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Does cellular glucose transport respond to a controlled diet and sulfonylurea therapy in type 2 diabetes mellitus?

Czy leczenie dietą i pochodną sulfonylomocznika wpływa na dokomórkowy transport glukozy u chorych na cukrzycę typu 2?

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Abstract

Introduction: The normalization of cellular glucose assimilation is the basic aim of therapy in diabetes mellitus. This process should be accompanied by a proportional increase of the cellular glucose transport (CGT). The level of CGT should react to therapy typically recommended in Type 2 diabetes mellitus (T2DM), composed of diet and sulfonylurea. In order to explore this clinically significant hypothesis, a clinical-experimental study was undertaken. Its aim was to determine the clinical pharmacotherapeutic significance of CGT measurements.

Material and methods: CGT testing was performed on peripheral blood lymphocytes. CGT was assessed with 2-[^{3}H(G)] glucose: before, and after the addition of sulfonylurea or sulfonylurea plus insulin to the incubation medium. Tests were performed at baseline in 28 persons with newly diagnosed, "therapeutically naive" T2DM and in 20 control subjects. In diabetic patients the tests for CGT were repeated after 3 months of routine diet and sulfonylurea therapy. In addition, the level of GLUT4 expression was assessed by flow cytometry before and after this therapy.

Results: Before treatment, CGT was significantly decreased in all subjects with T2DM. Incubated *in-vitro* cells responded directly to the addition of sulfonylurea with a moderate increase of CGT. This response was augmented by the addition of insulin to sulfonylurea in the incubation medium. The monitored three-month routine, controlled therapy with diet and sulfonylurea resulted in a significant increase of CGT process in all types of incubation tests.

Conclusions: The basal and reactive CGT is significantly decreased in lymphocytes of persons with T2DM before the introduction of therapy. Effective therapy with diet and sulfonylurea normalizes both types of CGT — basal and reactive. It is related to the near normalization of GLUT4 expression in the studied cells. This phenomenon may be used as a new marker for diabetes mellitus pharmacotherapy. **(Pol J Endocrinol 2010; 61 (1): 75–81)**

Key words: *sulfonylurea, cellular glucose transport, lymphocyte model for cellular glucose transport, GLUT4 expression, pharmacotherapeutic markers*

Streszczenie

Wstęp: Podstawowym celem w leczeniu cukrzycy jest normalizacja komórkowej asymilacji glukozy. Temu procesowi musi towarzyszyć proporcjonalne zwiększenie dokomórkowego transportu glukozy (CGT, *cellular glucose transport*). Wielkość CGT powinna zmieniać się w odpowiedzi na leczenie cukrzycy typu 2, które obejmuje dietę i stosowanie pochodnych sulfonylomocznika. Aby zbadać tę istotną z klinicznego punktu widzenia hipotezę, przeprowadzono badanie kliniczno-doświadczalne. Miało ono na celu określenie znaczenia pomiarów CGT w ocenie skuteczności farmakoterapii.

Materiał i metody: Dokomórkowy transport glukozy oceniono na podstawie poboru glukozy 2-[^{3}H(G)] przez limfocyty krwi obwodowej badanych osób przed i po dodaniu do medium pochodnej sulfonylomocznika lub pochodnej sulfonylomocznika i insuliny. Pomiar przeprowadzono wyjściowo u 28 chorych z nowo rozpoznaną cukrzycą typu 2, niestosujących wcześniej żadnego leczenia, i u 20 zdrowych ochotników stanowiących grupę kontrolną. W grupie chorych na cukrzycę pomiar CGT powtórzono po 3 miesiącach stosowania diety i pochodnych sulfonylomocznika. Ponadto przed rozpoczęciem terapii i po jej zastosowaniu oceniono poziom ekspresji GLUT4 za pomocą cytometrii przepływowej.

Wyniki: Przed leczeniem u wszystkich chorych na cukrzycę typu 2 CGT był istotnie zmniejszony. W komórkach inkubowanych *in vitro* bezpośrednią odpowiedzią na dodanie do medium pochodnej sulfonylomocznika był umiarkowany wzrost CGT. Odpowiedź ta była silniejsza, kiedy do inkubowanych komórek poza pochodną sulfonylomocznika dodano również insulinę. Po 3 miesiącach kontrolowanego leczenia dietą i pochodną sulfonylomocznika stwierdzono istotne zwiększenie CGT we wszystkich inkubowanych próbkach.

Wnioski: Przed zastosowaniem terapii podstawowy i reaktywny CGT w limfocytach osób chorych na cukrzycę typu 2 był istotnie obniżony. Skuteczne leczenie dietą i pochodną sulfonylomocznika spowodowało normalizację CGT — zarówno podstawowego, jak i reaktywnego. Powyższe korzystne zmiany były następstwem znacznej poprawy ekspresji GLUT4 w badanych komórkach. To zjawisko może zostać wykorzystane jako nowy wskaźnik w ocenie farmakoterapii cukrzycy typu 2. **(Endokrynol Pol 2010; 61 (1): 75–81)**

Słowa kluczowe: *pochodna sulfonylomocznika, dokomórkowy transport glukozy, limfocytarny model dokomórkowego transportu glukozy, ekspresja GLUT4, wskaźniki farmakoterapeutyczne*

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Introduction

The bilayer lipid membrane of the majority of cells in the human body is impermeable to monosacharides, and, therefore, glucose needs a biological transport system consisting of specialized transport proteins — glucotransporters [1, 2]. These proteins provide the physiological balance between the glucose cell supply and its further metabolism [3, 4].

Cellular glucose transport (CGT) is a complex multistage process. Individual stages of the process are coordinated by specialized regulatory systems. In physiological conditions, the sensitive quantitative coordination between the activity of the molecular CGT system and the glucose intracellular metabolism is related to glycaemia. Insulin is among the most important regulators of this relationship [5].

In diabetes mellitus, however, this physiological regulation becomes impaired. Glucose utilization by the cells is diminished despite the existence of hyperglycaemia. This is mostly because of disturbances of insulin regulatory action on CGT due to its deficiency or to insulin resistance. This pathogenetic phenomenon concerns CGT efficiency. Determination of the CGT process may point to a new specific pathophysiological background of hyperglycaemia which could be important for more precise differentiation of type 2 diabetes mellitus subtypes. It may also serve as an index of therapeutic efficiency.

In order to answer these pathophysiologically and clinically important questions, special research experiments were designed and performed. The investigations included:

— the comparative assessment of CGT in healthy volunteers and in newly diagnosed type 2 diabetes mellitus patients who had not previously been treated in any way;
— the assessment of the influence of type 2 diabetes mellitus treatment with diet and sulfonylurea compound on CGT;
— the study of changes of GLUT4 expression — the glucotransporter playing the most important role in CGT — which could take place as an effect of type 2 diabetes mellitus routine treatment with diet and sulfonylureas.

Peripheral blood lymphocytes of patients with type 2 diabetes mellitus and of healthy subjects were used as the cell model for the CGT *in vitro* testing.

Material and methods

Clinical characteristics of examined patients

Type 2 diabetes mellitus group

A group of 28 patients with type 2 diabetes mellitus who were not previously treated pharmacologically was qualified for the study. Clinical diagnosis of type 2 diabetes was made according to World Health Organization criteria. The qualification of the study population was accepted by the University Bioethics Council. The chosen subjects had no pathological disorders other than type 2 diabetes mellitus.

Control group

The control group was made up of 20 healthy volunteers with no family history of diabetes mellitus Qualification was based on a complete clinical examination in accordance with the full protocol of Warsaw Medical University clinical standards.

In patients with type 2 diabetes, the eight-point daily glycemia profile and HbA_{1c} measurements were performed. In the control group, an oral glucose tolerance test (75.0 g) was performed according to WHO standards.

Results of the initial clinical examination of both groups are presented in Table I.

Plan of the CGT process and GLUT4 expression testing experiments

CGT assessment in peripheral blood lymphocytes *in vitro*:

— The lymphocyte suspension samples were tested in accordance with the study plan, as taken from:
  • 20 healthy control subjects,
  • 28 type 2 diabetes mellitus patients with no previous treatment of hyperglycaemia, before and after a period of three-month controlled therapy with diet and sulfonylurea.
— The influence of sulfonylurea treatment on the CGT was studied in three independent series of lymphocyte incubation tests:
  • with neither sulfonylurea nor insulin added,
  • with sulfonylurea „in substantia" added,
  • with both sulfonylurea „in substantia" and insulin added.

Table I. *Comparative clinical characteristics of the 2 study groups — diabetes mellitus type 2 and control group*

Tabela I. *Porównawcza charakterystyka kliniczna 2 badanych grup — chorych na cukrzycę typu 2 i grupy kontrolnej*

Parameter	Diabetes mellitus type 2 group (before the treatment) n = 28		Control group n = 20		P
	Mean	SD	Mean	SD	
Age (years)	58.0	7.0	48.6	11.4	0.003
Sex M/F	20/8		8/12		
BMI [kg/m^2]	28.49	2.21	27.68	2.44	0.235
Blood pressure [mm Hg]:					
— systolic	135.4	14.8	127.3	13.3	0.060
— diastolic	79.3	8.0	77.0	9.2	0.365
Resting heart rate [min]	80.0	7.4	80.4	8.7	0.865
FPG [mmol/L]	8.05	1.03	5.13	0.66	< 0.001
FSI [μU/ml]	12.15	7.88	7.92	3.76	0.019
HOMA-IR	4.368	2.806	1.844	1.008	< 0.001
HbA_{1c} (%)	6.83	0.86			–
Creatinine, serum [mg/ml]	1.020	0.310	0.944	0.177	0.291
AspAT [IU/dL]	25.5	8.1	20.6	5.9	0.024
AlAT, [IU/dL]	26.9	13.6	24.0	10.4	0.426

FPG — fasting plasma glucose; FSI — fasting serum insulin; HOMA-IR — homeostatic model assessment — insulin resistance; AspAT — aspartate transaminase, serum; AlAT — alanine transaminase, serum

The CGT level was measured at the 15th, 30th, and 60th minutes of incubation.

In all cases and samples, the viability of lymphocytes was tested with the trypan blue method.

GLUT4 expression:

GLUT4 expression was measured in the lymphocytes of the same subjects who had CGT determined:

— in 10 healthy control subjects;
— in 10 type 2 diabetes mellitus patients;
— before and after sulfonylurea therapy.

Laboratory methods

Measurement of deoxy-D-glucose uptake by lymphocytes

The incubation tests were performed according to previously described methodology [6–8], adapted to our research laboratory.

To the 290 μl of suspension containing 300 000 lymphocytes, 1.5 μl of deoxy-D-glucose, 2-[^{3}H(G)] – 185––370 GBq (5–10 Ci) mmol (NEN Life Science Products, Inc.) and 7.5 μl of PBS solution (Phosphate Buffer Saline) was added. Deoxy-D-glucose, 2-[^{3}H(G)] uptake was measured after 15, 30, and 60 minutes of incubation:

Glucotransporter 4 expression measurements with the use of flow cytometry

Mononuclear cells were isolated from blood samples on Gradisol L (fluid density of 1.077 g/L, „AQUA-MEDICA") and washed twice in 0.9% NaCl.

To mark the population of cells that presented GLUT 4 protein expression, monoclonal antibody (MoAb) anti-GLUT4 was used together with single colour, indirect immunofluorescence technique.

For data acquisition and analysis, a FascCalibur flow cytometer (BectonDickinson, USA) with CellQuest software (Becton-Dickinson) was used. The results were given as the percentage of cells presenting the expression of the investigated protein (9).

Viability test

Testing lymphocytes with trypan blue showed that the experimental methods used had not influenced lymphocyte viability.

Statistical analysis

Comparison of clinical, demographic, CGT, and GLUT4 expression parameters in the examined groups was performed using the Student, Mann Whitney, or Chi-square tests, as appropriate. Variations in glucose upta-

Table II. *CGT efficiency measured in incubation tests,* **in vitro***, for peripheral blood lymphocytes taken from 20-control subjects expressed in pg/300 000 lymphocytes*

Tabela II. *Skuteczność CGT mierzona w testach inkubacyjnych,* **in vitro***, w limfocytach krwi obwodowej pobranych od 20 zdrowych osób z grupy kontrolnej wyrażona w pg/300 000 limfocytów*

Sample	Model	Incubation time [min]	CGT intensity [pg/300 000 lymphocytes]				
			Min	Max	Median	Mean	SD
1	Lymphocytes, no drugs added	15′	62.4	156.0	128.5	123.1	25.7
		30′	88.0	305.6	237.2	228.7	58.8
		60′	127.9	593.4	479.5	442.8	137.8
2	Lymphocytes, gliclazide in substantia added	15′	94.5	268.0	219.8	203.6	49.1
		30′	212.6	547.4	451.0	417.1	107.1
		60′	289.2	860.3	726.3	664.1	186.8
3	Lymphocytes, gliclazide in substantia and insulin added	15′	118.1	399.4	346.8	308.8	87.2
		30′	285.5	855.5	704.2	647.7	181.5
		60′	292.7	876.8	721.7	666.7	186.4
p		sample 2 *v.* 1			15′ < 0.001		
					30′ < 0.001		
					60′ < 0.001		
		sample 3 *v.* 2			15′ < 0.001		
					30′ < 0.001		
					60′ 0.469		

ke in peripheral blood lymphocytes were analyzed with Student's test [10].

Results

CGT assessment in peripheral blood lymphocytes — control group and diabetes mellitus group before therapy with diet and sulfonylurea

1. CGT incubation tests with lymphocytes of control subjects. The tests showed the existence of active CGT. Its effects clearly increased with the duration of incubation. The addition of sulfonylurea to the incubation environment enhanced the CGT by a statistically significant degree for all investigated incubation periods. The addition of sulfonylurea and insulin together intensified the CGT at periods of 15 and 30 minutes. This phenomenon did not appear at the incubation period of 60 minutes (Table II).
2. Incubation tests in type 2 diabetes patients.

When sulfonylurea „in substantia" was not added to the incubation environment, the CGT values were lower than were those in healthy control subjects. The influence of sulfonylurea and sulfonylurea together with insulin added to the incubation environment was distinctly weaker in samples from diabetic patients not treated pharmacologically, in comparison with healthy control subjects (Table III).

Assessment of the CTG in peripheral blood lymphocytes after prolonged therapy with diet and sulfonylurea

Diabetes mellitus metabolic compensation parameters, as compared to those measured before the start of typical treatment and after 12 weeks of treatment with diet and sulfonylurea, improved significantly.

Fasting plasma glucose concentration significantly decreased (on average by 1.77 mmol/L, $P < 0.001$). This was associated with a decrease of the HbA_{1c} level (on average by 0.69%, $P < 0.001$). Fasting plasma insulin concentration increased at the same time, on average, by 2.65 μU/ml ($P < 0.022$). The HOMA-IR index did not change.

In type 2 diabetes mellitus treated typically for 12 weeks with diet and sulfonylurea, the CGT intensity was significantly and consistently higher than before therapy. The significant therapeutic reactivity of the CGT was clearly observed (Table IV).

GLUT4 expression measurement

The results of GLUT4 expression measurement in peripheral blood lymphocytes for control subjects and for type 2 diabetic patients under study, measured before the start of the diet and sulfonylurea treatment and after 12 weeks of such routine therapy, are presented in Table V.

Table III. *CGT intensity measured in incubation tests,* **in vitro**, *for peripheral blood lymphocytes taken from 28 type 2 diabetes patients, at baseline, expressed in pg/300 000 lymphocytes*

Tabela III. *Intensywność CGT mierzona w testach inkubacyjnych,* **in vitro**, *w limfocytach krwi obwodowej pobranych od 28 chorych na cukrzycę typu 2, wyjściowo wyrażone w pg/300 000 limfocytów*

Sample	Model	Incubation time [min]	CGT intensity [pg/300 000 lymphocytes]				
			Min	Max	Median	Mean	SD
1	Lymphocytes, no drugs added	15′	20.4	147.9	108.7	106.4	25.3
		30′	62.3	225.6	188.4	179.4	32.3
		60′	79.2	355.3	265.2	263.1	47.8
2	Lymphocytes, gliclazide in substantia added	15′	20.5	179.6	116.1	113.5	25.8
		30′	41.4	248.8	205.4	199.0	36.3
		60′	89.4	380.3	332.6	316.2	57.8
3	Lymphocytes, gliclazide in substantia and insulin added	15′	66.9	231.3	187.4	186.4	28.4
		30′	79.5	373.1	311.9	293.3	56.7
		60′	84.5	384.3	323.7	306.0	57.1
p		sample 2 *v.* 1			15′ 0.118		
					30′ < 0.001		
					60′ < 0.001		
		sample 3 *v.* 2			15′ < 0.001		
					30′ < 0.001		
					60′ < 0.001		

Table IV. *CGT intensity measured in incubation tests,* **in vitro**, *for peripheral blood lymphocytes taken from 28 diabetes type 2 patients, after the completion of 12 weeks gliclazide treatment expressed in pg/300 000 lymphocytes*

Tabela IV. *Intensywność CGT mierzona w testach inkubacyjnych,* **in vitro**, *w limfocytach krwi obwodowej pobranych od 28 chorych na cukrzycę typu 2 po zakończeniu 12-tygodniowego leczenia gliklazydem i wyrażona w pg/300 000 limfocytów*

Sample	Model	Incubation time [min]	CGT intensity [pg/300 000 lymphocytes]				
			Min	Max	Median	Mean	SD
1	Lymphocytes, no drugs added	15′	72.4	212.9	153.0	162.4	34.6
		30′	141.2	397.2	293.2	308.1	57.5
		60′	289.9	611.5	567.0	543.6	62.1
2	Lymphocytes, gliclazide in substantia added	15′	77.7	261.3	224.2	210.3	36.7
		30′	151.2	569.0	405.4	412.0	97.3
		60′	298.5	957.0	663.4	727.2	147.5
3	Lymphocytes, gliclazide in substantia and insulin added	15′	144.3	405.5	360.4	349.3	54.5
		30′	295.0	891.0	617.4	689.6	145.2
		60′	296.5	932.7	644.1	709.7	150.0
p		sample 2 *v.* 1			15′ < 0.001		
					30′ < 0.001		
					60′ < 0.001		
		sample 3 *v.* 2			15′ < 0.001		
					30′ < 0.001		
					60′ < 0.001		

Table V. *The percentage of peripheral blood lymphocytes presenting GLUT4 expression for control group and for type 2 diabetic patients before and after 12 weeks of gliclazide treatment*

Tabela V. *Odsetek limfocytów krwi obwodowej, w których stwierdzono ekspresję GLUT4 w grupie kontrolnej i u chorych na cukrzycę typu 2 przed i po 12 tygodniach stosowania gliklazydu*

Percentage of lymphocytes with GLUT4 expression					
Control subjects		Type 2 diabetes mellitus patients			
Mean	SD	Before gliclazide treatment (W0)		After 12 weeks of gliclazide treatment (W12)	
		Mean	SD	Mean	SD
0.6	0.3	15.5	9.8	2.7	1.6

p (W0 *v.* W12) = 0.002; SD — standard deviation

In control subjects, GLUT4 expression was observed in 0.6% of lymphocytes. In type 2 diabetes mellitus patients before treatment, this value was much higher — 15.5% of lymphocytes presented GLUT4 expression. Under the influence of treatment with diet and sulfonylurea, the level of GLUT4 expression was diminished to a mean value of 2.7%.

Discussion

Clinical research of CGT concerns only selected areas. It has already provided valuable information regarding the activity and regulation of CGT and glucotransporter expression in humans under various conditions, both physiological and pathological [8, 9, 11–13]. In the majority of such studies, myocytes or adipocytes were used as the experimental model. At the same time, other cell models were practically ignored. The therapeutic significance of CGT abnormalities were rarely undertaken.

There are examples of CGT pharmacological research made on animal models, which could be analyzed in connection with the presented study. Tsiani E. et al. investigated the influence of sulfonylurea on the CGT intensity and on glucose transporter distribution in rat muscle tissue cells *in vitro*. They demonstrated that sulfonylurea causes a significant increase in CGT, independently from insulin. This phenomenon correlated with the increase in GLUT1 expression in the plasma membrane of the tested cells [12].

Interesting information concerning sulfonylurea influence on CGT in rat muscles was also presented by Pulido et al. [13, 14]. Sulfonylurea increased the CGT and enhanced GLUT4 translocation to the cell membrane. This direct effect was stimulated by insulin. Sulfonylurea compound administered to rats with streptozocine induced diabetes mellitus, normalizing both GLUT4 expression and the CGT in skeletal muscle tissue [13].

The results of the CGT study indicate abnormalities in CGT function in type 2 diabetic patients. Experiments revealed that typical therapy with diet and sulfonylurea significantly affects the CGT.

Comparison of the CGT measured in incubation tests at time periods of 15, 30, and 60 minutes in healthy subjects and in type 2 diabetic patients both untreated and subjected to 12 weeks of typical diet and sulfonylurea treatment provides important information on this issue. It was found that in untreated type 2 diabetic patients, the CGT in all *in vitro* testing measurements was significantly diminished compared with the values for control subjects. In untreated type 2 diabetes mellitus, two phenomena coexisted: hyperglycaemia and significant decrease of CGT. After 12 weeks of routine controlled diet and sulfonylurea therapy, the previously decreased CGT increased to a level similar to that observed in control subjects. This phenomenon correlated with a significant decrease of glycaemia and the HbA_{1c} percentage. It could be regarded, therefore, as a measure and an aim of the therapy.

This opinion is supported by the observation of GLUT4 expression by peripheral blood lymphocytes in relation to the therapy of diabetes mellitus. Several studies so far have revealed that GLUT1, GLUT3, GLUT4, and GLUT9 are present in granulocytes and monocytes [15–17]. In lymphocytes, the expression of GLUT1, GLUT3, and GLUT9 has been shown [9, 15, 16]. Surprisingly, in the study results available to date, no GLUT4 expression in lymphocytes from healthy people has been observed [17, 18]. The expression of the GLUT1 gene in lymphocytes from rat spleen increases under the influence of physical exercise [19, 20]. On the other hand, it has been shown that sustained hypoglycaemia affects GLUT genes expression in human blood leukocytes, especially in granulocytes and monocytes. In granulocytes the level of GLUT4 increases in this situation by 73%, as compared with control samples, whereas

a simultaneous reduction of GLUT1 and GLUT3 is observed. No such effect has been observed in the lymphocyte population [18].

In type 2 diabetes mellitus patients, two effects were noted simultaneously: an increase in the CGT and a decrease in GLUT4 expression - both close to the values observed in healthy people. The respective decrease of GLUT4 expression was from the mean value of 15.5% to the mean value of 2.7% cells ($P < 0.001$) as measured with flow cytometry technique. This is also a definitely positive therapeutic effect. Our observations suggest that therapy directly affected the CGT system.

Conclusions

To summarize, routine therapy with diet and sulfonylurea in type 2 diabetes mellitus simultaneously exerted a positive influence on both CGT efficiency and GLUT4 expression.

In conclusion, it could be stated, that:

1. The incubation tests show that lymphocytes constitute a useful model for CGT investigations. In healthy subjects, the increase of CGT intensity is proportional to the incubation time. CGT values in lymphocytes from healthy control subjects and tested in vitro additionally increase under the influence of sulfonylurea and insulin.
2. In type 2 diabetes mellitus, therapeutically naive, the CGT is diminished in comparison to the healthy control group. This difference is statistically significant. The reaction of CGT to the addition of sulfonylurea or insulin is also reduced.
3. A prolonged routine controlled diet and sulfonylurea therapy, lasting 12 weeks, caused a statistically significant increase in CGT. In type 2 diabetic patients treated with diet and sulfonylurea, the increase in CGT reaches the level encountered in control subjects. This change coexists with a decrease in GLUT4 expression. Under the influence of diet and sulfonylurea routine therapy, the elevated GLUT4 expression exhibited before treatment decreased to the level observed in control subjects.

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