UDC 616.379-008.64+616-006.6+577.156.6 doi: https://doi.org/10.15407/ubj92.06.113

ACTIVATION OF THE PI3K/Akt/mTOR/p70S6K1 SIGNALING CASCADE IN PERIPHERAL BLOOD MONONUCLEAR CELLS IN PATIENTS WITH TYPE 2 DIABETES

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Received: 17 April 2020; **Accepted:** 13 November 2020

Modern research shows that patients with diabetes mellitus have an increased risk of cancer. PI3K/Akt/mTOR/p70S6K1 signaling pathway plays an important role in the pathogenesis of cancer and diabetes. The aim of this study was to determine the state of PI3K/Akt/mTORC1/p70S6K signaling cascade activity in peripheral mononuclear blood cells (PBMC) of patients with type 2 diabetes (T2D) relatively to the insulin and insulin-like growth factor (IGF-1) concentrations in blood plasma. Enzyme-linked immunosorbent assay was used to examine the levels of insulin and IGF-1 in blood plasma as well as the content of phosphorylated forms of Akt (Ser473), PRAS40 (Thr246), and p70S6K (Thr389) in PMBC. It was shown that in the blood plasma of patients with T2D, the levels of insulin and IGF-1 were increased. Phosphorylation and activation of Akt by the mTORC2 protein kinase complex was not observed. At the same time, the relative degree of phosphorylation of mTORC1 inhibitor, PRAS40, and its substrate, p70S6K, was higher in PMBC of T2D patients in comparison with control values. These data suggest that phosphoinositide-dependent protein kinase 1 (PDK1) and, possibly, mitogen-activated protein kinase (MAPK) could mediate the effects of IGF-1 on Akt activation under type 2 diabetes.

K e y w o r d s: peripheral blood mononuclear cells, Akt, PRAS40, p70S6K, insulin, insulin-like growth factor, type 2 diabetes (T2D).

Recent research and clinical findings suggest an increased risk of cancer of certain types in patients with type 2 diabetes (T2D) [1]. The mechanisms of the association between these pathologies are recognized as the effects of ER-stress, obesity, hyperinsulinemia, hyperglycemia, cytokine imbalance, and oxidative stress [2].

The signaling cascade PI3K/Akt/mTOR/p70S6K mediates the effects of insulin and insulin-like growth factor (IGF-1) in mammalian cells. Functional disturbances of this cascade lead to severe chronic diseases such as insulin resistance, T2D and cancer. Protein kinase Akt is a key effector kinase of this cascade, which enhances insulin-dependent translocation of GLUT-4 and glucose transport, activates mTORC1 (mammalian target of rapamycin complex 1) and p70S6K (ribosomal protein S6 kinase). Akt is activated by the upstream phosphoinositide-dependent protein kinase1 (PDK1) via Thr308-phosphorylation and additionally by mTORC2-dependent phosphorylation on Ser473, which is necessary for its maximum activation [3, 4]. PRAS40 is a 40 kDa proline-rich Akt substrate. Phosphorylation of PRAS40 leads to its dissociation from regulatory-associated protein of mTOR (Raptor) in the mTORC1-complex that promotes mTOR activation. mTOR controls cell growth and homeostasis, including protein synthesis, lipogenesis, glucose metabolism, autophagy, lysosome biogenesis, proliferation and survival, in response to external

signals such as levels of amino acids, glucose, energy, oxygen and growth factors. The substrate of mTORC1 is p70S6K protein kinase, which controls protein synthesis, ribosome biogenesis, cell cycle, and apoptosis [3]. The PI3K/Akt signaling pathway plays an important role in the activation of macrophages and lymphocytes, secretion of cytokines, and in the initiation of inflammatory processes in these cells [5].

The peripheral blood mononuclear cells (PBMC), which mainly consist of lymphocytes (up to 40% of the total amount of leukocytes) and monocytes/macrophages (~ 11%), are involved in the pathogenesis of diabetes and its complications. The activity of protein kinases of PI3K/Akt/mTOR/p70S6K signaling pathway in these cells may reflect the changes of its activation, polarization and cytokine secretion in patients with T2D [6].

The aim of the work was to determine the concentrations of insulin and IGF-1 in blood in association with the activity of PI3K/Akt/mTORC1/p70S6K cascade kinases in peripheral blood mononuclear cells of patients with type 2 diabetes.

Materials and Methods

The study was conducted in accordance with the guidelines of the Declaration of Helsinki (1975) and its revised version of 1983. All patients signed the informed consent to conduct further diagnostic and research studies with biomaterials.

26 patients were examined. Patients were divided into groups: I – healthy (control group) (n = 12), II – patients with T2D (n = 14). Patients were grouped accordingly to age and BMI.

Immediately after collection, blood was centrifuged using Histopaque 1077 Sigma (USA). The obtained white blood cells were washed and frozen at -80°C. The cells were lysed in a buffer for extraction from kits, containing protease and phosphatase inhibitors. Studies were conducted in triplets. The concentration of protein in lysates was determined using the Novagen kit "BCA protein assay kit" (USA).

Insulin and IGF-1 levels were determined by enzyme-linked immunosorbent assay (ELISA) using the automatic analyzer Stat fax 303+ (USA) and diagnostic kits Insulin ELISA, EIA-2935 and IGF-1 600 ELISA, EIA-4140 (DRG, Germany). The amounts of phosphorylated Akt1/2/3 (p-Ser473), p70S6K1 (p-Thr389) and PRAS40 (p-Thr246) were determined using a microplate reader of "Bio-tek Instruments" company (USA) at a wavelength of 450 nm with the diagnostic ELISA kits (85-86046, 85-86053, KHO0421, respectivel (Invitrogen, USA). T2D compensation was assessed by determining the level of HbA1c using ion-exchange chromatography and BIO-RAD D-10 analyzer, the BIO-RAD (USA) reagents.

The data obtained were analyzed using Statistica 12.0 (StatSoft Inc., USA). The data are presented in the tables as M ± SD. To compare the data between experimental groups, one-way ANOVA and Student's t-test were used. Values of $P < 0.05$ were considered as significant.

Results and Discussion

The study included people over 55 years of age. The duration of diabetes in patients of group 2 was up to 10 years, without significant gender difference. The HbA1c level in patients of group II was more than 7.5%, also without gender difference (Table 1).

Significantly higher levels of IGF-1 and, especially, insulin were observed in patients with T2D, compared to the control group (Table 2). Increased insulin levels in patients with diabetes confirm condition of hyperinsulinemia, which is associated with obesity, insulin resistance and T2D. The level of IGF-1 is connected with blood insulin content, because hyperinsulinemia increases the bioavailability of IGF-1 by reducing the content of IGF-1-binding globulin-1 [6]. Insulin also increases the expression

Table 1. Characteristics participants of the study

Indicators	Group I (control), n = 12		Group II, (patients with T2D), n = 14	
	Men (n = 6)	Women (n = 6)	Men (n = 8)	Women (n = 6)
Age, (years)	58.21 ± 1.64	61.0 ± 3.18	60.14 ± 2.74	58.30 ± 1.28
BMI, (kg/m^2)	31.12 ± 2.34	31.22 ± 1.23	32.42 ± 1.25	31.19 ± 1.47
Duration of DM, (years)	–	–	8.42 ± 4.23	9.21 ± 3.12
HbA1c, (%)	5.65 ± 0.65	5.97 ± 0.41	7.84 ± 1.12*	8.33 ± 1.38*

Note: *the difference from the relevant control group is significant ($P < 0.05$)

Table 2. The concentrations of insulin, IGF-1 in the blood and amount of phosphorylated Akt (p-Ser473), PRAS40 (p-Thr246) and p70S6K1 (p-Thr389) in PBMC of patients with T2D

Indicators	Group I, (control) $n = 12$	Group II, (patients with T2D), $n = 14$
Insulin, (mIU/ml)	7.72 ± 0.58	24.38 ± 1.99*
IGF-1, (ng/ml)	141.60 ± 6.86	175.54 ± 5.46*
Phospho-Akt (Ser473), (conv.units/mg prot.)	0.009 ± 0.003	0.009 ± 0.005
Phospho-PRAS40, (units/mg)	1.16 ± 0.02	1.71 ± 0.07*
Phospho-p70S6K, (conv.units/mg prot.)	0.012 ± 0.001	0.017 ± 0.003*

Note: *the difference in comparison with control group is significant ($P < 0.05$); conv. units/mg prot. - conventional unit/mg of protein

of IGF-1 in the liver followed by activation of IGF-1 receptor and stimulation of cell growth. In addition, hyperinsulinemia activates growth hormone receptor in the liver that causes increased secretion of growth hormone, which further stimulates the IGF-1 synthesis and proliferative processes [7].

Among patients of group II, 4 received metformin monotherapy, 10 received combination therapy with metformin and sulfonylurea derivatives.

To evaluate the interplay between insulin and IGF-1 levels with the activity state of PI3K/PDK1/Akt/mTOR/p70S6K cascade in the blood cells, Akt activation via phosphorylation at the Ser473 residue mediated by mTORC2 complex was studied. There were no significant changes of phospho-Akt amount in patients with diabetes compared to control group ($P > 0.05$). However, we observed significant (~1.5 times) increase in the content of phosphorylated PRAS40 and p70S6K in patients with T2D (Table 2) that indicates the higher level of activity of insulin-dependent Akt/mTOR/p70S6K signaling under T2D.

The effects of insulin and IGF-1 in the cell are transduced through the insulin receptor substrate (IRS1), PI3K/PDK1/Akt/mTOR/p70S6K, and Ras/MAPK signaling cascades. Ligand-induced activation of IGF-1 receptor (IGF-1R) leads to association with adaptor proteins complex Shc/Gab-1 that results in increased expression of genes involved in the control of cell proliferation. A distinctive feature of insulin receptor (IR) activation is enhanced IRS-1-phosphorylation associated with induction of genes involved in the control of metabolic pathways [7]. In addition, there are data that IGF-1 can activate the mTOR through MAPK [8].

Insulin and IGF-1 activate phosphoinositide-3-kinase (PI3K) and PDK-1, with further activation of Akt [9]. Akt is a key enzyme in the IRS/PI3K/PDK1/Akt/mTOR/p70S6K signaling pathway, which controls the activity of transcription factors, cell cycle regulators, apoptosis and cell survival [10, 11]. There is tight relationship between PDK1, Akt and the mTOR complexes through the two sites of phosphorylation - Thr308 and Ser473. Energy charge, the ratio of ATP/AMP concentration, can also determine the active and inactive state of Akt and the sites of phosphorylation. The high ATP/AMP ratio activates mTORC1 and energy-consuming synthetic processes. Under nutrient-deprived conditions, active FoxO (forkhead box protein O) enhances expression of Rictor, thus promoting mTORC2 activity [12]. There are increasing evidences that selective mTORC1 inhibition can elicit increased phosphorylation of Akt at Ser473 and thus attenuates the signal effects on tumor cell proliferation [13, 14].

It is known that in patients with T2D, mTORC1 and p70S6K activities are increased, that lead to phosphorylation of IRS-1, disruption of the insulin signaling pathway and, as a result, to insulin resistance [7, 15]. Increasing content of phosphorylated PRAS40 and p70S6K confirms the activation of the PI3K/Akt/mTORC1 signaling pathway in patients with T2D perhaps as a consequence of hyperinsulinemia. Phosphorylation of Akt1-1 substrate - PRAS40 regulates mTOR1 activity [16, 17]. PRAS40 binds Raptor, preventing the mTORC1 kinase from interaction with its substrates, and p70S6K, in particular. Phosphorylation of PRAS40 causes its sequestration from the mTORC1 complex and, thus, activation of the latter. Activated mTORC1 phosphorylates the hydrophobic motif of its downstream substrate p70S6K1 in order to initiate translation [18, 19]. Ribosomal protein S6 kinase is also involved in the PI3K-pathway and regulates protein synthesis and biogenesis of ribosomes [20].

We observed significant increase of p70S6K1-phosphorylation in PBMC of patients with diabetes. Together, these data indicate the activation of the Akt/mTOR/p70S6K kinases of the insulin cascade. It is known that p70S6K1 phosphorylates Rictor to inhibit mTORC2 [21, 22] that may affect the Akt Ser473 phosphorylation.

Thus, despite the absence of Akt activation by mTORC2, the activation of the final kinases of Akt/mTORC1/p70S6K1 cascade in blood cells was observed. Obviously, the Akt and the downstream kinases are activated through Akt phosphorylation on Thr308 by PDK1. Apparently, one can talk about the reciprocal interdependence of phosphorylation and activation of Akt. That is why, the activation of kinase, depending on the cellular context, does not occur according to the scheme Thr308 and Ser473, but - Thr308 or Ser473. It is also possible that the MAPK signaling pathway, mediating the effects of IGF-1, can be also involved in the insulin cascade activation.

PBMC, are involved in the pathogenesis of cancer, diabetes, atherosclerosis and other serious diseases. It was suggested that peripheral blood monocytes might be more important in the development of insulin resistance than skeletal muscle or adipose tissue [23]. Therefore, the study of signaling mechanisms in these cells and their disfunctions may be important for the diagnosis and prognosis of the development of diabetes and other chronic diseases.

Conclusions

1. The PI3K/Akt signaling cascade in PBMC of patients with type 2 diabetes is functionally active. Insulin signaling is not impaired. The activity of the PI3K/AkT/mTOR/p70S6K1 cascade in the blood cells of patients with T2D is significantly higher than in the control group that is probably due to high levels of insulin and IGF-1 in the blood.

2. mTORC2 seemingly is not involved in enhancing Akt/mTORC1/p70S6K1 activation in PBMC of patients with T2D. Phosphorylation of PRAS40 and p70S6K1 indicates mTORC1 activation. Activation of Akt and mTORC1 in PBMC of patients with T2D can be via insulin-mediated PDK1 or IGF-1-mediated MAPK cascade.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

Funding. The study is a fragment of the research project “Epidemiology of oncological diseases in patients with diabetes mellitus and the effect of antihyperglycemic drugs on oncogenesis markers” (registration number 0117U005263), included into the complex research work of the Ivano-Frankivsk National Medical University - “Pathogenetic mechanisms of development of changes in organs of the respiratory, endocrine, nervous systems in the modeled pathological conditions and their correction” (registration number 0117U001758).

АКТИВАЦІЯ СИГНАЛЬНОГО КАСКАДУ PI3K/Akt/mTOR/p70S6K1 В МОНОНУКЛЕАРНИХ КЛІТИНАХ ПЕРИФЕРИЧНОЇ КРОВІ ПАЦІЄНТІВ З ЦУКРОВИМ ДІАБЕТОМ 2-го ТИПУ

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Новітні дослідження довели підвищений ризик раку у хворих на цукровий діабет. Сигнальний шлях PI3K/Akt/mTOR/p70S6K1 відіграє важливу роль у патогенезі раку та діабету. Метою цього дослідження було визначення стану активності кіназ каскаду PI3K/Akt/mTOR/p70S6K1 у периферичних мононуклеарних клітинах крові (PBMC) пацієнтів із діабетом 2-го типу (T2D) відносно концентрації інсуліну та інсуліноподібного фактора росту (IGF-1) у плазмі крові. Методом імуноензимного аналізу досліджували рівень інсуліну та IGF-1 у плазмі крові, а також вміст фосфорильованих форм Akt (Ser473), PRAS40 (Thr246) та p70S6K (Thr389) у PBMC. Показано, що в плазмі крові хворих на T2D рівень інсуліну та IGF-1 підвищувався. Фосфорилування та активація Akt-комплексом протеїнкінази mTORC2 не спостерігались. У той же час відносний рівень фосфорилування інгібітора mTORC1, PRAS40 та його субстрату, p70S6K був вищим у PBMC хворих на T2D порівняно з контрольними значеннями. Ці дані свідчать про те, що

фосфоінозитидзалежна протеїнкіназа 1 (PDK1) і, можливо, мітогенактивована протеїнкіназа (MAPK) можуть опосередковувати ефекти IGF-1 щодо активації Akt за діабету 2-го типу.

Ключові слова: мононуклеарні клітини периферичної крові, Akt, PRAS40, p70S6K, інсулін, інсуліноподібний фактор росту, цукровий діабет 2-го типу.

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