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ACTIVITY OF RESPIRATORY CHAIN CYTOCHROME COMPLEXES AND CYTOCHROMES CONTENT IN THE RAT KIDNEY MITOCHONDRIA UNDER DIFFERENT NUTRIENTS CONTENT IN A DIET

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An important role in ensuring the functioning of the respiratory chain belongs to the cytochrome part, which includes complexes III (ubiquinol-cytochrome c oxidoreductase) and IV (cytochrome c oxidase). The key components of these enzymatic complexes are heme-containing cytochromes, the number of which depends on the balance of heme synthesis and catabolism. δ-Aminolevulinate synthase catalyzes the first step of the heme biosynthetic pathway, while heme oxygenase is the key enzyme of heme degradation. It is known that nutritional imbalances drive many risk factors for chronic kidney disease. That is why our research aimed to study the activity of ubiquinol-cytochrome c oxidoreductase and cytochrome oxidase complexes, the level of cytochromes $a+a_3$, b, c, and c_1, and the activity of key enzymes of heme metabolism in the mitochondria of rat kidneys under conditions of different content of protein and sucrose in animal diet. The obtained results showed a decreased activity of ubiquinol-cytochrome c oxidoreductase and cytochrome oxidase complexes and reduced levels of mitochondria cytochromes $a+a_3$, b, c, and c_1 in the kidney mitochondria under the conditions of nutrient imbalance, with the most pronounced changes found in animals kept on a low-protein/high-sucrose diet. A decrease in δ-aminolevulinate synthase activity with a simultaneous 2-fold increase in heme oxygenase activity was found in kidney mitochondria of animals kept on a low-protein/high-sucrose diet compared to those kept on full-value diet indicating an intensification of heme catabolism along with inhibition of its synthesis. The obtained results testify the energy imbalance under the conditions of low-protein/high-sucrose which in turn can lead to the progression of kidney injury.

K e y w o r d s: nutrients, ubiquinol-cytochrome c oxidoreductase, cytochrome oxidase, cytochromes, δ-aminolevulinate synthase, heme oxygenase.

The kidneys control many biological mechanisms such as fluid, electrolyte, pH balance, blood pressure, excretion of toxins and waste, vitamin D metabolism, and hormone synthesis [1]. The kidneys balance many by-products of the dietary components metabolism, whereas the nutritional imbalances drive many risk factors for chronic kidney disease (CKD) [2]. It has been shown that the dysfunction of kidney mitochondria, in particular, disruption of the energy production processes, leads to disturbances of the reabsorption and filtration capacity of the kidneys, which underlies the pathogenesis of different nephropathies [3]. The structural and functional organization of the cytochrome part of the respiratory chain is decisive for maintaining the functioning of the mitochondrial energy supply system under pathological conditions. Previous studies examining dietary effects on kidney health are generally of short duration and manipulate a single macronutrient. On the other hand, there is growing evidence from studies on a wide range of species that, rather than macronutrients acting singly, their interactive effects (their balance) are of higher importance for health. Many of these macronutrient studies used a nutritional geometry approach, where the effects of macronutrients, calories, and food were interrogated across a broad spectrum of diets differing in macronutrient content [4].

Dietary protein has the greatest influence on kidney function, where chronic low protein intake

causes a decrease in glomerular filtration rate and kidney mass and an increase in immune infiltration and structural damage to the kidney [5]. Recent clinical data support a positive relationship between carbohydrate intake and CKD risk [6]. Persistent chronic hyperglycemia is known to contribute to the development and progression of diabetic nephropathy [7]. Diabetic nephropathy is characterized by specific renal morphological and functional alterations [8]. Excess glucose is responsible for inducing redox imbalance and both systemic and intrarenal inflammation, playing a critical role in the pathogenesis of kidney disease [9]. The pathogenesis of the disease is complex, multifactorial and not fully elucidated; many factors and mechanisms are involved in the development, progression and clinical outcomes of the disease. In this situation, there is an elevated expression of non-insulin-dependent, such as renal cells, that have GLUT1 and GLU2 glucose transporters, leading to the increased entry of glucose into renal cells, stimulated by hyperglycemia, and the sodium-glucose co-transporter (SGLT) 1 and SGLT 2, responsible for tubular reabsorption [10]. These glucose transporters do not regulate glucose entry into cells, leading to glucotoxicity. Glucotoxicity is caused by an inability of the cells to compensate for the increased glucose uptake in case of hyperglycemia. Increased stimulation of glucose oxidation pathways in non-insulin-dependent cells leads to the activation of alternative pathways with increased production of ROS and oxidative stress in the hyperglycemic state [11]. Several reports indicate that hyperglycemia-induced generation of superoxide within the mitochondria plays a critical role in the development of diabetic complications [12]. Nevertheless, the influence of hyperglycemia on the renal mitochondrial respiratory complex function has not been thoroughly investigated. A few studies have indicated that diabetes induces alterations in the activities of mitochondrial respiratory complexes and mitochondrial respiration in the kidney [13]. At the same time, the research into the mechanisms of the development of metabolic disorders in the kidneys under the conditions of nutritional imbalance has become especially relevant [14].

An important role in ensuring the functioning of the respiratory chain belongs to the cytochrome part, which includes the III (ubiquinol-cytochrome *c* oxidoreductase) and IV (cytochrome oxidase) complexes. The key components of these enzymatic complexes are cytochromes. Since cytochromes are heme-containing proteins, their number is largely determined by the balance of heme synthesis and catabolism processes [15]. The key enzyme of the metabolic pathway of heme synthesis is δ-aminolevulinate synthase (ALAS; EC 2.3.1.37), which catalyzes the first reaction of the heme biosynthetic pathway – the condensation of glycine and succinyl coenzyme A with the formation of δ-aminolevulinic acid [16]. Heme oxygenase (HO; EC 1.14.99.3) – the key enzyme of heme degradation – provides oxidative cleavage of heme with the formation of equimolar amounts of carbon monoxide (CO) and biliverdin, which are considered cytoprotective molecules [17].

The research aimed to study the activity of enzymes of the III and IV respiratory chain complexes, the levels of cytochromes a+a_3, b, c, and c_1, and the activity of the heme metabolism key enzymes in the mitochondria of rat kidneys under the conditions of the high-sucrose and low-protein diet

Materials and Methods

Experimental design and procedures. In the study, 10-12-week-old white nonlinear rats weighing 130-140 g were used. The animals were separated into solitary plastic cages and ad libitum access to water. Animals were housed at a controlled temperature of 19±2°C with a 12-hour light-dark cycle. The animals were monitored daily, weighed three times/week. The experiment was conducted in accordance with the rules set by the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986) and approved by bioethics commission of the Educational and scientific institute of the biology, chemistry and biological resources of Yuriy Fedkovych Chernivtsi National University (protocol No 2 dated 24.12.2021).

The animals were divided into the following experimental groups: I – animals receiving full-value semi-synthetic ration (C); II – animals receiving low-protein diet (LPD); III – animals receiving high-sucrose diet (HSD); IV – animals receiving low-protein high-sucrose diet (LPD/HSD).

Animals consumed a semi-synthetic diet AIN-93 in accordance with the recommendations of the American Institute of Nutrition [18] on the principle of pair-feeding (Table).

Diets were manufactured at Educational and Scientific Institute of Biology, Chemistry and Natural Resources, stored at 4°C.

Animals received feed of 30 g/100 g BW. In order to estimate the total macronutrients from the diet, a weighed quantity of food was added daily at the same time of day (09:00 to 10:00 hours). The animals of the control group consumed 4.2 g of protein/100 g BW, 3.0 g of fat/100 g BW, and 3 g of sucrose/100 g BW. The animals of the group II consumed isoenergetic ration containing 1.4 g of protein/100 g BW, 3 g of sucrose/100 g BW and 3.0 g of fat/100 g BW. The animals of the group III consumed high-sucrose diet containing 4.2 g of protein/100 g BW, 12 g of sucrose/100 g BW and 3.0 g of fat/100 g BW, balanced by all other essential nutrients [2]. The animals of the group IV consumed isoenergetic ration containing 1.4 g of protein/100 g BW, 12 g of sucrose/100 g BW), and balanced by all other essential nutrients. The animals were maintained on the corresponding diet for four weeks. Cervical dislocation was performed under the light ether anesthesia on day 29 of the experiment [19].

The mitochondrial fraction of the kidney homogenate was separated by differential centrifugation (Heraeus Biofuge, Germany) in the following buffer medium: 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl; pH 7.4 at 0-3°C [20]. Mitochondrial protein concentration was determined using the Bradford method.

Complex III (ubiquinol:cytochrome c oxidoreductase) activity was assessed in assay buffer containing 50 mM Tris-HCl, 4 mM NaN_3, 0,1 mg/ml of bovine serum albumin (BSA), and 0.05% (vol/vol) Tween 20. The reaction was started by adding of 0.25 mM decylubiquinol and 0.0625 mM cytochrome *c*. The reaction was initiated by the addition of 100 µl of a mitochondria suspension containing 1 mg of protein. Reduction of cytochrome *c* with decylubiquinol was determined at 550 nm [21]. Ubiquinol cytochrome *c* oxidoreductase activity is expressed as nmol/min·mg of protein.

Cytochrome oxidase activity was determined by a method based on the ability of cytochrome oxidase to oxidize dimethylparaphenylenediamine and α-naphthol (NADI reagent) to form a colored product [22]. Its concentration is proportional to cytochrome oxidase activity. Cytochrome oxidase activity was assessed in the following buffer medium: 0.1 M phosphate buffer, pH 7.4, 0.1% α-naphthol solution in 22% ethyl alcohol, 0.1% dimethyl-p-phenylenediamine hydrochloride, 0.002% cytochrome *c*. Cytochrome oxidase activity is expressed as nmol/min·mg of protein. The samples were measured spectrophotometrically at λ 550 nm.

Determination of cytochrome contents. The determination of the contents of cytochromes $a+a_3$, *b*, *c* and c_1 in the suspension of isolated mitochondria was carried out by the difference spectra between the reduced and oxidized states at room temperatures [23].

ALAS activity was assayed based on the reaction with acetylacetone, ALA formation was measured as a pyrrole that is detected quantitatively using Ehrlich's reagent by measuring color development at 556 nm. The enzyme assays were performed in the final volume of 1 ml containing 50 mM Tris-HCl buffer (pH 7.5), 5 mM glycine, 1 mM succinyl-CoA,

Table. Ingredient composition of the diets (g/kg diet)

Ingredient	Diet			
	C	LPD	HSD	LPD/HSD
	3601.0 kcal/kg	3601.0 kcal/kg	3797.7 kcal/kg	3797.7 kcal/kg
Cornstarch, g/kg	620.7	714.1	320.7	414.1
Casein, g/kg	140	46.6	140	46.6
Sugar, g/kg	100	100	400	400
Fiber (cellulose microfiber), g/kg	50	50	50	50
Mineral Mix, g/kg[1]	35	35	35	35
Vitamin Mix, g/kg[1]	10	10	10	10
L-Cystine, g/kg	1.8	1.8	1.8	1.8
Choline bitartrate, g/kg	2.5	2.5	2.5	2.5
Soy Oil, g/kg	40	40	40	40

None. [1]Mineral and Vitamin Mix – Based on the AIN-93G vitamin and mineral mixes

0.5 mM pyridoxal-5-phosphate, and 1 mg mitochondrial protein. The assays were terminated by the addition of trichloroacetic acid [24]. ALAS activity is expressed as nmol/min·mg of protein.

Heme oxygenase activity was assessed by bilirubin production in mitochondrial fraction, using 0.5-mg protein. Bilirubin production was measured spectrophotometrically and expressed as picomoles of bilirubin per mg of protein per hour ($\varepsilon_{453\ nm}$ = 40 $mM^{-1}\cdot cm^{-1}$) [25].

Data analysis and statistics. The data were compared and analyzed by using unpaired T-test. Characteristics of the study group were expressed as mean±SD for normal distribution. For all statistical calculations, significance was considered to be a value of $P < 0.05$.

Results and Discussion

The results of our research showed that under all the studied conditions, there was a decrease in the activity of ubiquinol-cytochrome c oxidoreductase, an enzyme of Complex III of the respiratory chain (Fig. 1), and cytochrome oxidase, which reflects the activity of Complex IV of the respiratory chain (Fig. 2). In particular, in animals kept on a low-protein diet, the activity of the studied enzymes of the cytochrome part of the respiratory chain decreases by 20-40% compared to the control group. Probably, under the conditions of dietary protein deficiency, the synthesis of individual subunits of the respiratory chain enzymes is disturbed, which results in the dysfunction of the entire enzymatic complex.

At the same time, in animals kept on a high-sucrose diet, the activity of ubiquinol-cytochrome *c* oxidoreductase and cytochrome oxidase decreased approximately by half compared to the control (Fig. 1, Fig. 2). The maximum 2.7-fold decrease in cytochrome oxidase activity compared to the control was found in animals kept on a low-protein/high-sucrose diet (Fig. 2), while the ubiquinol-cytochrome *c* oxidoreductase activity remained at the same level as in animals kept on a diet with an excess of sucrose (Fig. 1). Changes in the activity of the cytochrome part of the respiratory chain enzymes in case of excessive sucrose intake can be associated with several reasons. The decrease in the activity of Complexes III and IV can be caused by oxidative damage to mitochondrial proteins and occurrence of hyperglycemia, as was found in our earlier studies [26, 27].

It has been shown that the early stage of hyperglycemia is accompanied by Complex III assembly defects followed by an intensification of ROS generation leading to an impairment of mitochondrial and renal function [15]. Moreover, hyperglycemia is associated with an increase in the levels of Rieske and core 2 proteins – structural components

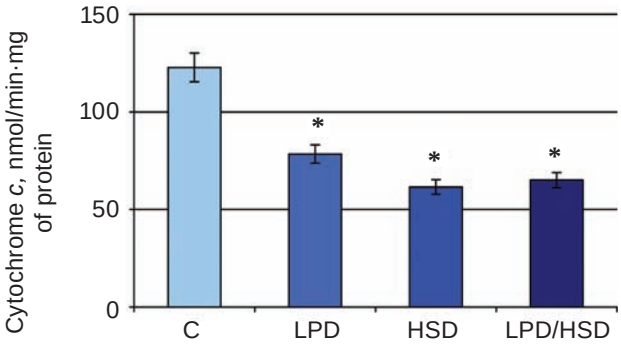

*Fig. 1. Activity of ubiquinol-cytochrome c oxidoreductase in rat kidney mitochondria under the conditions of different nutrient contents in a diet (M ± m, n = 9): C – animals receiving complete semi-synthetic ration; LPD – animals receiving low-protein ration; HSD – animals receiving high-sucrose diet; LPD/HSD – animals receiving low-protein high-sucrose. *The difference is statistically significant compared with the control value (P < 0.05)*

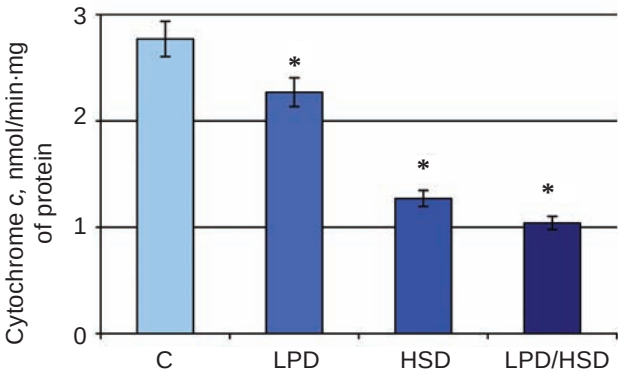

*Fig. 2. Activity of cytochrome oxidase in rat kidney mitochondria under the conditions of different nutrient contents in a diet (M ± m, n = 9): C – animals receiving complete semi-synthetic ration; LPD – animals receiving low-protein ration; HSD – animals receiving high-sucrose diet; LPD/HSD – animals receiving low-protein high-sucrose. *The difference is statistically significant compared with the control value (P < 0.05)*

of Complex III, which is considered a compensatory mechanism for restoring the activity of ubiquinol-cytochrome *c* oxidoreductase. Besides, taking into account that reduced ubiquinone is the coenzyme of ubiquinol-cytochrome *c* oxidoreductase, and its levels in the kidneys are decreased under the conditions of nutrient imbalance [26], this can be considered as another cause of the reduced activity of the respiratory chain complexes. Since the important structural components of Complexes III and IV are heme-containing proteins – cytochromes, one of the reasons for the drop in the ubiquinol-cytochrome *c* oxidase and cytochrome oxidase activity under the studied conditions could be changes in their levels.

The results of our research show that under the conditions of a low-protein diet, there is a decrease in the levels of all mitochondrial cytochromes. In particular, the cytochromes $a+a_3$, *b*, and c_1 levels decrease by 20-55%; the cytochrome *c* levels decrease approximately by half compared to the control (Fig. 3). The changes in the cytochromes levels can be caused by the disturbances in the synthesis of the corresponding protein subunits under the conditions of protein deficiency, or/and by changes in heme metabolism. Similar changes in the levels of cytochromes are characteristic of animals kept on a high-sucrose diet (Fig. 3). In animals kept on a low-protein/high-sugar diet, the levels of cytochromes *b* and c_1 – the structural components of ubiquinol-cytochrome *c* oxidoreductase, remained at the levels of other experimental groups. However, under the specified conditions, we found an approximately 2-fold decrease in the cytochromes $a+a_3$ levels and a 2.5-fold reduction of the cytochrome c levels compared to the control (Fig. 3).

The reduction in the cytochrome *c* levels is likely associated with its increased release into the cytosol under the conditions of oxidative stress, and the decrease in the cytochromes $a+a_3$ levels is caused by the changes in the activity of heme o-synthase and heme a-synthase – enzymes catalyzing the sequential transformation of heme *b* into heme *a* [28]. Considering that the activity of ubiquinol-cytochrome *c* oxidoreductase in the group of the low-protein/high-sucrose diet does not differ significantly from the indices in the high-sucrose diet group, and the activity of cytochrome oxidase, which components are cytochromes $a+a_3$, decreases, then it is the change in the cytochromes levels that is the determining factor of alterations in the activity of Complexes III and IV enzymes.

It is known that the quantitative content of mitochondrial cytochromes is largely determined by the intensity of the breakdown and synthesis of their heme, so another reason for the changes may be an alteration of the activity of key enzymes of heme

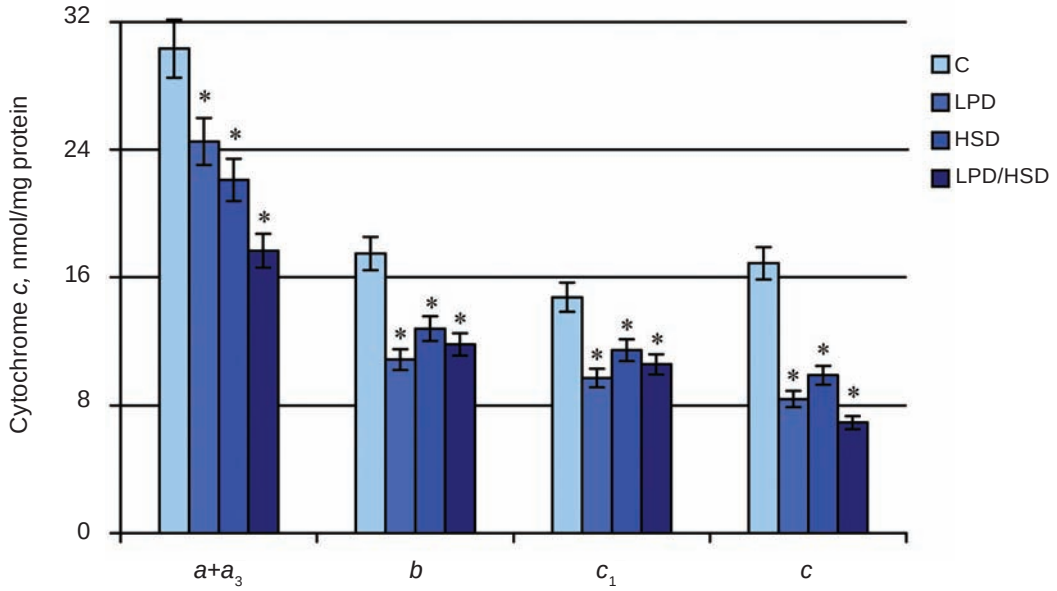

*Fig. 3. Cytochromes levels in rat kidney mitochondria under the conditions of different nutrient contents in a diet (M ± m, n = 9): C – animals receiving complete semi-synthetic ration; LPD – animals receiving low-protein ration; HSD – animals receiving high-sucrose diet; LPD/HSD – animals receiving low-protein high-sucrose. *The difference is statistically significant compared with the control value (P < 0.05)*

metabolism. The key enzymes of heme metabolism are δ-aminolevulinate synthase, which catalyzes the condensation reaction of glycine and succinyl-coenzyme A with the formation of the precursor of heme – δ-aminolevulinic acid [29], and heme oxygenase, which provides oxidative cleavage of heme with the formation of an equimolar amount of carbon monoxide and biliverdin, and subsequently bilirubin and ferritin via iron release from the heme moiety [3]. The research results have shown that the activity of δ-aminolevulinate synthase and heme oxygenase does not change significantly in animals that consumed a low-protein diet (Fig. 4, Fig. 5).

Therefore, the found decrease in the levels of mitochondrial cytochromes probably is not related to a disruption of heme metabolism but caused by changes in the synthesis of the protein part of cytochromes as a result of a dietary protein deficiency. At the same time, the excessive sucrose consumption resulted in the decrease of δ-aminolevulinate synthase activity by approximately 30% compared to the control (Fig. 4). It has been shown that an increase in the blood glucose levels, which is characteristic of animals kept under the conditions of excessive sucrose intake, suppresses the transcription of the δ-aminolevulinate synthase gene [30]. Similar changes in the activity of δ-aminolevulinate synthase are found in the kidneys of rats kept on a low-protein/high-sucrose diet (Fig. 4). The research results showed that the maximum (approximately 2-fold) increase in heme oxygenase activity compared to the control was seen in animals kept on a high-sucrose diet (Fig. 5). Taking into account the biological effects of heme oxygenase, the found increase in its activity has a pronounced compensatory effect. Apparently, the increase in the heme oxygenase activity occurs in response to changes in the structural organization of hemoproteins of the respiratory chain to prevent the accumulation of non-specifically bound heme showing pro-oxidant properties.

It is known that the products of the heme oxygenase reaction – biliverdin and carbon monoxide (CO) exhibit a pronounced protective effect under the conditions of hyperglycemia: biliverdin and bilirubin act as antioxidants [31]; they increase insulin sensitivity and suppress inflammatory reactions [32]. The role of CO remains debatable, but it has been shown that carbon monoxide increases insulin secretion, participates in the regulation of Ca^{2+} signals [33], lowers glucose levels and increases oxygen consumption [34]. Moreover, the activation of heme oxygenase induces the synthesis of ferritin with subsequent chelation of the released iron and reduction of the oxidative stress manifestations [35]. Therefore, the found increase in the heme oxygenase activity against the background of the decrease in the δ-aminolevulinate synthase activity can be considered as one of the possible mechanisms for reducing

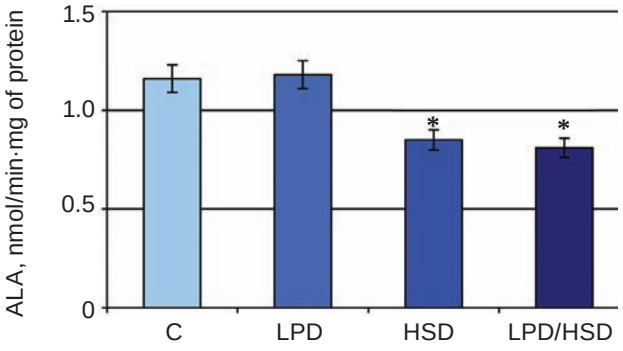

*Fig. 4. Activity of ALAS in rat kidney mitochondria under the conditions of different nutrient contents in a diet (M ± m, n = 9): C – animals receiving complete semi-synthetic ration; LPD – animals receiving low-protein ration; HSD – animals receiving high-sucrose diet; LPD/HSD – animals receiving low-protein high-sucrose. *The difference is statistically significant compared with the control value (P < 0.05)*

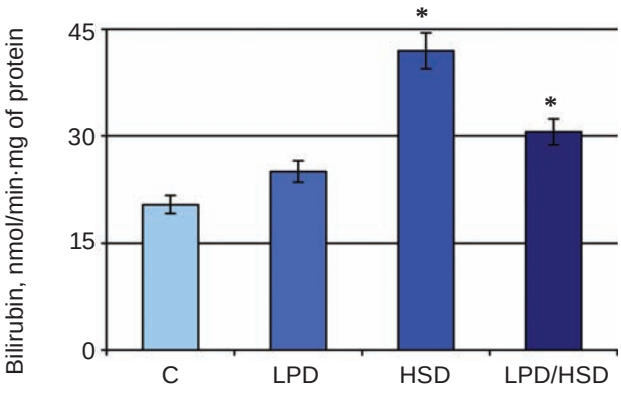

*Fig. 5. Activity of heme oxygenase in rat kidney mitochondria under the conditions of different nutrient contents in a diet (M ± m, n=9): C – animals receiving complete semi-synthetic ration; LPD – animals receiving low-protein ration; HSD – animals receiving high-sucrose diet; LPD/HSD – animals receiving low-protein high-sucrose. *The difference is statistically significant compared with the control value (P < 0.05)*

the levels of mitochondrial cytochromes under the conditions of a high-sucrose diet.

The obtained results regarding the levels of cytochromes, the activity of cytochrome enzymes and key enzymes of heme metabolism in the kidney can be considered as prerequisites for deepening the energy imbalance under the conditions of different content of protein and sucrose in a diet, which in turn can lead to the progression of kidney injury.

The obtained results open up prospects for developing a strategy for correcting energy metabolism disorders in conditions of nutritional imbalance.

Conflict of interest. The 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.

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АКТИВНІСТЬ ЕНЗИМІВ ЦИТОХРОМНОЇ ДІЛЯНКИ ДИХАЛЬНОГО ЛАНЦЮГА МІТОХОНДРІЙ НИРОК ЩУРІВ ЗА УМОВ РІЗНОЇ ЗАБЕЗПЕЧЕНОСТІ ХАРЧОВОГО РАЦІОНУ НУТРІЄНТАМИ

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Важливу роль у забезпеченні функціонування дихального ланцюга відіграє цитохромна ділянка, до складу якої входять III (убіхінол-цитохром-*c*-оксидоредуктаза) і IV (цитохромоксидаза) комплекси, що беруть участь у генерації електрохімічного потенціалу ($\Delta\mu H^+$) через транспорт електронів від убіхінолу. Ключовими компонентами цих ензиматичних комплексів є гемовмісні цитохроми, кількість яких значною мірою визначається балансом між процесами синтезу та катаболізму гема. δ-Амінолевулінатсинтаза каталізує перший етап шляху біосинтезу гема, гемоксигеназа є ключовим ензимом деградації гема. У роботі досліджена активність ензимів убіхінол-цитохом *c*-оксидоредуктазного та цитохромоксидазного комплексів, вміст цитохромів $a+a_3$, *b*, *c*, c_1 та активність ключових ензимів метаболізму гема у мітохондріях нирок щурів за умов різної забезпеченості раціону протеїном та сахарозою. Встановлено, що під час нутрієнтного дисбалансу у мітохондріях нирок щурів спостерігається зниження активності ензимів убіхінол-цитохром *c*-оксидоредуктазного та цитохромоксидазного комплексів, при цьому максимальні зміни були у тварин, які споживали низькопротеїновий/високосахарозний раціон. Активність убіхінол-цитохром *c*-оксидоредуктази знижувалась приблизно вдвічі на тлі зниження цитохромоксидазної активності у понад 2,5 раза порівняно з контролем. У нирках тварин усіх дослідних груп спостерігалося зниження вмісту мітохондріальних цитохромів $a+a_3$, *b*, *c*, c_1, при цьому мінімальні значення були у тварин, яких утримували на низькопротеїновому/високосахарозному раціоні. Водночас спостерігалося зниження активності δ-амінолевулінатсинтази в середньому у 1,5 раза при одночасному підвищенні активності гемоксигенази приблизно вдвічі порівняно з контрольними значеннями, що свідчило про посилення процесів катаболізму гема на тлі сповільнення його синтезу за досліджуваних умов. Встановлене нами підвищення активності гемоксигенази на тлі зниження активності δ-амінолевулінатсинтази може розглядатися як один із можливих механізмів зниження вмісту мітохондріальних цитохромів за умов нутрієнтного дисбалансу, що у свою чергу може призводити до порушення активності ензимів цитохромної ділянки. Отримані результати щодо вмісту цитохромів, активності ензимів цитохромної ділянки та ключових ензимів метаболізму гема у нирках щурів можуть розглядатися як передумови для поглиблення енергетичного дисбалансу за різної забезпеченості раціону сахарозою та протеїном, що у свою чергу може призводити до прогресування пошкодження нирок.

Ключові слова: нутрієнти, убіхінол-цитохром *c*-оксидоредуктаза, цитохромоксидаза, цитохроми, δ-амінолевулінатсинтаза, гемоксигеназа.

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