Endokrynologia Polska/Polish Journal of Endocrinology
Tom/Volume 61; Numer/Number 2/2010
ISSN 0423–104X

Investigations of cellular glucose transport and its regulation under the influence of insulin in human peripheral blood lymphocytes

Badania transportu glukozy i jego insulinozależnej regulacji w ludzkich limfocytach krwi obwodowej

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Abstract

Introduction: We investigated the effects of insulin on glucose transport in human peripheral lymphocytes using flow cytometry. We hypothesized that lymphocytes could be used as tools to study insulin action at the cellular level and facilitate the investigation of mechanisms that lead to insulin resistance.

Material and methods: Blood was withdrawn from 25 healthy subjects. The expression of glucose transporter (GLUT) isoforms in plasma membrane and the rates of glucose transport were determined with and without insulin (10 to 100 mU/L). Anti-CD3 phycoerythrin monoclonal antibody was used for lymphocyte gating. GLUT1, GLUT3, and GLUT4 isoforms were determined after staining cells with specific monoclonal antibodies to GLUT1, 3, and 4. Glucose transport was monitored with deoxy-D-glucose, 2-[^{3}H(G)] — 185–370 GBq.

Results: Insulin increased the uptake of deoxy-D-glucose and the expression of GLUT1, GLUT3, and GLUT4 isoforms in the plasma membrane. The optimal effects were always reached at 50 mU/L of insulin with the increase in GLUT1, 3, and 4 expression of 12%, 44%, and 38%, respectively. Mean baseline values of deoxy-D-glucose uptake were 3409 ccpm at 15 min., 6587 ccpm at 30 min., and 12525 ccpm at 60 min. of investigation. The maximal uptake in insulin-stimulated conditions was reached with 50 mU/L of insulin and went up to 12450 ccpm at 15 min., 37482 ccpm at 30 min., and 37916 ccpm at 60 min. of investigation ($p < 0.01$).

Conclusions: Peripheral blood lymphocytes may become an interesting model system to study the effects of insulin on cellular glucose transport. Flow cytometry is suitable for this investigation and may be used as a method to estimate the influence of insulin on GLUTprotein translocation and the dynamics of glucose uptake by lymphocytes. **(Pol J Endocrinol 2010; 61 (2): 182–187)**

Key words: *lymphocytes, insulin, glucose transport, flow cytometry, GLUT1, GLUT3, GLUT4*

Streszczenie

Wstęp: Celem pracy było zbadanie możliwości wykorzystania techniki cytometrii przepływowej do oceny wpływu insuliny na dokomórkowy transport glukozy w limfocytach krwi obwodowej człowieka. Celem badania było również potwierdzenie hipotezy, że limfocyty mogą stanowić interesujący model badawczy do oceny komórkowej aktywności insuliny ułatwiający wykrycie mechanizmu prowadzącego do insulinooporności.

Materiał i metody: Krew do badań pobierano na czczo na heparynę od 25 zdrowych ochotników, dotychczas nie leczonych farmakologicznie. Ekspresję izoform transporterów glukozy (GLUT) w błonie komórkowej oraz tempo transportu glukozy oznaczono bez i w obecności insuliny (od 10 do 100 mj./l). W celu oznaczenia obecności limfocytów wykorzystano przeciwciała barwione fikoerytryną anty-CD3. Obecność izoform GLUT ustalono dzięki zastosowaniu przeciwciał monoklonalnych (MoAb) anty: GLUT1, GLUT3 oraz GLUT4. Transport glukozy monitorowano z użyciem deoksy-D-glukozy, 2-[^{3}H(G)] — 185–370 GBq.

Wyniki: Insulina spowodowała wzrost wychwytu zarówno deoksy-D-glukozy, jak i ekspresji izoform GLUT1, GLUT3 i GLUT4 w błonie komórkowej. Optymalny efekt reakcji osiągnięto przy stężeniu insuliny 50 mj./l, uzyskując wzrost ekspresji GLUT1, 3 i 4 odpowiednio o 12%, 44% oraz 38%. Ilość pobranej deoksy-D-glukozy w warunkach podstawowych wynosiła średnio 3409 ccpm w 15 min, 6587 ccpm w 30 min oraz 12 525 ccpm w 60 min badania. Przy zastosowanej dawce insuliny 50 mj./l uzyskano maksymalne wartości średnie: 12 450 ccpm w 15 min, 37 482 ccpm w 30 min oraz 37 916 ccpm w 60 min badania ($p < 0,01$).

Wnioski: Limfocyty krwi obwodowej mogą stanowić interesujący model doświadczalny badań wpływu insuliny na transport glukozy. Cytometria przepływowa jest cenną metodą pozwalającą na oszacowanie wpływu insuliny na translokację białek GLUT w błonie komórkowej i wzrost tempa wychwytu glukozy przez limfocyty. **(Endokrynol Pol 2010; 61 (2): 182–187)**

Słowa kluczowe*: limfocyty, insulina, transport glukozy, cytometria przepływowa, GLUT1, GLUT3, GLUT4*

The study was part of a project lead under the grant from MniSW nr 2PO5B04330.

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Introduction

Impairment of insulin activity is a major pathomechanism of hyperglycaemic states [1]. Despite the broad spectrum of information concerning insulin-dependent regulation of glucose levels in human tissues, the role of cellular and molecular mechanisms is not fully explained [2]. This is mostly due to the fact that acquiring samples of insulin-dependent human tissues is very difficult. Acquisition of skeletal muscles and adipose tissue, i.e. the main tissues involved in insulin-dependent oxidation of glucose, is difficult and demands the use of invasive methods. Consequently, it is necessary to find an easily available experimental model. Peripheral blood lymphocytes can constitute an appropriate subject of such studies because the processes of their intracellular oxidation of glucose are rapidly intensified under the effect of insulin [3–7].

There are, however, serious obstacles in the measurement techniques based on the use of radioisotopes currently used for the assessment of the influence of insulin on these cells. First of all, in order to acquire a sufficient amount of lymphocytes, a large amount of drawn blood is needed (20 mL), which constitutes a potential problem if numerous samples are needed. Secondly, the employed methods require radiolabelled isotopes or the Western blot method, which is time-consuming and laborious and, therefore, less useful in routine application. Thirdly, all the mentioned techniques require (before measurement) the absolute isolation of cells from the blood through adherence to the vessel surface; this process activates the cells and can influence their reactivity [8].

Flow cytometry technique, as compared to earlier employed methods, possesses a range of advantages:

— it requires a small amount of drawn blood (about 5 mL);
— it is based on immunolabelling and fluorescence phenomenon, which provides accuracy and selectiveness and, what is more, its application is not complicated and the results are available within a short time;
— it does not require the isolation of lymphocytes from the rest of the leukocytic cell line; therefore, they remain in an acceptable state for metabolic activity measurements [9].

This study assesses cellular glucose transport and its regulation under the influence of insulin in peripheral blood lymphocytes with the use of flow cytometry (as an alternative method), which classifies this group of cells as a potential experimental model for the cellular activity of insulin.

Material and methods

Studied group

The studied group consisted of 25 healthy volunteers (mean age 42 ± 4 years, BMI index 24 ± 1.5 kg/m^2) with no family history of diabetes. Qualification was based on a complete medical examination accordingly to the full protocol of the Warsaw Medical University clinical standard.

Laboratory methods

Incubation of cells

An overnight fast blood sample (5 mL) was drawn from the antecubital vein and collected in a heparinised tube. Lymphocytes were isolated within two hours of venipuncture by density gradient centrifugation in Gradisol L („Aqua-Medica", Poland) (1200 × g, 20 min.). Afterwards the lymphocytes were collected and washed twice with 0.9% NaCI solution (500 × g, 10 min.). After isolation, the lymphocytes were washed twice in a transport solution (20 mM Hepes, 150 mM NaCI, 5 mM KCI, 5 mM $MgSO_4$, 1.2 mM KH_2PO4, 2.5 mM $CaCl_2$, 2 mM pyruvate, pH 7.4) and an adequate volume of transport solution was added to obtain a density of 10^6 cells/mL. This suspension was incubated for 60 minutes in a buffer with different concentrations of human insulin (0, 10, 25, 50, 100, and 200 mU/mL; Sigma Diagnostics, St. Louis, MO) depending on the experimental conditions.

Measurement of deoxy-D-glucose uptake by lymphocytes

The incubation tests were performed according to the previously-described methods [10–13] adapted to our research laboratory.

The 1.5 μL of deoxy-D-glucose, 2-[^{3}H(G)] — 185–370 GBq (5–10 Ci) mmol (NEN Life Scence Products, Inc.) and 7,5 μL of PBS solution was added to the 290 μL of suspension containing 300 000 lymphocytes. PBS (Phosphate Buffer Saline) was added. Deoxy-D-glucose, 2-[^{3}H(G)] uptake was measured after 15, 30 and 60 minutes of incubation:

At the measurement time points, as defined in the protocol, the transport was stopped by the addition of 2 vol of 50 mM glucose in PBS. Then the lymphocytes were washed three times in the same solution and the cells were lysed with 0.1 mM NaOH/0.1% SDS (sodium dodecylsulphate). The following day, after cells lysis (within 24 h), the radioactivity was determined using a scintillation counter (Wallac 1450 MicroBeta Trilux). Each procedure was performed in triplicate.

Glucotransporter expression measurements with flow cytometry

To mark the population of cells that present GLUT1, 3, or 4 protein expression monoclonal antibodies (MoAb), anti-GLUT1, 3, and 4 were used together with single colour, indirect immunofluorescence technique.

For each staining process, a sample of 10^6 mononuclear cells was taken. The cells were incubated for 30 minutes with 5 μL of anti-GLUTantibody in an ice bath at 4°C and washed with PBS + 0.01% NaN_3. The cells were then incubated for 25 minutes (ice bath, temp. 4°C) with 10 μL of secondary, non-specific antibody bonding to the immunoglobulin fragment $F(ab')_2$ and conjugated with fluorochrome — fluorescein isothiocyanate (FITC). Later, the MNC cells were washed again with PBS + + 0.01% NaN_3 and suspended in 500 μL of FACS Flow (Becton-Dickinson).

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

Viability test

Testing lymphocytes with trypan blue showed that the experimental methods used had not influenced lymphocyte viability. Individual dead cells were observed in the samples both before the experiments and after their completion.

Statistical analysis

The results are presented in the form of the mean margin of statistical error (x ± SD). Glucotransporter expression, as in earlier published works from our laboratory [14, 15], was formulated as the percentage of cells displaying expression of a studied receptor.

The results of deoxy-D-glucose uptake by lymphocytes were compared with the use of nonparametric difference sign test. In the statistical analysis, $P \leq 0.05$ was assumed as the level of statistical significance.

Results

Deoxy-D-glucose uptake

The first phase of the study was to establish the circumstances of linear capture in time. Figure 1 presents the influence of increasing concentrations of insulin on the capture of deoxy-D-glucose after 15, 30, and 60 minutes of incubation. Significant changes in the absence of insulin were not noted. The addition of 10 mU/L of insulin did not trigger a statistically significant increase of dexoy-D-glucose capture compared to the trial at the 60th minute of the examination (12344 ccpm and 12525 ccpm, respectively) (Table I).

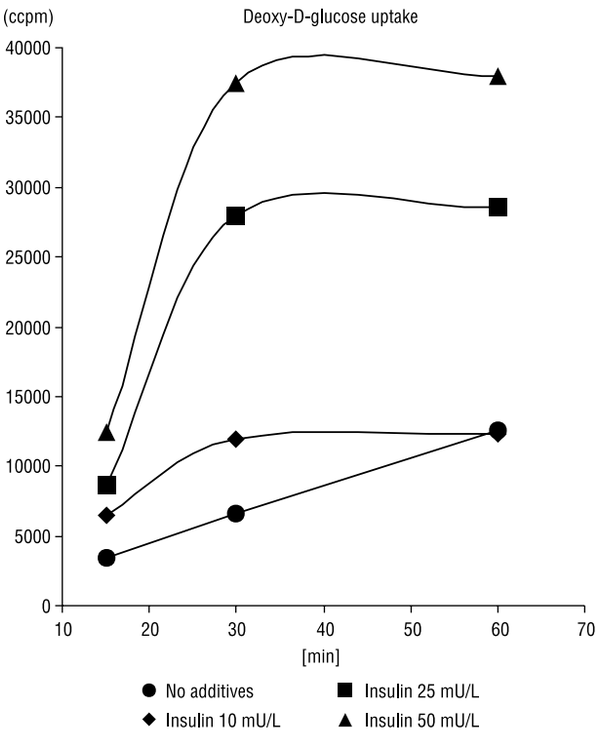

Figure 1. *Deoxy-D-glucose uptake by lymphocytes in basal conditions and insulin stimulated 10, 25, and 50 mU/L ($p < 0.05$)*

Rycina 1. *Wychwyt deoksy-D-glukozy przez limfocyty w warunkach podstawowych i po podaniu insuliny w dawce 10, 25 i 50 mj./l ($p < 0,05$)*

With the concentration of insulin at 25 or 50 mU/L, deoxy-D-glucose capture reached the plateau phase after 30 minutes of examination. Observations at this level were used to mark the curve of dose-dependent capture of deoxy-D-glucose under the influence of insulin (Fig. 2). An increase in insulin concentration to 25 mU/L triggered a 2.3 × increase of deoxy-D-glucose capture. An increase in insulin concentration to 50 mU/L caused a 3× increase of glucose capture ($P < 0.05$). Higher concentrations of insulin did not have any influence on deoxy-D-glucose capture (38137 ccpm with the level of insulin at 100 mU/L after 60 minutes of observation) (Table I).

Flow cytometry

In peripheral blood lymphocytes acquired from healthy volunteers, GLUT1, GLUT3, and GLUT4 expression was

Table I. *Influence of insulin on deoxy-D-glucose uptake by peripheral blood lymphocytes*

Tabela I. *Wpływ insuliny na wychwyt deoksy-D-glukozy przez limfocyty krwi obwodowej*

Time (min) [ccpm]	No additives [ccpm]	Insulin 10 mU/L [ccpm]	Insulin 25 mU/L [ccpm]	Insulin 50 mU/L [ccpm]	Insulin 100 mU/L
15	3409	6493	8678	12 450	13 354
30	6587	11 875	27 903	37 482	37 791
60	12 525	12 344	28 560	37 916	38 137

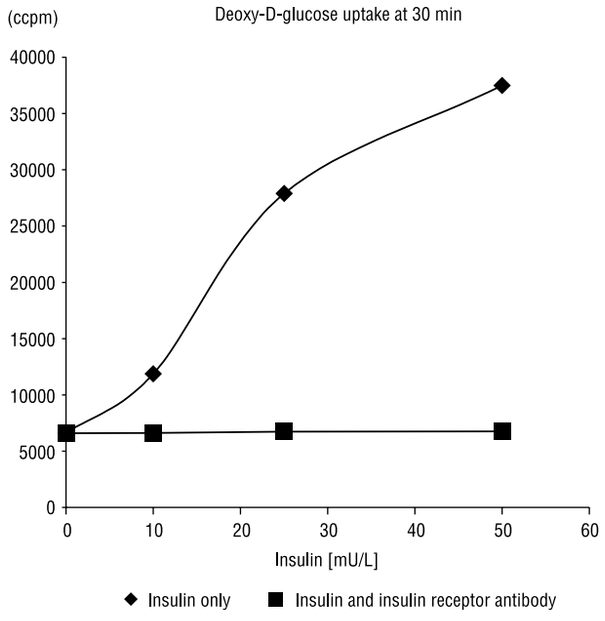

Figure 2. *The influence of insulin on deoxy-D-glucose uptake (data from 30th min. of observation). Cells were incubated with and without the presence of insulin receptor antibodies (IRA; 5 mg/mL n = 5) (p < 0.01)*

Rycina 2. *Wpływ insuliny na wychwyt deoksy-D-glukozy (dane z 30. minuty obserwacji). Komórki inkubowano bez i w obecności przeciwciał przeciw receptorom insulinowym (IRA; 5 mg/ml n = 5) (p < 0,01)*

discovered, with the following values in standard conditions: 22 ± 0.9%, 28 ± 1.2%, and 12 ± 0.6% (Table II).

Insulin caused a significant increase in the amount of GLUT3 and GLUT4 proteins in cellular membrane, by 55% and 40%, respectively (P < 0.01). The influence of insulin on the GLUT1 protein was slight (15%) (Fig. 3).

In order to establish whether 100 mU/L insulin concentration caused the maximal stimulating effect, lymphocytes were incubated with 200 mU/L of insulin and GLUT4 isoforms were marked on the cellular surface: the stimulation effect in both concentrations was the same (16.8 ± 0.9% and 16.4 ± 0.6% in the concentrations of insulin at 100 and 200 mU/L, respectively) (Table II).

Table II. *Influence of insulin on glucotransporter expression on the surface of peripheral blood lymphocytes*

Tabela II. *Wpływ insuliny na ekspresję transporterów glukozy na powierzchni limfocytów krwi obwodowej*

Insulin mU/L	GLUT1 (%)	GLUT3 (%)	GLUT4 (%)
0	22	28	12
10	23.32	33.04	13.92
25	23.76	36.68	15.84
50	24.64	40.32	16.56
100	25.3	43.4	16.8
200	–	–	16.4

Validation of used methods

The results of the experiment performed in order to validate the insulin-dependent capture of glucose and GLUTtranslocation are presented in Figures 2 and 3. Blocking of the insulin activity inhibited insulin-dependent GLUTtranslocation and capture of 2-[$^{+}$H(G)].

Discussion

The subject of our study was the measurement of insulin influence on glucose transport in lymphocytes with the use of flow cytometry technique.

Glucose transport is an important phase of cellular metabolism because of the control of oxidation percentage of this carbohydrate [16, 17]. In skeletal muscles and adipose tissue, three isoforms of transporter are present: GLUT1, GLUT3, and GLUT4 [18, 19]. Insulin causes an increase in glucose capture mainly in the translocation stimulation mechanism of GLUT4 transporter from intracellular resources to cellular membrane [18].

Flow cytometry allows determination of the degree of GLUT protein isoforms gathering in the cellular membrane and hence is an important assessment method of insulin effectiveness on the process of translocation. Expression of GLUT isoforms present on the surface of lymphocytes in metabolically healthy persons and per-

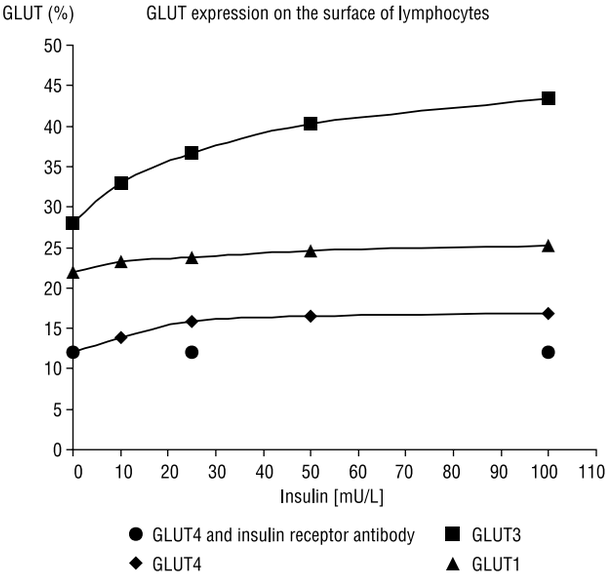

Figure 3. *The influence of insulin on the expression of GLUT1, GLUT3, and GLUT4 on the surface of peripheral blood lymphocytes. The cells, which were stained with anti-GLUT4, were incubated with or without the presence of insulin receptor antibodies (IRA; 5 mg/mL n = 5) (p < 0.01)*

Rycina 3. *Wpływ insuliny na ekspresję GLUT1, GLUT3 i GLUT4 na powierzchni limfocytów krwi obwodowej. Komórki, które zostały wybarwione przy użyciu przeciwciał i anty-GLUT4 inkubowano bez lub w obecności przeciwciał przeciw receptorom insulinowym (IRA; 5 mg/ml n = 5) (p < 0,01)*

sons with type 2 diabetes mellitus has been examined in two studies conducted in our centre to date. In the first [3] we confirmed the presence of two isoforms (GLUT1 and GLUT3), while in the second [14] we showed a two-fold higher expression of GLUT4 protein on the surface of lymphocytes in persons with type 2 diabetes mellitus in relation to the control group. In earlier studies concerning mononuclear cells of peripheral blood [7, 20, 21], the authors noted expression of GLUT1 and GLUT3 on lymphocytes acquired from healthy persons and persons with type 1 diabetes mellitus, and GLUT4 [20] in healthy persons. None of these studies marked the quantitative influence of insulin on GLUT proteins.

The results of our study show that GLUT1, GLUT3, and GLUT4 isoforms are present in human lymphocytes. The level of GLUT3 and GLUT4 isoforms in cellular membrane increases in a dose-dependent manner under the influence of insulin of physiological concentrations, while the response of GLUT1 is slight. What is more, GLUT3 isoform presents the highest sensitivity to insulin influence, which suggests that this transporter is most responsible for the insulin-dependent glucose transport to these cells (Fig. 3). It should be underlined that these results concern metabolically healthy persons, which does not mean that they can be related to persons with diabetes. These results correspond to the insulin-dependent reaction of the tissues essential because of the amount of glucose oxidation, i.e. muscles and adipose tissue [16]. For example, the increase of insulin level in the range of physiological values causes a 60% growth of GLUT4 gathering in the cellular membrane of muscles [22, 23].

The influence of insulin on the speed of glucose transport to lymphocytes was examined in our experiment with the use of deoxy-D-glucose [3], previously used by other authors for the measurement of glucose transport in myoblasts [13], mononuclear cells of peripheral blood [7], monocytes [21], and lymphocytes [11, 12], where its selectiveness towards GLUT was shown. However, none of these studies assessed the effects of insulin influence on the transport of deoxy-D-glucose through cellular membrane.

We have shown that insulin causes increased deoxy-D-glucose capture by lymphocytes. The effect of stimulation was dependent on the administered dose, mean effective doses remained in the range of physiological values (20 mU/L), and reached their peak at an insulin level of 50 mU/L. The blocking of insulin activity caused by the antibody of insulin receptor caused the inhibition of insulin-dependent deoxy-D-glucose capture, which additionally confirmed the significant influence of insulin on the cellular glucose transport in lymphocytes (Fig. 2).

The speed of glucose capture by human lymphocytes has been observed in four studies [3, 4, 7, 11]. In one of these studies, glucose capture was measured in an indirect way by measurement of glucose depletion from incubation surface [4]; in other studies, cells radiolabelled with 2-deoxy-D-glucose were incubated [3, 7, 11]. In these studies it was shown that glucose capture reaches its peak at the 30^{th} minute of the examination (later on, the plateau phase was observed), with insulin concentration exceeding 100 mU/L [4, 7]. These results are similar to those acquired in our study with the use of flow cytometry, but they differ in terms of the insulin concentrations used for the examination. In quoted works, the examined cells were acquired from patients with type 1 diabetes mellitus, i.e. displaying insulin resistance, which is a probable cause of the necessity of employment of considerably higher concentrations of insulin required for the achievement of maximal deoxy-D-glucose capture.

In humans [24] and in rats [25, 26], glucose capture in peripheral tissues (muscles and adipose tissue) can be accurately measured with the method of hyperinsulinemic euglycaemic clamp and radiolabelled glucose infusion. In these experiments, the increase of insulin level in serum to 100 mU/L caused an increase of the

speed of peripheral glucose capture by 350% to 400% [24–26]. Similar effects were acquired from in vitro examinations performed on isolated tissues. For example, in muscles and adipose tissue an increase of insulin concentration in the range of physiological norms (0–100 mU/L) increased the speed of glucose transport (measurement conducted with the use of radiolabelled analog of glucose) by about 300% to 400% [22, 23, 27–31]. The range of these effects is close to the effects observed in lymphocytes, which quantitatively suggests that these cells can constitute a good experimental model for studies of insulin influence on glucose metabolism.

As a result of the difficulties of acquisition of human cells for the examination of insulin activity on the cellular level, lymphocytes could prove to be a useful, easily available tool in studies on insulin activity disorders leading to insulin resistance, encountered, for example, in type 2 diabetes mellitus, obesity, and endocrinopathies. The insulin concentrations employed in this examination remained within the range of values normally observed in human serum.

The data acquired in the studies of lymphocytes can be combined with the results of earlier in vivo examinations with the use of methods assessing the sensitivity of a whole organism to insulin (such as hyperinsulinemic euglycaemic clamp and oral glucose-tolerance test) and can facilitate the study of the pathomechanism leading to insulin resistance. Such a method was employed in the case of binding of insulin receptor in patients with type 2 diabetes mellitus [32]. It suggests that in these cases insulin resistance was an effect of post-receptor defect of insulin activity.

Our results show that flow cytometry is a valuable method allowing the estimation of insulin influence on GLUTprotein translocation in cellular membrane and the increase of the speed of glucose capture by peripheral blood lymphocytes.

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