting PDHb into PDHa in the former, but not in the

latter, possibly explaining low PDHa activity and its recovery in the two cases.

In vitro, insulin at $5 \mu\text{U ml}^{-1}$ is highly effective in raising PDH activity in cells of controls; in cells of patients it is ineffective, but in combination with gliclazide at 10 ng ml^{-1} (alone at this concentration the drug is devoid of effects on PDH in these cells and in those of controls), it is as effective as insulin alone in cells of controls. Since in both cell groups activation takes only 15 min, the difficulties with phosphatase suggested above should not imply a quantitative enzyme defect.

PDHP phosphatase is considered to be the insulin target [15–17, 27–30] and in circulating lymphocytes of controls and patients insulin alone [8, 22, 24, 25, 32], or in combination with gliclazide at 10 ng ml^{-1} affects PDH only when combined with intact cells. It follows that the control exerted by insulin over PDH takes place through an intermediate, generated by plasma membrane components, that finds its target in PDHP phosphatase, in line with reported evidence [16, 17, 28, 35–41].

In cells of controls insulin at $2.5 \mu\text{U ml}^{-1}$ is less efficient than at $5 \mu\text{U ml}^{-1}$ in raising PDH activity and at $1.25 \mu\text{U ml}^{-1}$ it is ineffective; however, in combination with gliclazide at 10 ng ml^{-1} , at any of the above concentrations, it is as effective as insulin alone at a concentration of $5 \mu\text{U ml}^{-1}$. In cells of patients insulin alone at any of the above concentrations is ineffective on PDH unless gliclazide at 10 ng ml^{-1} is present, in which case here too it is as effective as insulin alone at $5 \mu\text{U ml}^{-1}$ in cells of controls. It follows that cells of patients exposed to insulin at any of the above concentrations, or cells of controls exposed to insulin at $1.25 \mu\text{U ml}^{-1}$ are in a comparable situation in that in both cases they do not respond to insulin but are potentially capable of doing so. Presumably the mechanism behind unresponsiveness to insulin at $1.25 \mu\text{U ml}^{-1}$ in cells of controls (unless gliclazide at 10 ng ml^{-1} is present), must be responsible for unresponsiveness to insulin over a wider range of concentrations in cells of patients. The role of gliclazide at 10 ng ml^{-1} appears to be to overcome physiological as well as pathological unresponsiveness to insulin, thereby overcoming differences related to the type of cell or insulin concentration. This implies that for PDH to be affected by insulin, receptive structures on the cell membrane must be primed by insulin. Once primed, only if the insulin concentration is sufficiently high, will intermediates be generated and their efficiency will be related to the insulin concentration.

In cells of controls and patients priming should take place at any of the above concentrations; however, because in cells of patients intermediate generation does not follow (cell exposure to insulin at 2.5 and $5 \mu\text{U ml}^{-1}$), it can be concluded that patients are defective only for this step. In view of the effects gliclazide has in controls and patients, this is the step that constitutes the actual drug target. However, in both cell groups with gliclazide concentrations lower than 10 ng ml^{-1} , insulin at $1.25 \mu\text{U ml}^{-1}$ again has difficulties in giving rise to intermediates and these difficulties are greater in cells of patients than in those of controls. In fact, the dose-effect curve related to the drug is sigmoidal in both cases but more so in patients. This suggests the gliclazide target (i.e. the defective step in cells of patients)

is constituted by a multiple binding site structure(s) on which the drug behaves as a homotropic cooperative positive modulating factor with a comparable $E_{\max}$ in cells of controls and patients but with a reduced potency in the latter, possibly due to reduced affinity of the multiple binding site structure for the drug. In controls, once structures are primed, insulin alone is capable of mimicking the effects of gliclazide, providing its concentration is at least $2.5 \mu\text{U ml}^{-1}$; thus, physiological or pathological unresponsiveness to insulin (i.e. at $1.25 \mu\text{U ml}^{-1}$ or at $2.5 \mu\text{U ml}^{-1}$ and above, respectively) should also involve difficulties for the above mentioned structure in binding insulin, that are progressively overcome by insulin itself at increasing concentrations in controls, but not in patients.

In cells of patients exposed to insulin alone at $50 \mu\text{U ml}^{-1}$ PDH is finally stimulated and its activity rises close to the values of unstimulated cells of controls. However, in these circumstances PDH activation is not maximal (since in these cells higher activity values can be reached), PDH is activated instead of inhibited as is the case in similarly exposed cells of controls [8] and gliclazide at 10 ng ml^{-1} is ineffective. If gliclazide ineffectiveness is because the structures targeted by insulin at $50 \mu\text{U ml}^{-1}$ are not targeted by gliclazide, this would imply that in both cell groups insulin at concentrations around $50 \mu\text{U ml}^{-1}$ targets different structures from those it targets at around $5 \mu\text{U ml}^{-1}$. In this case reversed PDH response to insulin at $50 \mu\text{U ml}^{-1}$ in cells of patients would support the view that in these cells structures targeted by insulin at high concentrations are also impaired. If instead these structures are the same as those targeted by insulin at around $5 \mu\text{U ml}^{-1}$, their insensitivity to gliclazide at 10 ng ml^{-1} could be attributed to high insulin levels, that partially overcome (PDH is not maximally stimulated) the inadequate binding potency for insulin suggested above.

In vitro, at higher concentrations than $10\text{--}15 \text{ ng ml}^{-1}$, gliclazide alone also affects PDH in cells of controls and patients, presumably by means of plasma membrane components (as is the case for insulin, in lysed cells the drug has no effect on PDH) and evidence supports this view [6]. Here too, there are differences between the two cell groups. In cells of patients at $50\text{--}500 \text{ ng ml}^{-1}$ the drug determines an increase of PDH activity up to the basal levels of cells of controls; in the latter cells, activation takes place at 25 ng ml^{-1} , is maximal at 50 ng ml^{-1} and from 100 ng ml^{-1} progressive inhibition, significant at 500 ng ml^{-1} , occurs. Further, in both cell groups the structures that are targeted by gliclazide at these concentrations, have multiple binding sites (Hill coefficients differ and are not equal to 1) and in cells of patients are defective in that $E_{\max}$ is 20% lower than in cells of controls, whereas potency is only slightly lower. The defect should thus be referable to an intrinsic impaired capacity of the structures in responding to drug stimuli and not to reduced binding affinity. This is the opposite of that described above for the defect affecting structures targeted by gliclazide at concentrations below 10 ng ml^{-1} , thereby supporting the view that the drug has two targets on the plasma membrane and both are impaired in cells of patients.

In treated patients blood gliclazide levels are in the range from $100\text{--}1000 \text{ ng ml}^{-1}$ and gliclazide, similarly to that of other sulphonylurea drugs, is 85–97% bound to serum

proteins [42]. This means that in treated patients gliclazide concentrations vary within a range that allows the drug to enhance insulin control over PDH (effects raised below 15 ng ml^{-1}) or to be effective on the enzyme on its own (50 ng ml^{-1} upwards). In the latter case the underlying impairment at the plasma membrane level, characterizing these cells, determines the success of treatment. In fact were the drug to retain the biphasic effect it has in controls, it would be difficult in patients under treatment to keep blood drug levels within the relatively narrow range that *in vitro* determines enzyme activation, i.e. $25\text{--}50 \text{ ng ml}^{-1}$ (below 25 ng ml^{-1} it would be ineffective and above 50 ng ml^{-1} it would inhibit an already inhibited enzyme). From the aforesaid, which of the above suggested two mechanisms is operative would depend on the actual drug free fraction. In this context it is noteworthy that in skeletal muscle of NIDDM patients the control exerted by insulin on glycogen synthase, the rate limiting enzyme in glucose consumption through non-oxidative pathways, is impaired but improves in gliclazide treated patients [1–3, 43, 44]. This supports the view that the effects that gliclazide has, *in vitro*, in potentiating insulin control over PDH, apply *in vivo* as well. In this respect it is of interest that blood insulin levels in patients before and after treatment remain unaltered.

Although the use of PDH in circulating lymphocytes, as a surrogate marker for PDH activity in tissues actively involved in the preservation of glucose homeostasis, remains to be established, the results of this study might contribute to understanding of the mechanisms through which sulphonylureas behave as hypoglycaemic agents.

The authors are indebted to Professor S. Bedino (Department of Medicina e Oncologia Sperimentale, Sezione di Biochimica, University of Turin) for the help given in the analysis of the kinetic parameters and in graph preparation. This study was supported by a research grant from 'Ministero dell' Università e della Ricerca Scientifica e Tecnologica' (Italy).

References

- 1 Prince MJ, Olefsky JM. Direct *in vitro* effect of a sulphonylurea to increase human fibroblast insulin receptors. *J Clin Invest* 1980; **66**: 608–611.
- 2 DeFronzo RA, Simonson DC. Oral sulphonylurea agents suppress hepatic glucose production in non-insulin-dependent diabetic individuals. *Diabetes Care* 1984; **7** Suppl 1: 72–80.
- 3 Gerich JE. Oral hypoglycemic agents. *N Engl J Med* 1989; **321**: 1231–1245.
- 4 Henquin JC. The fiftieth anniversary of hypoglycaemic sulphonamides. How did the mother compound work?. *Diabetologia* 1992; **35**: 907–912.
- 5 Müller G, Wied S. The sulphonylurea drug glimepiride stimulates glucose transport, glucose transporter translocation and dephosphorylation in insulin-resistant rat adipocytes *in vitro*. *Diabetes* 1993; **42**: 1852–1867.
- 6 Müller G, Dearey EA, Pünter J. The sulphonylurea drug, glimepiride, stimulates release of glycosylphosphatidylinositol-anchored plasma-membrane proteins from 3T3 adipocytes. *Biochem J* 1993; **289**: 509–521.
- 7 Bahr M, Von-Holtey M, Müller G, Eckel J. Direct stimulation of myocardial glucose transport and glucose transporter-1 (GLUT1) and GLUT4 protein expression by the

- sulphonylurea glimepiride. *Endocrinology* 1995; **136**: 2547–2553.
- 8 Rinaudo MT, Curto M, Rabbone I, et al. Effect of sulphonylurea agents on pyruvate dehydrogenase activity in circulating lymphocytes from patients with non-insulin-dependent diabetes mellitus (NIDDM). *J Diab Comp* 1994; **8**: 221–225.
- 9 Mandarino LJ. Regulation of skeletal muscle pyruvate dehydrogenase and glycogen synthase in man. *Diabetes-Metabolism Rev* 1989; **5**: 475–486.
- 10 Eriksson J, Franssila-Kallunki A, Ekstrand A, et al. Early metabolic defects in persons at increased risk for non-insulin-dependent diabetes mellitus. *N Engl J Med* 1989; **321**: 337–343.
- 11 Beck-Nielsen H, Vaag A, Damsbo P, et al. Insulin resistance in skeletal muscle in patients with NIDDM. *Diabetes Care* 1992; **15**: 418–429.
- 12 Kelley DE, Mandarino LJ. Hyperglycemia normalizes insulin-stimulated skeletal muscle glucose oxidation and storage in non-insulin-dependent diabetes mellitus. *J Clin Invest* 1990; **86**: 1999–2007.
- 13 Kelley DE, Mokan M, Mandarino LJ. Intracellular defects in glucose metabolism in obese patients with NIDDM. *Diabetes* 1992; **41**: 698–706.
- 14 Mandarino LJ, Madar Z, Kolterman OI, Bell JO, Olefsky JM. Adipocyte glycogen synthase and pyruvate dehydrogenase in obese and type II diabetic subjects. *Am J Physiol* 1986; **251** (Endocrinol. Metab.14), E489–496.
- 15 Wieland OH, Siess E, Schulze-Wethmar FH, Von Fimcke HG, Winton B. Active and inactive forms of pyruvate dehydrogenase in rat heart and kidney. Effect of diabetes, fasting and refeeding on pyruvate dehydrogenase interconversion. *Arch Biochem Biophys* 1971; **143**: 593–601.
- 16 Wieland OH, Patzelt C, Löffler G. Active and inactive forms of pyruvate dehydrogenase in rat liver. Effect of starvation and refeeding and of insulin treatment on pyruvate dehydrogenase interconversion. *Eur J Biochem* 1972; **26**: 426–433.
- 17 Stansbie D, Denton RM, Bridges BJ, Pask HT, Randle PJ. Regulation of pyruvate dehydrogenase and pyruvate dehydrogenase phosphate phosphatase activity in rat epididymal fat-pads. *Biochem J* 1976; **154**: 225–236.
- 18 Hagg SA, Taylor SI, Ruderman NB. Glucose metabolism in perfused skeletal muscle. Pyruvate dehydrogenase activity in starvation, diabetes and exercise *Biochem J* 1976; **158**: 203–210.
- 19 Rinaudo MT, Curto M, Bruno R. Effect of insulin on pyruvate dehydrogenase in a mixture of plasma membranes and mitochondria from normal and alloxan treated rat brains. *Int J Biochem* 1986; **18**: 603–607.
- 20 Rinaudo MT, Curto M, Bruno R, Marino C, Mostert M. Effects of insulin on pyruvate dehydrogenase in circulating lymphocytes from normal and diabetic rats. *Int J Biochem* 1988; **20**: 667–674.
- 21 Mondon CE, Jones IR, Azhar S, Hollenbech CB, Reave GM. Lactate production and pyruvate dehydrogenase activity in fat and skeletal muscle from diabetic rats. *Diabetes* 1992; **41**: 1547–1554.
- 22 Rinaudo MT, Curto M, Bruno R, et al. The behaviour of pyruvate dehydrogenase in circulating lymphocytes from diabetic children. *Diabetologia* 1989; **32**: 245–248.
- 23 Pellacani A, Curto M, Piccinini M, et al. Pyruvate dehydrogenase (PDH) as a marker of impaired glucose tolerance (IGT). *Diabetologia* 1995; **122**: (suppl. 1), A 33.
- 24 Curto M, Piccinini M, Bruno R, Mostert M, Rinaudo MT. Insulin modulation of pyruvate dehydrogenase in human circulating lymphocytes. *Int J Biochem* 1988; **20**: 1211–1217.
- 25 Curto M, Piccinini M, Marino C, Mostert M, Bruno R, Rinaudo MT. Pyruvate dehydrogenase activation by insulin in human circulating lymphocytes and the possible pathway involved. *Int J Biochem* 1990; **22**: 99–106.
- 26 Wieland OH. The mammalian pyruvate dehydrogenase complex: Structure and regulation. *Rev Physiol Biochem Pharmacol* 1983; **96**: 123–170.
- 27 Hughes WA, Denton RM. Incorporation of ^{32}P into pyruvate dehydrogenase phosphate in mitochondria from control and insulin-treated adipose tissue. *Nature* 1976; **264**: 471–473.
- 28 Popp DA, Kiechle FL, Kotagal N, Jarett L. Insulin stimulation of pyruvate dehydrogenase in an isolated plasma membrane-mitochondrial mixture occurs by activation of pyruvate dehydrogenase phosphatase. *J Biol Chem* 1980; **255**: 7540–7543.
- 29 Thomas AP, Diggle TA, Denton RM. Use of toluene-permeabilized mitochondria to study the regulation of adipose tissue pyruvate dehydrogenase *in situ*. *Biochem J* 1986; **238**: 83–91.
- 30 Rutter GA, Diggle TA, Denton RM. Regulation of pyruvate dehydrogenase by insulin and polyamines within electroporated fat-cells and isolated mitochondria. *Biochem J* 1992; **285**: 435–439.
- 31 National Diabetes Data Group. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *Diabetes* 1979; **28**: 1039–1045.
- 32 Curto M, Piccinini M, Cerutti F, et al. The insulin signal and its effects on the pyruvate dehydrogenase complex in circulating lymphocytes of obese children. *Int J Biochem* 1992; **24**: 831–837.
- 33 Teague WM, Pettit FH, Wu TL, Silberman SR, Reed LJ. Purification and properties of pyruvate dehydrogenase phosphatase from bovine heart and kidney. *Biochemistry* 1982; **21**: 5585–5592.
- 34 Reich JG, Wangemann G, Falk M, Rohde K. A general strategy for parameter estimation from isosteric and allosteric kinetic data and binding measurements. *Eur J Biochem* 1972; **26**: 368–379.
- 35 Saltiel AR, Siegel MI, Jacobs S, Cuatrecasas P. Putative mediators of insulin action: Regulation of pyruvate dehydrogenase and adenylate cyclase activities. *Proc Natl Acad Sci USA* 1982; **79**: 3513–3517.
- 36 Gottschalk WK, Jarett L. The insulinomimetic effects of the polar head group of an insulin-sensitive glycopospholipid on pyruvate dehydrogenase in both subcellular and whole cell assays. *Arch Biochem Biophys* 1988; **15**: 175–185.
- 37 Jarett L, Seals JR. Pyruvate dehydrogenase activation in adipocyte mitochondria by an insulin generated mediator from muscle. *Science* 1979; **206**: 1407–1408.
- 38 Cheng K, Galasko G, Huang L, Kellog J, Larner J. Studies of the insulin mediator—II Separation of two antagonistic biologically active materials from fraction II. *Diabetes* 1980; **29**: 659–661.
- 39 Jarett L, Kiechle FL, Popp DA, Kotagal N, Gavin JR. Differences in the effects of insulin on the generation by adipocytes and IM-9 lymphocytes of a chemical mediator which stimulates the action of insulin on pyruvate dehydrogenase *Biochem Biophys Res Commun* 1980; **96**: 735–741.
- 40 Saltiel AR. Second messengers of insulin action. *Diabetes Care* 1990; **13**: 244–256.
- 41 Lilley K, Zhang C, Villar-Palasi C, Larner J, Huang L. Insulin mediator stimulation of pyruvate dehydrogenase phosphatase. *Arch Biochem Biophys* 1992; **296**: 170–174.
- 42 Campbell DB, Taylor A, Taylor D, et al. The biodisposition

- and pharmacodynamics of gliclazide in man. FADL Publishers, Servier Research & Development Ltd, Fulner Hall, Slough, Bucks, England 1987: 24–38.
- 43 Damsbo P, Vaag A, Hother-Nielsen O, Beck-Nielsen H. Reduced glycogen synthase activity in skeletal muscle from obese patients with and without type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 1991; **34**: 239–245.
 - 44 Bak JF, Schmitz O, Sorensen NS, Pedersen O. Postreceptor effects of sulphonylurea on skeletal muscle glycogen synthase activity in type II diabetic patients. *Diabetes* 1989; **38**: 1343–1350.

(Received 7 April 1997,
accepted 11 November 1997)