doi: https://doi.org/10.15407/dopovidi2018.05.100
UDK 616.379-008.64-002:577.2

**L.K. Sokolova, V.M. Pushkarev,**
**Y.B. Belchina, V.V. Pushkarev, M.D. Tronko**

V.P. Komisarenko Institute of Endocrinology and Metabolism of the NAMS of Ukraine, Kiev
E-mail: pushkarev.vm@gmail.com

# Effect of combined treatment with insulin and metformin on 5′AMP-activated protein kinase activity in lymphocytes of diabetic patients

*Presented by Corresponding member of the NAS of Ukraine M.D. Tronko*

*The activity of 5'adenosine monophosphate-activated protein kinase (AMPK), controlling the energy balance of a cell, in blood cells at the combined treatment of patients with type 2 diabetes with metformin and insulin was determined by enzyme immunoassay. It has been shown that metformin increases three-fold the AMPK activity in lymphocytes of patients with type 2 diabetes, compared with patients before treatment. Insulin and its analog reduced the activity of the protein kinase stimulated by metformin in the blood cells, acting as metformin antagonists. The mechanisms of drug interaction and the consequences of their antagonism are discussed.*

***Keywords:*** *AMPK, type 2 diabetes, metformin, insulin.*

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. Modern guidelines recommend early insulin therapy with the selection of an adequate effective dose of insulin followed by timeous intensification. Early insulin therapy together with oral hypoglycemic agents is also offered in the updated ADA guide (2018) for managing patients with T2D [1]. At the same time, combined therapy of the first-line hypoglycemic drug metformin (MF) with insulin can lead to negative consequences and even increase the death rate of patients [2—4]. These facts require a further study to understand the risks associated with the use of insulin and MF in patients with type 2 diabetes.

Taking the data obtained in clinical and experimental studies into account, we attempted to study the activity of the main energy sensor of cells — 5′AMP-activated protein kinase (AMPK) in lymphocytes from patients taking MF both as monotherapy and in combination with insulin preparations.

**Materials and methods.** The study was conducted in the diabetology department of the Institute. All patients signed informed consent to conduct the further diagnostic and research study.

Immediately after the collection, blood was centrifuged using a Histopaque 1077 (Sigma, USA), the lymphocytes collected were washed and frozen at –80 °C until use. The cells were lysed in the extraction buffer with inhibitors of proteases and phosphatases. To determine the amount of phospho-AMPK (phospho-threonine 172) enzyme-linked immunosorbent assay (ELISA) kit ab154468 (Abcam, UK) was used. The studies were carried out in triplets. The protein concentration in the lysate was determined using Novagen (USA) BCA protein assay kit. The measurements were carried out on a microplate reader (Bio-tek Instruments, USA) at a wavelength of 600 nm.

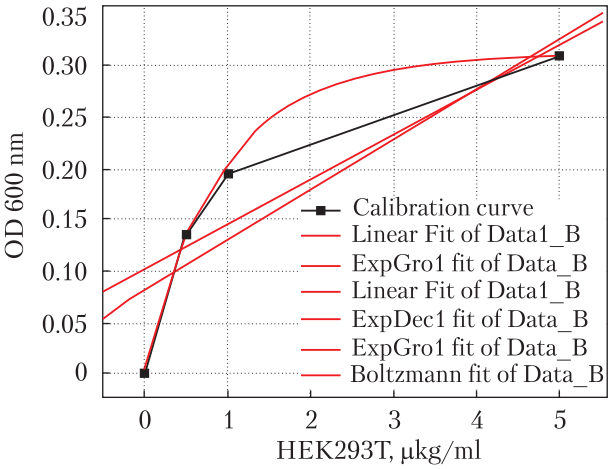


***Fig. 1.*** Calibration curve for the AMPK determination

To get the calibration curve for the AMPK determination, a kidney cell culture HEK293T of human embryonic kidney was used, which is recommended by the manufacturer as a positive control.

The OD values of samples obtained (0.005—0.04) are located on the calibration curve region almost perfectly coinciding with exponential theoretical curves, which indicates no scattering of the data (Fig. 1).

The results of the study are presented as $M \pm SD$, $n = 3$—6. 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 before the hypoglycemic therapy, 4 — patients with T2D receiving metformin at a dose of 1000 mg twice a day as a hypoglycemic therapy, 5 — patients on combination therapy — MF at a same dose and analog of insulin (glargine), 6 — patients on combination therapy — MF and human insulin. Insulin glargine is the first and only analog of human insulin, a long-acting drug, whose single administration provides 24-hour basal glycemic control.

AMPK activity was determined by the amount of the phosphorylated Thr172 of α-subunit of the protein. Fig. 2 shows that the level of phospho-AMPK in lymphocytes of patients with T1D (group 2) does not differ from control, and, in patients with T2D before treatment with hypoglycemic drugs (group 3), it is lower than in control samples. MF increases more than three-fold the activity of AMPK in blood cells of patients with T2D, which may indicate that the drug affects not only muscles, liver, and adipose tissue, but also blood cells. An increase in the activity of protein kinase in blood cells, including monocytes/macrophages, whose inflammatory process plays an important role in increasing the insulin resistance, may partly explain its attenuation by the action of metformin.

A stimulating effect of MF on AMPK activity in undifferentiated bone marrow precursor cells was also described [5].

Attention is drawn to the lack of the insulin effect on AMPK activity in patients with T1D (group 2), which indicates a certain independence of the hormone signal pathway (PI3K/Akt)

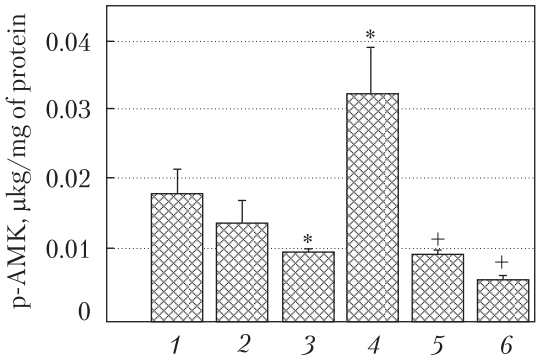


***Fig. 2.*** AMPK activity in lymphocytes of diabetic patients before treatment and after taking hypoglycemic drugs. *1* — control; *2* — T1D; *3* — T2D before treatment; *4* — metformin; *5* — metformin + analog of insulin; *6* — metformin + human insulin. $M \pm SD$, * — difference from control — significant, $P < 0.05$; + — difference from metformin effect — significant, $P < 0.05$

from the cascade of AMPK activation (liver kinase B1 (LKB1), calcium-/calmodulin-dependent kinase, kinase 2 (CAMKK2), and TGFβ-activated kinase 1 (TAK1)). At the same time, both insulin and its analog, insulin glargine, completely suppressed MF-stimulated AMPK activity in the lymphocytes of patients with T2D (Fig. 2, groups 5 and 6), reducing it to the level of a group of patients without treatment (group 3).

The latter fact is of particular interest, taking into account that the hyperglycemic effect of metformin is mainly related to the suppression of the glucose production in liver by activating the LKB1-AMPK pathway [6].

It is worth to note that MF realizes pleiotropic actions in multiple organs and exerts protective effects on cardiovascular diseases and pancreatic β-cells failure. Moreover, metformin therapy improves insulin secretion and protects against palmitic acid-induced pancreatic β-cell apoptosis, therefore supporting the advantageous effects of metformin on pancreatic β-cells. MF also exerts direct effects on the β-cell function such as insulin release, transcriptional regulation in pancreatic islets, and islet cell viability, in dependence on the glucose concentration [7]. Therefore, insulin inhibition of AMPK activity is perhaps a feedback mechanism regulating the synthesis and secretion of the hormone itself. It should be noted that insulin suppresses AMPK activity not only in lymphocytes, but also in liver, muscles, and possibly in other tissues and organs [8].

As for the mechanism of such inhibition, so far little is known. There is evidence that insulin can inhibit adrenergic agonist-stimulated AMPK [9]. Recent studies also suggest that insulin downregulates AMPK activity via Ser485/491 phosphorylation of the AMPK α-subunit [10].

Taking the obtained data into account, it is possible that the inhibition of AMPK and, accordingly, the effect of metformin, by insulin in their combined application, can explain complications in the cardiovascular and excretory systems [2—4]. On the other hand, many disadvantages of insulin treatment in type 2 diabetes seem to be minimized by concomitant treatment with metformin. MF and insulin versus insulin alone seems to cause favorable reductions in weight, HbA1c, and insulin dose [11].

**Conclusions.** Metformin increases the activity of AMPK in blood cells of patients with type 2 diabetes more than 3-fold that may suggest a direct effect of the drug on the monocyte/macrophage system.

Insulin and its analog completely suppress the metformin-stimulated AMPK activity in lymphocytes of patients with T2D, and, consequently, insulin can interfere with the therapeutic effects of metformin.

REFERENCES

1. Standards of medical care in diabetes — 2018. (2018). Diabetes Care., 41, Suppl. 1, pp. S1-S2. doi: https://doi.org/10.2337/dc18-Sint01
2. Hung, A. M., Roumie, C. L., Greevy, R. A., Grijalva, C. G., Liu, X., Murff, H. J., Ikizler, T. A. & Griffin, M. R. (2016). Comparative effectiveness of second-line agents for the treatment of diabetes type 2 in preventing kidney function decline. Clin. J. Am. Soc. Nephrol., 11, No. 12, pp. 2177-2185.
3. Roumie, C. L., Greevy, R. A., Grijalva, C. G., Hung, A. M., Liu, X., Murff, H. J., Elasy, T. A. & Griffin, M. R. (2014). Association between intensification of metformin treatment with insulin vs sulfonylureas and cardiovascular events and all-cause mortality among patients with diabetes. JAMA, 311, No. 22, pp. 2288-2296
4. Roumie, C. L., Min, J. Y., D'Agostino McGowan, L., Presley, C., Grijalva, C. G., Hackstadt, A. J., Hung, A. M., Greevy, R. A., Elasy, T. & Griffin, M. R. (2017). Comparative safety of sulfonylurea and metformin monotherapy on the risk of heart failure: a cohort study. J. Am. Heart Assoc., 6, No. 4, e005379. doi: https://doi.org/10.1161/JAHA.116.005379
5. Molinuevo, M. S., Schurman, L., McCarthy, A. D., Cortizo, A. M., Tolosa, M. J., Gangoiti, M. V., Arnol, V. & Sedlinsky, C. (2010). Effect of metformin on bone marrow progenitor cell differentiation: in vivo and in vitro studies. J. Bone Miner. Res., 25, No. 2, pp. 211-221.
6. Meng, S., Cao, J., He, Q., Xiong, L., Chang, E., Radovick, S., Wondisford, F. E. & He, L. (2015). Metformin activates AMP-activated protein kinase by promoting formation of the αβγ heterotrimeric complex. J. Biol. Chem., 290, pp. 3793-3802.
7. Yang, X., Xu, Z., Zhang, C., Cai, Z. & Zhang, J. (2017). Metformin, beyond an insulin sensitizer, targeting heart and pancreatic β cells. Biochim. Biophys. Acta., 1863, No. 8, pp. 1984-1990. doi: https://doi.org/10.1016/j.bbadis.2016.09.019
8. Valentine, R. J., Coughlan, K. A., Ruderman, N. B. & Saha, A. K. (2014). Insulin inhibits AMPK activity and phosphorylates AMPK $Ser^{485/491}$ through Akt in hepatocytes, myotubes and incubated rat skeletal muscle. Arch. Biochem. Biophys., 562, pp. 62-69. doi: https://doi.org/10.1016/j.abb.2014.08.013
9. Omar, B., Zmuda-Trzebiatowska, E., Manganiello, V., Göransson, O. & Degerman, E. (2009). Regulation of AMP-activated protein kinase by cAMP in adipocytes: roles for Phosphodiesterases, Protein kinase B, Protein kinase A, Epac and lipolysis. Cell Signal., 21, No. 5, pp. 760-766.
10. Kido, K., Yokokawa, T., Ato, S., Sato, K. & Fujita, S. (2017). Effect of resistance exercise under conditions of reduced blood insulin on AMPKα $Ser^{485/491}$ inhibitory phosphorylation and AMPK pathway activation. Am. J. Physiol. Regul. Integr. Comp. Physiol., 313, No. 2, pp. R110-R119.
11. Hemmingsen, B., Schroll, J. B., Wetterslev, J., Gluud, C., Vaag, A., Sonne, D. P., Lundstrøm, L. H. & Almdal, T. (2014). Sulfonylurea versus metformin monotherapy in patients with type 2 diabetes: A Cochrane systematic review and meta-analysis of randomized clinical trials and trial sequential analysis. CMAJ Open., No. 2, pp. E162-E175.

Received 24.01.2018

*Л.К. Соколова, В.М. Пушкарев,*
*Ю.Б. Бельчина, В.В. Пушкарев, Н.Д. Тронько*

ГУ "Институт эндокринологии и обмена веществ
им. В.П. Комиссаренко НАМН Украины", Киев
E-mail: pushkarev.vm@gmail.com

ВЛИЯНИЕ КОМБИНИРОВАННОГО ЛЕЧЕНИЯ ИНСУЛИНОМ
И МЕТФОРМИНОМ НА АКТИВНОСТЬ 5′АМФ-АКТИВИРУЕМОЙ
ПРОТЕИНКИНАЗЫ В ЛИМФОЦИТАХ БОЛЬНЫХ ДИАБЕТОМ

Методом иммуноферментного анализа определяли активность 5′аденозинмонофосфатактивируемой протеинкиназы (АМРК), контролирующей энергетический баланс клетки, в клетках крови при комбинированном лечении больных диабетом 2-го типа метформином и инсулином. Показано, что метформин в три раза повышает активность АМРК в лимфоцитах больных диабетом 2-го типа по сравнению с пациентами до лечения. Инсулин и его аналог снижали стимулируемую метформином активность протеинкиназы в

клетках крови, выступая антагонистами метформина. Обсуждаются механизмы взаимодействия препаратов и последствия их антагонизма.

***Ключевые слова:*** *AMPK, диабет 2-го типа, метформин, инсулин.*

*Л.К. Соколова, В.М. Пушкарьов,*
*Ю.Б. Бєльчина, В.В. Пушкарьов, М.Д. Тронько*

ДУ "Інститут ендокринології та обміну речовин
ім. В.П. Комісаренка НАМН України", Київ
E-mail: pushkarev.vm@gmail.com

ВПЛИВ КОМБІНОВАНОГО ЛІКУВАННЯ ІНСУЛІНОМ
ТА МЕТФОРМІНОМ НА АКТИВНІСТЬ 5′АМФ-АКТИВОВАНОЇ
ПРОТЕЇНКІНАЗИ В ЛІМФОЦИТАХ ХВОРИХ НА ДІАБЕТ

Методом імуноферментного аналізу визначали активність 5′аденозинмонофосфатактивованої протеїнкінази (AMPK), що контролює енергетичний баланс клітини, в клітинах крові при комбінованому лікуванні хворих на діабет 2-го типу метформіном і інсуліном. Показано, що метформін утричі підвищує активність AMPK у лімфоцитах хворих на діабет 2-го типу в порівнянні з пацієнтами до лікування. Інсулін і його аналог знижували стимульовану метформіном активність протеїнкінази в клітинах крові, виступаючи антагоністами метформіну. Обговорюються механізми взаємодії препаратів та наслідки їх антагонізму.

***Ключові слова:*** *AMPK, діабет 2-го типу, метформін, інсулін.*