ного церулоплазмина и экспрессии мРНК TNFα в тканях печени этих животных.

Таким образом, полученные данные свидетельствуют о протективном эффекте метформина на углеводный, липидный обмен, а также уровень экспрессии провоспалительных факторов, связанных с NFkB-сигнальным путем в тканях печени мышей, которые находились на диете с высоким содержанием фруктозы.

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<u>ENGLISH VERSION:</u> INFLUENCE OF METFORMIN ON LIPID METABOLISM AND CHRONIC INFLAMMATION IN THE LIVER TISSUES OF MICE ON A DIET RICH IN FRUCTOSE*

Shlykova O.A. , Mikityuk M.V. , Bobrova N.L.. , Izmailova O.V. , Mamontova T.V. , Baranova A.F. , Vesnina L.E. , Kaidashev I.P.

Higher State Educational Establishment of Ukraine "Ukrainian Medical Stomatological Academy", Poltava

The effect of metformin on lipid metabolism and the expression of pro-inflammatory factors in the liver tissue of mice that were on a diet rich in fructose (DRF) (60 g fructose / 100 g of food) has been studied. The concentrations of total cholesterol (TC), triglycerides (TG), ceruloplasmin (CP), glucose in serum and mRNA expression of inhibitor of NF-kB (IkBα), mRNA of tumor necrosis factor α (TNFα) have been determined in homogenates of liver tissues in 3-months-aged mice of BALBc line weighing 25-30 g. It has been demonstrated that increasing the content of fructose in the diet leads to increased concentrations of glucose, TC and TG in the blood of mice. Manifestations of systemic inflammatory response become stronger, as evidenced by the increase in CP in the blood serum and the expression level of mRNA of TNFα in liver tissues of animals that were on a DRF. Metformin in the dose of 50 mg / kg per day reduced the production of triglycerides in the blood serum of mice that were on a DRF; it contributed to the normalization of hepatic gluconeogenesis. Administering metformin caused anti-inflammatory effect by lowering serum CP and expression of mRNA of TNFα in liver tissues of these animals. Thus, the data suggest the protective effect of metformin on lipid metabolism and the expression of pro-inflammatory factors associated with NF-kB-signaling pathway in the liver tissues of mice which were on a DRF.

Keywords: diet rich in fructose, metformin, expression, proinflammatory factors.

Numerous studies display that diet rich in fructose (DRF) causes various pathological changes, including oxidative stress, impaired glucose tolerance, insulin resistance, type 2 diabetes, liver conditions, hypertension

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and other cardiovascular diseases [16]. It is shown that lipid metabolism in the development of stress of hepatocytes in animals on DRF occurs in the result of fructose metabolism overload [11]. It is assumed that the increased production of lipids in the liver enhances mitochondrial beta-oxidation of fatty acids, generation of peroxidation products that stimulate IkB-kinase (IKK), and therefore the activation of NFkB [7]. It is known that molecular mechanisms of chronic low-level systemic inflammation (CSI) are closely associated with the activation of proinflammatory nuclear transcription factors (NTF), primarily NFkB [5].

In recent years, the concept of permanent NF-kB activation (long-term and low-intensity) has been formulated as a possible model of pathological process that determines the relationship between insulin resistance, chronic inflammation, hypertension, endothelial dysfunction and dyslipidemia (I.P. Kaidashev, 2012). In this regard, the search for methods to reduce the transcriptional activity of NF-kB (diet, exercise, therapeutic drugs) is relevant, since it will eliminate the molecular basis of the metabolic / insulin resistance syndrome and reduce the risk of cardiovascular diseases [2]. Previous studies have shown that activation of metformin reduces NFkB, possibly by inhibiting the activation of IKK [1].

The aim of the research is to study the influence of metformin on blood glucose, lipid metabolism and the level of expression of proinflammatory factors in the liver tissues of mice that were on a diet rich in fructose.

Materials and methods of the research

The experiment was performed on mice of BALBc line aged 3 months, weighing 25-30 g, kept in polypropylene cages under controlled conditions of 12 hours light / 12 hours dark cycle. Animals were on a standard diet (60% of vegetable amylum) and with unlimited access to water for one week. The study was conducted in accordance with authorization of the Bioethics Commission of Higher State Educational Establishment of Ukraine "Ukrainian Medical Stomatological Academy".

In a week after acclimation period, the animals were divided into four equal groups: group 1 – mice on a standard diet and water; group 2 – mice on a standard diet and water, which were administered metformin (per os) in a dose of 50 mg / kg from the 85th day of the experiment for 49 days [14, 15]; group 3 – mice on a DRF (60 g fructose / 100 g of food); group 4 – mice on a DRF, which were administered metformin (per os) in a dose of 50 mg / kg from the 85th day of the experiment for 49 days.

The standard diet comprised the grain mixture (wheat, oats, barley, and millet), maize, sunflower seeds, peas, corn granules, nuts, and dried fruit. Mineral component were: calcium, phosphorus, potassium, sodium. Vitamins: A, B1, B2, D3, E, N. The nutrient content: crude protein – 11%; crude fat – 4.0%; crude fiber – 10.0%; calcium – 0.15%, phosphorus – 0 1%; moisture – 10%.

On the 134th day of the experiment, after 12-hour fasting period, the concentration of glucose in blood serum of animals was determined before and in 120 minutes after the glucose load (2 g / kg) (test the glucose tolerance). The concentration of glucose (GL) was determined in blood from the tail vein using "GLYUKOFOT" glucometer with standard indicator strips "GEMOGLAN" (PIE "NORM", Ukraine).

On the 135th day of the experimental period, the animals were euthanized by cervical dislocation. Blood was collected in dry plastic vials. Serum was separated by centrifugation at 400 g for 10 minutes. In blood serum, concentration of total cholesterol (TC) and triglycerides (TG) using the reagents kit (LACHEMA, Czech Republic), the concentration of ceruloplasmin (CP) by enzymatic oxidation of p-phenyldiamine were determined [3]. The sampling of liver tissue was performed and homogenised on ice in refrigerated 0.1M phosphate buffer, pH 7.4. In liver homogenate, the content of RNA messenger (mRNA), NF-kB inhibitor (IkBα) and mRNA of tumor necrosis factor α (TNFα), isolated using the reagents kit "Ribo-sol-B" (AmpliSens, Russia), were determined. For obtaining cDNA in the reverse transcription reaction, primer oligo (dT) 18 and reverse transcriptase M-MuLV (SibEnzyme, Russia) were used. Gene expression was analyzed by PCR method in "real time", using oligonucleotide primers for cDNA IkBα: 5'-TGAAGGACGAGGAGTACGAGC-3'; 5'-TTCGTGGATGATTGCCAAGTG-3' and TNFα: 5'-CCCTCACACTCAGATCATCTTCT-3'; 5'-GCTACGACGTGGGCTACAG-3', in the presence of the dye SYBR Green I ("Synthol", Russia), using the detecting amplifier DT-322 ("DNA Technology", Russia) via relative quantitative analysis. The gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene: 5'-AACTTTGGCATTGTGGAAGG-3'; 5'-GGATGCAGGGATGATGTTCT-3'.

For data analysis, relative Ct method with the calculation formula ΔCt was used [18].

The results were processed statistically using the program STATISTICA 6.0. ("StatSoft", USA). The data are presented as the sample average (M), the sample standard deviation (S / D). The critical level of significance when testing the statistical hypotheses was set at 0.05.

Results and discussion

As our research has shown, the gradual increase in body weight of mice in the studied groups during the trial period was observed. Table 1 displays that the final average body weight of mice (g) in group 1 was 32,9 ± 2,0; in groups 2, 3 and 4: 34,7 ± 2,33; 29,9 ± 5,29; 31,1 ± 2,08, respectively. It should be noted that the average value of the final weight of mice in groups did not differ significantly from each other.

Table 1.
Change in body weight in the studied groups of animals (M ± SD).

Parameter	Groups			
	1	2	3	4
Initial body weight, g	28,4±2,2	31,2±1,56	25,9±2,1	29,2±1,13
Body weight in 84 days, g	32,6±2,8	33,7±0,77	28,8±3,2	30,3±1,36
Body weight in 134 days, g	32,9±2,0	34,7±2,33	29,9±5,29	31,1±2,08

When determining the glucose tolerance, a statistically significant increase in concentration of GL (in fasting state) in the blood of mice on DRF was observed, unlike the mice of group 1 (p = 0.0059) (Fig.1). Under the influence of metformin, glucose concentration in the blood serum of mice from group 4 was reduced in comparison with group 3 (p = 0.052) (Figure 1)

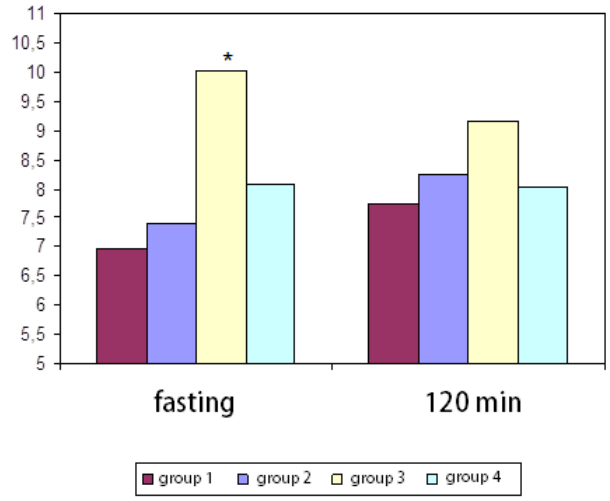

Figure 1. Glucose level in groups of studied animals in fasting state and in 120 min after the glucose load (2 g / kg).

*Note: * – p <0.05 as compared with group 1.*

In our studies, the lipogenesis processes in liver cells varied under the influence of DRF, as evidenced by the increase in TC to 3.05 ± 0.57 mmol / l and triglyceride to 2.15 + 0.42 mmol / l in the serum of group 3 as compared with mice of group 1, who were on a standard diet with no added fructose (p <0.05) (Table 2).

Table 2.
Changes in the level of total cholesterol and triglycerides in the serum of studied animals (M + SD).

Parameter	Groups			
	1	2	3	4
TC, mmol / l	1,6 ± 0,38	1,75 ± 0,31	3,05 ± 0,57*	2,6 ± 0,46
TG, mmol / l	1,13 ±0,57	1,3 ± 0,35	2,15 ± 0,42*	1,48 ± 0,23**

*Note: * – p <0.05 as compared with group 1;*
*** – p <0.05 as compared with group 3.*

Metformin reduced the intensity of lipogenesis in liver cells of group 4, as evidenced by reduction in triglycerides (1,48 ± 0,23 mmol / l) as compared to group 3 (2,15 ± 0,23 mmol / l) (p = 0.049), the TC level did not significantly change (p = 0.2) (Table 2).

At the same time, according to histological studies, no clinically significant structural changes in liver tissues were observed in the studied groups of mice. The indicators of liver mass coefficient in the studied groups of animal did not change as well, amounting in group 1 – 0,056 ± 0,008, group 2 – 0,054 ± 0,003, group 3 – 0,06 ± 0,009, group 4 – 0,061 ± 0,01.

At the next stage of work, manifestations of systemic inflammatory response were assessed. As markers of the inflammatory process, the level of CP in blood serum and the expression of mRNA of TNFα and IκBα in liver homogenates were determined. In previous studies of I. P. Kaidashev et al., 2008, it was assumed that the contents of CP in serum may be a marker of increased risk of inflammation and metabolic syndrome, and along with other indicators, it will allow assessing the level of systemic inflammatory response [4]. As seen from the data presented in Figure 2, the increase in fructose content in the diet led to a significant increase in the level of ceruloplasmin in serum of group 3 – 297.65 + 17.07 mg / l as compared with group 1 – 221.9 + 18,5 mg / l (p = 0.0004).

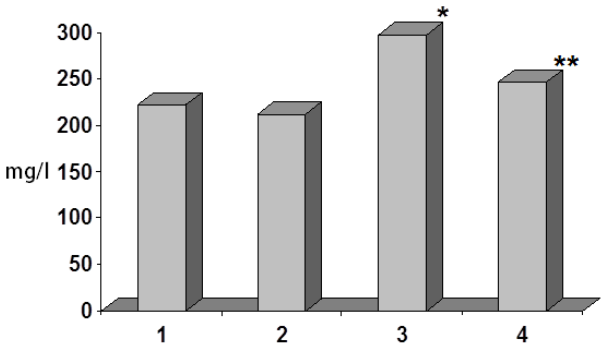

Figure 2. Changes in the level of ceruloplasmin in groups of studied animals.

*Note: * – p <0.05 as compared with group 1;*
*** – p <0.05 as compared with group 3.*

The expression of mRNA of TNFα and IkBα in liver tissues of mice by increasing the content of fructose in the diet, as well as under exposure to metformin, was studied.

In the group of animals on a DRF, expression of mRNA of TNFα increased and expression of mRNA of IkBα did not virtually change (Table 3).

Under the action of metformin, the manifestations of chronic inflammation reduced due to decrease of ceruloplasmin level in serum of animals from group 4 (246,67 ± 15,41 mg / l) as compared to group 3 (297,65 ± 17,07 mg / L) (p = 0.0044) (Figure 2). Under the influence of metformin, expression of mRNA of TNFα in liver cells decreased. Metformin had virtually no effect on expression of mRNA of IκBα (Table 3).

Table 3.
Changes in the relative mRNA expression of mRNA of TNFα and IkBα in liver tissues of studied animals (M ± SD).

Parameter	Groups			
	1	2	3	4
Expression of TNF-alpha, ΔCt	14,67 ±0,74	11,62 ±0,78	12,42 ±0,94*	14,75 ±1,04**
Expression of IkB-alpha, ΔCt	11,2 ±1,04	10,0 ±0,28	10,62 ±0,88	11,2 ±1,97

*Note: * – p <0.05 as compared with group 1;*
*** – p <0.05 as compared with group 3.*

In our study it has been demonstrated that the increase of fructose content in the diet leads to increased concentrations of glucose, TC and TG in the blood of mice. Manifestations of systemic inflammatory response are exacerbated, as evidenced by the increase in the level of ceruloplasmin in serum and the level of expression of mRNA of TNFα in liver tissues of animals that were on DRF. At the same time, we observed no increase in body mass and structural changes in the liver tissue of mice by high fructose intake with food.

The data on the changes in the process of lipogenesis in liver cells of mice on a DRF are consistent with studies of H. Basciano et al, 2005, in which it is shown that large admission of fructose to the liver activates the metabolism of glucose and the way of its assimilation, leads to a significant increase in the rate of de novo lipogenesis, the synthesis of triglycerides (TG), which is due to the high flux of glycerol and acyl molecule units of fructose catabolism. These metabolic disorders appear to be one of the reasons underlying the induction of insulin resistance, which is often observed in humans and animals with large consumption of dietary fructose [8].

No increase in body weight of mice, as well as the lack of changes in weight and structure of the liver in our studies may be due to the high content of dietary fiber in the diet of mice. Numerous experimental and clinical studies have shown beneficial effects of a diet high in dietary fiber for metabolic syndrome [6, 9, 10].

Strengthening the systemic inflammatory response in our study is confirmed by an increased concentration of serum ceruloplasmin and expression of mRNA of TNFα in the liver, and as is known, TNFα is a marker of activation of NFkB. There is evidence to show that hepatic insulin resistance induced by NFkB activation is associated with increased expression of TNFα, IL-6 and IL-1 [12].

In our previous studies it has been suggested that metformin affects NFkB-signaling pathway, possibly by changing the activity of IKK [1]. IKK activation is one of the key mediators of insulin resistance, as evidenced by the increased sensitivity to insulin at directed disorders in IKK gene [17]. Considering the fact that in this study the activation of NFkB in the liver of mice may be associated with overexpression of IKK, causing resistance of liver and skeletal muscle to the action of insulin and signs of systemic inflammatory response (increase of serum IL-6) [13], we continued to study the effect of metformin on NFkB-signaling pathway in the experiment.

In our study, metformin at 50 mg / kg reduced the production of triglycerides in liver cells of mice that were on DRF, facilitated normalization of hepatic gluconeogenesis. Administering metformin has anti-inflammatory effect, by reducing the concentration of serum ceruloplasmin and expression of mRNA of TNFα in liver tissues of these animals.

Thus, the findings suggest that metformin has protective effect on carbohydrate and lipid metabolism, as well as the level of expression of proinflammatory factors associated with NFkB-signaling pathway in liver tissues of mice that were on a diet rich in fructose.

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