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Effect of combined treatment with insulin, metformin, and gliclazide on the expression and activity of Akt, mTOR, and p70S6K protein kinases in lymphocytes of diabetic patients

Presented by Corresponding Member of the NAS of Ukraine M.D. Tronko

The expression of the main effector protein kinase Akt of the PI3K signaling pathway and the activities of mTOR and p70S6K protein kinases in lymphocytes of patients with diabetes mellitus are determined at the combination treatment with insulin and hypoglycemic drugs. It is shown that the Akt expression is reduced in blood mononuclear cells of patients with diabetes of the 2nd and, to a less extent, type 1. The activities of mTOR and p70S6K are also reduced in lymphocytes of patients with type 2 diabetes. Possible mechanisms of inhibiting the expression and activity of protein kinases are discussed.

Keywords: *Akt, mTOR, p70S6K, diabetes mellitus, metformin, insulin, gliclazide.*

Type 2 diabetes (T2D) is characterized by a phosphoinositide 3 kinase (PI3K)/Akt signaling disorder, which leads to a decrease of the glucose uptake and the insulin resistance in peripheral tissues [1]. mTOR is a serine/threonine protein kinase, which belongs to the PI3K-related kinase family and plays a key role in the cell growth regulation, as well as in lipid and glucose metabolism. Growth factors and insulin stimulate the mTORC1 complex through the PI3K signaling pathway. Activated mTORC1 promotes the phosphorylation of ribosomal S6 kinase (p70S6K), which is involved in fundamental cellular processes, including protein and lipid synthesis, cell growth, and metabolism [2].

Type 2 diabetes mellitus (T2D) is a progressive disease with a steady decrease in the function of pancreatic β-cells, which ultimately determines the inevitability of insulin therapy. Insulin therapy together with oral hypoglycemic agents is offered in the ADA guide for managing patients with T2D [3].

We study effects of combined therapy of the first-line hypoglycemic drug metformin (MF) with insulin and gliclazide (diabeton MR) upon the expression of main effector kinase Akt of PI3K pathway and the activity of its downstream kinases mTOR and p70S6K.

Materials and methods. The study was conducted in the diabetology department of the Institute. All patients signed the informed consent to conduct further diagnostic and research studies with biomaterials. Immediately after collection, blood was centrifuged using Histopaque 1077 (Sigma, USA), the lymphocytes collected were washed and frozen at –80 °C until use. The cells were lysed in an extraction buffer with inhibitors of proteases and phosphatases. The protein concentration in the lysate was determined, using the Novagen (USA) BCA protein assay kit.

Reagents. Polyclonal antibodies to Akt/PKB PH domain, phospho-mTOR (Ser2448), phospho-p70 S6 Kinase 1/2 (Thr412/Thr397) were from Millipore Corp. (USA). Horseradish peroxidase conjugated second antibodies were from the Sigma (USA). Complexes of proteins with antibodies were visualized, using ECL reagent (Amersham Life Science, UK).

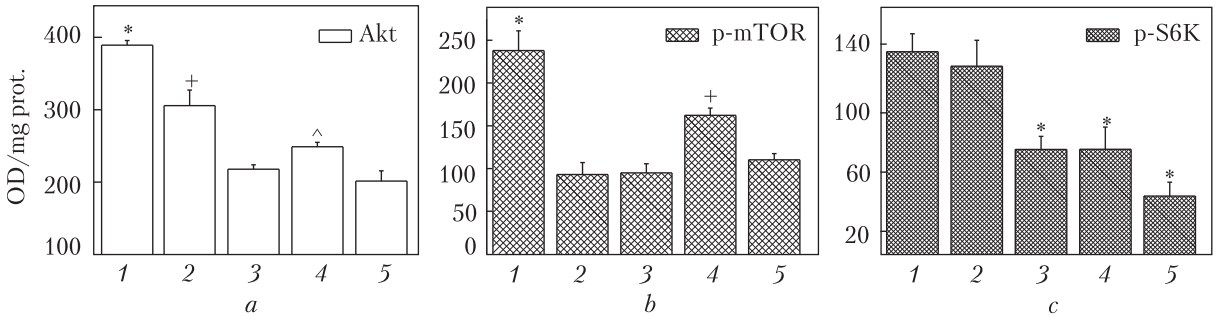
Preparation of cell lysates and Western blot analysis were performed as previously described [4]. Total cell lysates were boiled in a sample buffer (100 mM Tris-HCl, 4 % sodium dodecyl sulfate, 0.2 % bromophenol blue, 20 % glycerol, 10 % dithiothreitol) and separated by SDS-PAGE 12.5 % gels. ~30 μg of protein were applied per each lane. Proteins were transferred onto nitrocellulose membranes with 0.2 μm pores (Millipore Corp., USA) by semidry blotting. Membranes were blocked with Tris-buffered saline (TBS)/0.1 % Tween 20 containing 5 % nonfat dry milk or 5 % BSA and incubated with primary antibodies, at 4 °C overnight. After washing three times with TBS/0.1 % Tween 20, the blots were incubated with horseradish peroxidase-conjugated species-specific secondary antibody for 1 h at ambient temperature and then again washed three times. Complexes were visualized, using ECL reagents (Amersham, Life Science, UK). Developed X-ray film was scanned using GelPro 3.1 software and normalized by the protein amount of the sample and β-actin amount in each lane.

The results of the study are presented as $M \pm SD$, $n = 3$. To compare the data groups, Student's t-test was used. Values of $P \leqslant 0.05$ were considered as significant.

Results and their discussion. The patients were divided into groups: the control group (1) consisted of healthy individuals who did not have diabetes mellitus, representative by age, 2 — patients with type 1 diabetes on insulin therapy, 3 — patients with type 2 diabetes on combination therapy receiving original metformin and gliclazide, 4 — patients with T2D receiving metformin as a hypoglycemic therapy and insulin, 5 — patients on combination therapy — original metformin and insulin.

Expression of Akt is reduced in lymphocytes of patients with diabetes, both 1st (T1D) and 2nd (T2D) types. However, the amount of protein kinase in lymphocytes of patients with T1D who received insulin is higher than in patients with T2D (Fig., *a*). In turn, in patients with T2D, who received metformin and pharmasulin, the expression of Akt is slightly higher than in patients taking original metformin with gliclazide and original metformin with insulin.

Decrease in the activation of Akt in patients with T2D is natural and associated with the impaired insulin signal transduction from insulin receptor substrates (IRS1/2) to PI3K as a result of the substrate inhibitory phosphorylation by various protein kinases, including mTOR and p70S6K [1]. A decrease in the expression, however, probably reflects a deeper change in signaling mechanisms as a result of the prolonged pathological process. This can be evidenced by the fact that samples 3 and 5 are taken from patients with 15 and 21 years average of the disease duration, respectively, while sample 4 is from a patient with an 8-year disease (see Fig., *a*, *3*, *5*).



Effects of combined therapy of the first-line hypoglycemic drug metformin with insulin and gliclazide upon the expression of main effector kinase of PI3K pathway — Akt, and activity of its downstream kinases mTOR and p70S6K. *1* — control group; *2* — patients with type 1 diabetes on insulin therapy; *3* — patients with type 2 diabetes on combination therapy receiving original metformin and gliclazide; *4* — patients with T2D receiving generic metformin and insulin; *5* — patients on combination therapy – original metformin and insulins. $M \pm SD$, $n = 3$. Akt: * — difference between control and other group significant; + — significant difference between group 2 and 3—5; ^ — significant difference between group 4 and 3, 5 ($P < 0.05$). mTOR: * — difference between control and other group significant; + — significant difference between group 4 and 3, 5 ($P < 0.05$). S6K: * — difference between control and group 3—5 significant ($P < 0.05$)

20 % decrease in the Akt quantity in patients with T1D (see Fig., *a*, *2*), apparently, can be explained by the suppression of the insulin signaling due to a decrease in the hormone amount.

The mTOR activity is significantly reduced in patients with diabetes (see Fig., *b*) that may be due to the inhibition of activity and, possibly, the expression of Akt that activates mTOR in response to insulin. As in the case of Akt, the duration of the disease may affect the activity of this protein kinase (see Fig., *b*, *4*).

As was shown earlier, metformin activates AMPK in lymphocytes of patients with T2D [5], and this may be the reason for an mTOR activity decrease, because AMPK inhibits mTOR through phosphorylation and activation of mTOR inhibitor — TSC2 (tuberous sclerosis 2) and phosphorylation of RAPTOR (Regulatory-associated protein of mTOR), which causes its binding to proteins 14-3-3 [6].

Given a decrease in the mTOR activity, it can be assumed that not only the expression, but also the activity of Akt in lymphocytes is suppressed, since mTORC2 activates Akt, by phosphorylating it on Ser473 [7]. The activity of PDK1, which phosphorylates Akt at another activating site Thr308, can also be reduced in diabetes by the interruption of the insulin signal transduction via IRS.

mTORC1 and mTORC2 have been implicated in integrating signals from growth factors, energy status, oxygen, and amino acids with the rate of autophagy, and regulation of protein synthesis. Growth factors and insulin activate both complexes through PI3K/PTEN/Akt signaling network. In addition, amino acids activate mTORC1 via small G-proteins such as Rag family of GTPases and GTP-bound Ras homolog enriched in brain (Rheb) on the lysosomal membrane [8]. Because mTORC1 is a key nutrient sensor, integrating diverse extra- and intra-cellular cues to downstream signaling pathways in response to the nutrient availability, it is natural that this signaling is dysregulated in diabetes [9].

mTORC1 plays additional roles in the metabolic regulation through the p70S6K1/2-mediated phosphorylation of the CAD enzyme complex to stimulate pyrimidine biosynthesis, ATF4-

dependent stimulation of the mitochondrial tetrahydrofolate cycle to enhance the purine biosynthesis [10], and stimulation of lipid and sterol biosynthesis through the activation of sterol regulatory binding element proteins [11]. p70S6K is an AGC kinase of the RSK family that is required for the cell growth and G1 cell cycle progression. It is phosphorylated and activated by mTOR in mitogenic pathways downstream of PI3K. p70S6K phosphorylates the S6 protein of the 40S ribosomal subunit and is involved in the translational control of 5' oligopyrimidine tract mRNAs. Activity is controlled by multiple phosphorylation events located within the catalytic, linker, and pseudosubstrate domains. Mouse knockout shows an increased insulin senstivity, resulting in the protection against diet-induced obesity [7]. mTORC1–S6K1 mediates various extrinsic signals that regulate the cell growth and metabolism. Activation of mTORC1–S6K1 signaling by nutrients has received broad attention because of its implication in obesity and insulin resistance. Phosphorylation of IRS1 at sites Ser307 and Ser636/Ser639, which antagonize the IRS1 signaling, is elevated in animal models of obesity and in muscle from type 2 diabetic patients. S6K1 might have the major role in the insulin resistance under conditions of nutrient overload [5, 12].

The activity of p70S6K1/2 was reduced only in patients with T2D (see Fig., *c*, *3—5*), which can be explained by the decreased activities of PI3K and mTOR, phosphorylating S6K in the main activating sites of Thr412 and Thr389. The high activity of this kinase in patient T1D can be associated with its activation by other protein kinases [13] or more likely with the absence of an inhibitory effect on mTOR of adenosine monophosphate-activated protein kinase (AMPK), which is activated by metformin [14].

Conclusions. 1. Expression of Akt is reduced in lymphocytes of patients with diabetes of both types.

2. Activity of mTOR and its downstream kinase S6K in lymphocytes of patients with T2D is reduced and may reflect the effectiveness of hypoglycemic drugs.

3. Expression of Akt and the mTOR activity can be associated with the duration of the disease.

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ВПЛИВ КОМБІНОВАНОГО ЛІКУВАННЯ ІНСУЛІНОМ, МЕТФОРМІНОМ ТА ГЛІКЛАЗИДОМ НА ЕКСПРЕСІЮ І АКТИВНІСТЬ ПРОТЕЇНКІНАЗ Akt, mTOR І p70S6K У ЛІМФОЦИТАХ ХВОРИХ НА ЦУКРОВИЙ ДІАБЕТ

Визначали експресію основної ефекторної протеїнкінази сигнального шляху PI3K — Akt та активність протеїнкіназ mTOR і p70S6K в лімфоцитах хворих на цукровий діабет при комбінованому лікуванні інсуліном і цукрознижувальними препаратами. Показано, що експресія Akt знижена в мононуклеарах крові хворих на діабет 2-го і, меншою мірою, 1-го типу. Активність mTOR і p70S6K також знижена в лімфоцитах хворих на діабет 2-го типу. Обговорюються можливі механізми пригнічення експресії і активності протеїнкіназ.

Ключові слова: *Akt, mTOR, p70S6K, цукровий діабет, метформін, інсулін, гліклазид.*

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ВЛИЯНИЕ КОМБИНИРОВАННОГО ЛЕЧЕНИЯ ИНСУЛИНОМ, МЕТФОРМИНОМ И ГЛИКЛАЗИДОМ НА ЭКСПРЕССИЮ И АКТИВНОСТЬ ПРОТЕИНКИНАЗ Akt, mTOR И p70S6K В ЛИМФОЦИТАХ БОЛЬНЫХ САХАРНЫМ ДИАБЕТОМ

Определяли экспрессию основной эффекторной протеинкиназы сигнального пути PI3K — Akt и активность протеинкиназ mTOR и p70S6K в лимфоцитах больных сахарным диабетом при комбинированном лечении инсулином и сахароснижающими препаратами. Показано, что экспрессия Akt снижена в мононуклеарах крови больных диабетом 2-го и, в меньшей степени, 1-го типа. Активность mTOR и p70S6K также снижена в лимфоцитах больных диабетом 2-го типа. Обсуждаются возможные механизмы подавления экспрессии и активности протеинкиназ.

Ключевые слова: *Akt, mTOR, p70S6K, сахарный диабет, метформин, инсулин, гликлазид.*