

ЕКСПЕРИМЕНТАЛЬНІ РОБОТИ

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THE EFFECT OF N-STEAROYLETHANOLAMINE ON PLASMA LIPID COMPOSITION IN RATS WITH EXPERIMENTAL INSULIN RESISTANCE

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A model of insulin resistance (IR), induced by prolonged high fat diet with high content of saturated fats was used to investigate the effect of N-stearoylethanolamine (NSE) on the composition of free fatty acids (FFA), plasma lipoprotein spectrum and content of proinflammatory cytokine TNF α in rats. The results of this work showed a rise in the content of monounsaturated fatty acids (18:1 n-9) and a reduction in the level of polyunsaturated fatty acids (20:4 n-6) in plasma of rats with experimental IR. These findings are accompanied by the increased TNF α production and significant changes in plasma lipoprotein profile of rats with the fat overload. Particularly, a decreased high-density lipoprotein (HDL) cholesterol level and increased low-density (LDL) and very low-density lipoprotein (VLDL) cholesterol level were detected. The NSE administration to obese rats with IR restored the content of mono- and polyunsaturated FFA, increased HDL cholesterol content and reduced LDL cholesterol level. In addition, the IR rats treated with NSE showed normalization in the serum TNF α level. Our results showed the restoration of plasma lipid profile under NSE administration in rats with obesity-induced IR. Considering the fact that plasma lipid composition displays the lipid metabolism in general, the NSE actions may play a significant role in the prevention of IR-associated complications.

Key words: N-stearoylethanolamine, blood plasma of rats, free fatty acids composition, lipoprotein spectrum, TNF α , obesity, experimental insulin resistance.

In numerous experimental and clinical studies it was found that the excessive consumption of saturated fats favors the development of obesity and dyslipidemia, which causes insulin resistance as well as the development of a chronic inflammatory process. Thus, the obesity-induced increase of the free fatty acids (FFA) pool leads to evaluated production of malonyl-KoA – an allosteric inhibitor of the protein responsible for the transport of fatty acids (FA) to mitochondria and as a result, inhibition of FA β -oxidation [1]. The enhanced pool of saturated FFA through activation of Toll-like receptors triggers the intensification of proinflammatory cytokines formation. Simultaneously, intensification of oxygenase pathway of polyunsaturated FA (PUFA) metabolism causes the formation of eicosanoids and leukotrienes, a considerable part of which displays pro-inflammatory properties [2].

It is known that an increase of saturated FFA pool that inhibits insulin signaling by reducing the

activity of phosphatidylinositol-3-kinase, Akt-kinase and by decreasing the insulin receptor expression [3]. The elevated FFA pool induces the accumulation of triacylglycerols (TAG) and considerable changes in phospholipid fatty acid composition, thus, influencing membrane properties and activity of membrane-bound receptors [4]. The insulin resistance (IR) progression causes the disturbance of plasma lipoprotein profile that triggers the development of cardiovascular diseases. Primarily, the overproduction of very low density lipoproteins (VLDL) enriched in TAG initiate significant changes in other lipoproteins ratio (the increase of low-density lipoprotein (LDL) and the decrease of high-density lipoprotein (HDL) level) [5].

Nowadays more attention is focused on the study of biological effects of lipid mediators N-acyl-ethanolamines (NAE) that regulate energy metabolism by cannabinoid and noncannabinoid receptors. It is known that polyunsaturated (anandamide),

monounsaturated (N-oleylethanolamine) and saturated NAEs (N-palmitoylethanolamine, N-stearoylethanolamine) exert opposite effects on lipogenesis. In particular, anandamide activates CB1 receptors and stimulates synthesis of fatty acids in hepatocytes of rats with obesity, while monounsaturated and saturated NAE by activation of peroxisome proliferator-activated receptors (PPAR α) stimulate lipolysis in the liver and lowers TAG content in blood plasma [6]. Earlier it was found that saturated N-stearoylethanolamine (NSE) inhibits the expression of hepatic stearoyl-CoA-desaturase, the key enzyme in lipid biosynthesis that causes the reduction of TAG formation [7]. Moreover, in our previous work on the model of obesity-induced IR we showed that NSE normalizes the liver phospholipid and fatty acid composition that was accompanied by the decrease of plasma insulin content, improvement of glucose tolerance and insulin sensitivity [8, 9].

Therefore, the aim of our study was to investigate the effect of NSE on plasma FFA composition, lipoprotein spectrum and anti-inflammatory cytokines (TNF- α) level from rats with experimental IR.

Materials and Methods

Experimental model was induced on white outbred male rats with initial body weight 200–220 g. In the process of the experiment the animals were

kept in standard cages with free access to food and water in accordance with General Ethical Principles of Experiments on Animals (Ukraine, 2001). Insulin resistance was induced by the diet with prevailing content of fats (58% of the diet) during 6 months. The percentage of fat was increased by adding visceral lard to the diet of rats. Table 1 presents the fatty acid composition of the fat, where the amount of saturated and monounsaturated FA (MUFA) was, respectively, by 38% and by 35% higher compared to these indices in the content of granulated chow at the expense of increase of the amount of palmitic, stearic and oleic FA, while the content of cholesterol in the fat was only 0.57 mg/g. The control rats received standard pellet diet, which contained 4% of fats of the total diet.

The peroral glucose tolerance test was carried out after 24 weeks [10]. According to the results of the test rats with impaired glucose tolerance (glucose level remained at a high level (> 5 mmol) during 150 min after loading) were selected and divided in two groups: *IR* ($n = 9$) and *IR+NSE* ($n = 10$). The control rats with normal glucose tolerance (150 min after loading the glucose level was decreased to 3.8 mmol) were divided into groups *Control* ($n = 10$) and *NSE* ($n = 7$). The rats of the *NSE* and *IR+NSE* groups were given daily water suspension of NSE *per os* at a dose of 50 mg/kg of the body weight

Table 1. Fatty acid composition (% of total amount of fatty acids), content of cholesterol (mg/g) of lard and pellet diet

Fatty acids	Formula	Lard	Pellet diet
Saturated FA		52.56	38.4
Unsaturated FA		47.32	60.7
Sat./Unsat.		1.11	0.63
MUFA		37.92	28.12
DUFA		8.79	20.9
PUFA		0.61	11.57
Palmitic	16:0	24.21	14.76
Palmitoleic	16:1n-9	5.24	3.31
Stearic	18:0	23.44	13.52
Oleic	18:1n-9	30.17	18.92
Linoleic	18:2n-6	8.37	20.32
Linolenic	18:3n-6	0.56	6.43
Eicosatrienoic	20:3n-6	–	0.71
Arachidonic	20:4	–	4.27
Free cholesterol		0.57	–

during two weeks. The IR development in rats with fat overload was confirmed by the results of plasma insulin test (6.3 ± 0.4 ; $P < 0.05$, compared with the *Control* group of rats 2.3 ± 0.09), glycosylated hemoglobin (0.61 ± 0.05 ; $P < 0.05$ compared with the *Control* group of rats 0.46 ± 0.04) level and the calculation of HOMA-IR index (1.34 ± 0.12 ; $P < 0.05$ compared with the *Control* group of rats 0.39 ± 0.01) [9].

At the end of the experiment rats were decapitated under nembutal anesthesia. For biochemical research plasma was isolated from erythrocytes by centrifugation during 5 min at 450 g and at 4 °C. The obtained plasma was taken into plastic Eppendorf tubes and immediately frozen.

Insulin content in plasma was determined using a set of reagents for solid phase immunoenzyme analysis (ELISA) of DRG Company (Germany). Measurements were performed by the Stat Fax 2100 microplate reader.

The lipid extraction by Bligh and Dyer method [11] was performed to estimate lipid composition of plasma, fat and pellet diet. The content of general lipids in plasma was determined by weighing dry residue.

After that lipid extracts were separated by thin-layer chromatography in the system of solvents hexan : diethyl ether : glacial acetic acid (85 : 15 : 1) a fraction of free fatty acids was methylated with 3 M HCl in methanol [12].

Quantitative analysis of methyl esters was performed by gas-liquid chromatograph HRGC 5300 Carlo Erba Instruments (Italy). Individual fatty acids were identified using standards of the firms Sigma and Serva (Germany). The content of fatty acids was calculated in percents of their total quantity.

The content of free and etherified cholesterol in the rat plasma was determined by enzymatic method, using a standard set of the firm Philicite-Diagnotics (Dnipropetrovsk, Ukraine).

The LDL and HDL content in the plasma was determined by the commercial kit (Philicite-Diagnotics, Dnipropetrovsk, Ukraine), while the level of cholesterol in lipoproteins of IDL+VLDL – by the difference in the level of total cholesterol and in the composition of LDL and HDL.

The content of TNF α in the rat plasma was determined using a set of reagents for solid phase immunoenzyme analysis (Platinum ELISA) of the firm eBioscience, USA.

Experimental data were processed by generally accepted methods of variation statistics with the use of Student's *t*-test. The results were considered statistically significant, if $P < 0.05$.

Results and Discussion

The long-term saturated fats overload, which causes the increase of the FFA pool and their influx to the liver, favors the disturbance of lipoprotein profile of rat plasma (Table 2). It is known that FFA are a source of production of VLDL with high level of TAG, that is agreed with the obtained data concerning the increase of cholesterol content in the fraction VLDL+IDL (Table 2). The increase of ApoB-lipoproteins secretion in the mice liver according to the study of Zhang Y. L., et al. was associated with infusion of high concentrations of oleic acid (18:1n-9) [13]. Thus, we suggest that the high level of 18:1n-9 in the fat diet (Table 1) is one of the causes of increased VLDL level in IR group of rats. In the previous research we have fixed a considerable compensatory increase of free cholesterol level as a result of the increased content of both free monoenoic FA (MUFA) and those in the composition of liver phospholipids from rats with obesity [9, 15].

It is well-known that activation in the liver of VLDL production and/or disturbance of their removal from the blood flow also leads to formation of small LDL. Thus, in Table 2 it is shown that the level of LDL cholesterol in the IR rat plasma is by 55% higher compared with Control group of rats. Simultaneously, a tendency to the decrease of HDL content in the IR rat plasma was revealed in our research (Table 2); this may be connected with the decrease of TAG-enriched lipoproteins clearance and the increase of cholesterol esters transport from HDL to VLDL in exchange for TAG by cholesterol-transporting protein (ChTP). Thus, under IR-state the increase of ChTP activity results in production of TAG-enriched HDL, which are then modified with participation of liver lipase, and this leads to dissociation of structurally important protein ApoA-1 and to a decrease of HDL content [16].

A more than three-fold increase of the level of pro-inflammatory cytokine TNF α in the rat serum under fat overload compared with indications in control rats was found (Fig. 1). It is known from the literature data that an increase of TNF α secretion under adipose tissue hypertrophy induces lipolysis that causes the increase of FFA pool and their ex-

Table 2. Content of total lipids and cholesterol of lipoprotein fractions in rat plasma ($M \pm m$; $n = 7-10$)

Parameter	Group of animals			
	Control	NSE	IR	IR+NSE
Content of total lipids in plasma, mg/ml	4.5 ± 0.8	7.29 ± 0.86	10.67 ± 1.24* [@]	3.25 ± 0.59 ^{@.#}
Content of total cholesterol in plasma, mM	0.880 ± 0.032	0.720 ± 0.049*	1.21 ± 0.14* [@]	1.140 ± 0.058* [@]
Content of HDL in plasma, mM of cholesterol	0.080 ± 0.007	0.080 ± 0.026	0.06 ± 0.006	0.100 ± 0.007 [#]
Content LDL in plasma, mM of cholesterol	0.090 ± 0.015	0.060 ± 0.008	0.140 ± 0.017* [@]	0.110 ± 0.004 ^{@.#}
Content VLDL+HDL in plasma, mM of cholesterol	0.790 ± 0.004	0.064 ± 0.040	1.060 ± 0.106 [@]	0.790 ± 0.106

Values represented mean ± SEM. Values in "Control" ($n = 10$), "NSE" ($n = 7$), "IR" ($n = 9$), "IR+NSE" ($n = 10$) were comparable. * $P < 0.05$, compared to the "Control" rats; [@] $P < 0.05$, compared to the "NSE" group; [#] $P < 0.05$, compared to the "IR" group

cessive income to the liver, where they are involved in the TAG synthesis [17, 18]. Beside the activation of TAG synthesis in the liver, the overproduction of TNF α leads to the decrease of TAG-enriched VLDL clearance that is associated with its inhibiting effect on lipoprotein lipase [19]. Interesting to submit that changes in the TNF α content are related to the insulin resistance level. It is shown in the in vitro studies that TNF α inhibits the expression of several proteins involved in the insulin signaling pathway (IRS-1, glucose-4 transporter) and also PPAR γ receptor and adiponectin [20].

The NSE administration to rats with fat overload caused normalization of TNF α content in blood serum (Fig. 2), that was accompanied by the increase of the content of plasma HDL and the decrease of LDL level compared to those in IR group of rats (Table 2). Simultaneously, the decrease of VLDL+IDL level in the rat plasma under the effect of NSE was found (Table 2).

The data presented in the work of A. Artmann and co-authors [21] shows the capacity of NSE to activate PPAR α . It is known that activation of PPAR under dyslipidemic condition modulates the expression of transcriptional factors SEBP1-c (sterol regulatory element binding protein-1) and ChREBP (carbohydrate regulatory element binding protein), which regulate almost all genes involved in biosynthesis of FA, TAG and phospholipids [22, 23]. According to the data that we have already obtained (compensatory action of NSE on the liver phospholipid and fatty acid composition of rats with IR), we suggest

that NSE restores the distribution of cholesterol in lipoprotein fractions of rat plasma by influencing on the processes of hepatic *de novo* lipogenesis.

Previously on the model of nonspecific inflammation we found the decreased content of anti-inflammatory cytokines under the NSE effect [24]. According to the fact that activation of PPAR α and PPAR γ under IR inhibits activity of the transcription factor NF-kB, which stimulates production of inflammatory mediators [25, 26], the decrease of TNF α content under the effect of NSE may be one of confirmations of its interaction with PPARs.

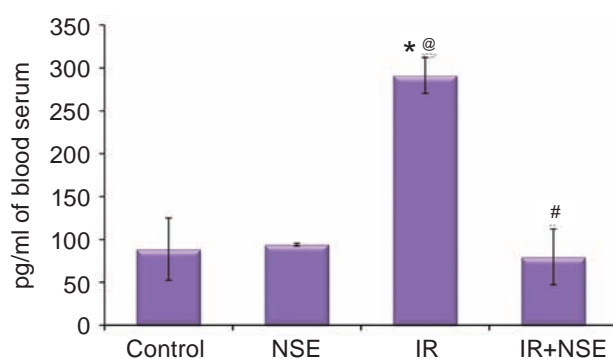


Fig. 1. Content of TNF α (pg/ml) in rat blood serum ($M \pm m$, $n = 7-10$). Here, on Fig. 2-3 * Values represented mean ± SEM. Values in "Control" ($n = 10$), "NSE" ($n = 7$), "IR" ($n = 9$), "IR+NSE" ($n = 10$) were comparable. * $P < 0.05$, compared to the "Control" rats; [@] $P < 0.05$, compared to the "NSE" group; [#] $P < 0.05$, compared to the "IR" group

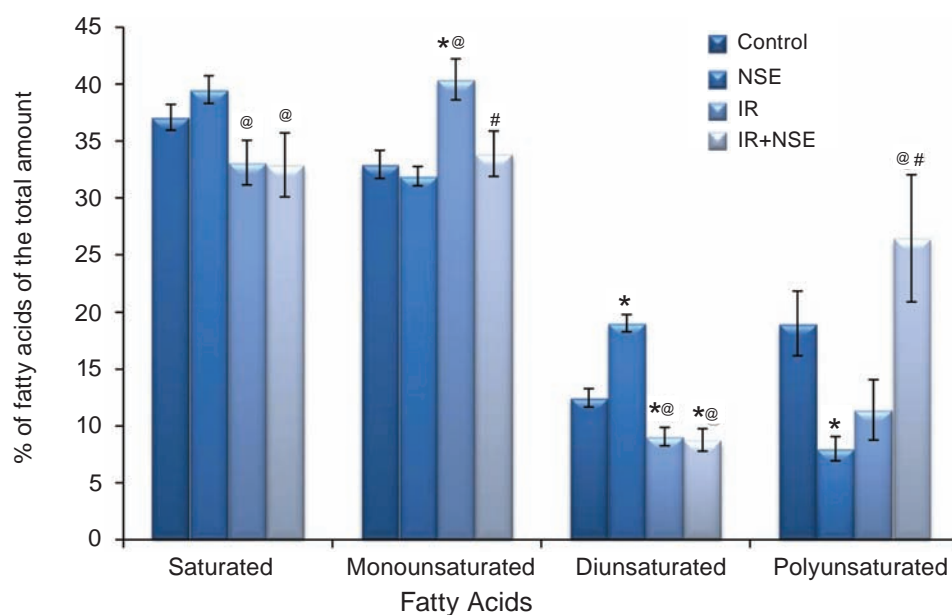


Fig. 2. Saturated and unsaturated (monounsaturated, diunsaturated, polyunsaturated) fatty acids in the phospholipid composition (% of the total amount of fatty acids) in the rat plasma ($M \pm m$, $n = 7-10$)

The investigation results have shown that the long-term high fat overload causes considerable changes in the fatty acid composition of rat plasma (Fig. 2), in particular, the increase of the content of MUFA (Fig. 2), primarily 18:1n-9, in plasma of IR group of rats. The decreased content of palmitic (16:0) and increased stearic (18:0) FFA level, the precursor for 18:1n-9 synthesis, suggest the activation of n-9 FA synthesis pathway in rat liver with fat overload. The administration of NSE to rats with experimental IR causes normalization of 18:0 and 18:1n-9 FFA content in the rat plasma (Fig. 3) that correlates with the change of FFA composition in the liver of rats with IR [15].

The increased pool of 18:1n-9 FFA, the main substrate for TAG synthesis, is accompanied by the increase of TNF α production, the decrease of PPAR α expression, activation of the lipid peroxidation processes and apoptosis in hepatocyte cells [27]. Accordingly, for the leading role of monounsaturated FFA in TAG synthesis, the decrease of their level under the effect of NSE can evidence for its protecting influence on tissues of rats with dyslipidemia.

The investigation of PUFA composition in the rat plasma has shown the increase of linolenic (18:3n-6), eicosatrienoic (20:3n-6) level, the decrease of linoleic (18:2n-6) and a tendency to decrease of arachidonic FFA content in IR group compared to Control (Fig.3). This finding is accordingly in sup-

port of the literature data [28] and results of clinical research, where a considerable decrease of 18:2n-6 and 20:4n-6 FFA level in the plasma of children with obesity was shown [29]. The increase of 18:3n-6 and 20:3n-6 content – the precursors for 20:4n-6 synthesis, with simultaneous decrease of the product was found, suggesting the disturbance of 20:4n-6 biosynthesis under experimental IR. Meanwhile, the decrease of 20:4n-6 in the rat plasma may be related to activation of oxidative processes and synthesis of pro-inflammatory eicosanoids under IR conditions [30]. The increased amount of lipid and protein peroxidation products in the rat liver with experimental IR was found in our previous study [9, 15].

The NSE administration to rats with IR caused the increase of 20:4n-6 level (Fig. 3) that may be associated with its antioxidant activities and its influence on FA metabolism. It is known that a decrease of 20:4n-6 content may be followed by the development of IR, because the Δ 5-desaturase activity that catalyzes the 20:4n-6 formation is regulated by insulin [31]. Thus, the increase of 20:4n-6 content under the NSE action correlated with the improvement of insulin sensitivity and normalization of 20:4n-6 biosynthesis. On the other hand, this effect of NSE may be associated with the inhibition of polyunsaturated lipids peroxidation [9].

Thus, the decrease of MUFA level in blood plasma of IR rats treated by NSE was accompa-

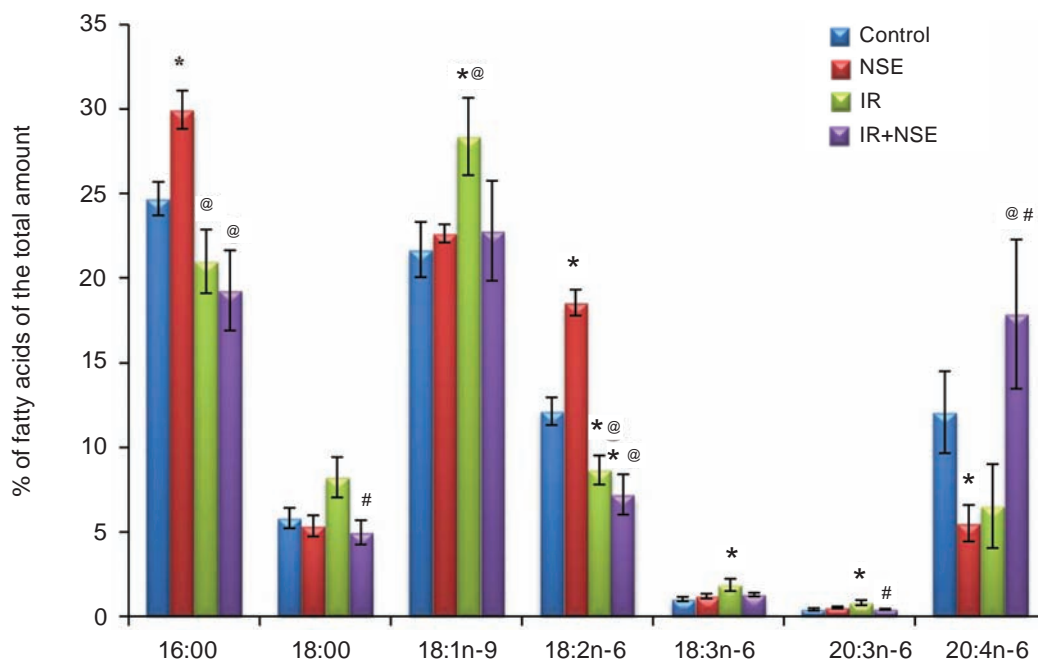


Fig. 3. The main free fatty acids (% of the total amount) in the rat plasma ($M \pm m$, $n = 7-10$)

nied by the reduction of pro-inflammatory cytokine TNF α and the restoration of cholesterol in lipoprotein fractions. Therefore, this effect of NSE may prevent the development of complications connected to IR.

ВПЛИВ N-СТЕАРОЇЛЕТАНОЛАМІНУ НА ЛІПІДНИЙ СКЛАД ПЛАЗМИ КРОВІ ЩУРІВ З ЕКСПЕРИМЕНТАЛЬНОЮ ІНСУЛІНОРЕЗИСТЕНТНІСТЮ

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На моделі інсулінорезистентності (ІР), індукованої довготривалим жирним навантаженням із переважним вмістом насичених та мононенасичених жирів, було вивчено вплив N-стеароїлетаноламіну (NSE) на склад вільних жирних кислот (ВЖК), ліпопротеїновий спектр плазми крові та вміст прозапального цитокіну TNF α в організмі щурів. За результатами проведених досліджень встановлено зростання рівня мононенасичених ВЖК (18:1n-9) та зниження

поліненасичених ВЖК (20:4n-6) у плазмі крові щурів за експериментальної ІР. Ці процеси супроводжувались збільшенням вмісту цитокіну TNF α та значними змінами у ліпопротеїновому профілі плазми крові. Зокрема, зафіксовано зниження вмісту холестеролу ліпопротеїнів високої щільності (ЛВЩ), зростання холестеролу ліпопротеїнів низької (ЛНЩ) та дуже низької щільності (ЛДНЩ). Введення NSE щурам з індукованою ожирінням ІР сприяло відновленню рівня моно- та поліненасичених ВЖК, зростанню вмісту холестеролу ЛВЩ та зниженню – холестеролу ЛНЩ. Крім того, за ІР в щурів, які отримували NSE, показана нормалізація рівня TNF α в сироватці крові. Одержанні результати свідчать про відновлення за дії NSE ліпідного профілю плазми крові щурів з ІР, індукованою аліментарним ожирінням. Оскільки ліпідний спектр плазми крові відображає метаболізм ліпідів у цілому, встановлений ефект NSE може мати певне значення для попередження ускладнень, пов'язаних із розвитком ІР.

Ключові слова: N-стеароїлетаноламін, плазма крові щурів, склад вільних жирних кислот, ліпопротеїновий спектр, TNF α , ожиріння, експериментальна інсулінорезистентність.

**ВЛИЯНИЕ
N-СТЕАРОИЛЭТАНОЛАМИНА
НА ЛИПИДНЫЙ СОСТАВ
ПЛАЗМЫ КРОВИ КРЫС С
ЭКСПЕРИМЕНТАЛЬНОЙ
ИНСУЛИНОРЕЗИСТЕНТНОСТЬЮ**

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На модели инсулинорезистентности (ИР), вызванной длительной жировой нагрузкой с преимущественным содержанием насыщенных и мононенасыщенных жиров, было изучено влияние N-стеароилэтанолamina (NSE) на состав свободных жирных кислот (СЖК), липопротеиновый спектр плазмы крови и содержание провоспалительного цитокина TNF α в организме крыс. В проведенных исследованиях установлено повышение уровня мононенасыщенных СЖК (18:1 n-9) и снижение уровня полиненасыщенных СЖК (20:4 n-6) в плазме крови крыс с экспериментальной ИР. Эти процессы сопровождались увеличением содержания цитокина TNF α и значительными изменениями липопротеинового профиля плазмы крови крыс. В частности, зафиксировано снижение содержания холестерина липопротеинов высокой плотности (ЛВП) и увеличение уровня холестерина липопротеинов низкой (ЛНП) и очень низкой плотности (ЛОНП). Введение NSE крысам с ИР способствовало восстановлению уровня моно- и полиненасыщенных СЖК, увеличению содержания холестерина ЛВП и снижению – холестерина ЛНП. Кроме того, у крыс с ИР, получавших NSE, отмечена нормализация уровня TNF α в сыворотке крови. Полученные результаты свидетельствуют о восстановлении при действии NSE липидного профиля плазмы крови крыс с ИР, индуцированной алиментарным ожирением. Принимая во внимание, что липидный состав плазмы крови отражает метаболизм липидов в целом, установленный эффект NSE может иметь определенное значение для предупреждения осложнений, связанных с развитием ИР.

Ключевые слова: N-стеароилэтаноламин, плазма крови крыс, состав свободных жирных кислот, липопротеиновый спектр,

TNF α , ожирение, экспериментальная инсулинорезистентность.

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