

The role of free radical oxidation in the kidneys in the nephroprotective action of eplerenone, a mineralocorticoid receptor antagonist, in experimental diabetes mellitus

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ABSTRACT

Aim. To study the effect of eplerenone on the activity of free radical oxidation and renal function in rats with experimental diabetes mellitus induced by streptozotocin.

Materials and methods. Experiments were carried out on 36 male Wistar rats. Diabetes mellitus (DM) was simulated by a single intraperitoneal injection of streptozotocin at a dose of 65 mg / kg. Eplerenone was injected into the stomach at a dose of 50 mg / kg.

Results. It was found that eplerenone in experimental diabetic nephropathy (DN) significantly attenuated proteinuria: the concentration of protein in the urine became 4 times lower than in untreated DN ($p < 0.001$). In the kidneys, eplerenone therapy normalized the structure and function of renal glomeruli and restored the podocyte number, which was reduced by 37.8% in the DN model. Free radical oxidation (FRO) in the kidneys of rats treated with eplerenone increased – the concentration of thiobarbituric acid reactive substances rose by 1.5 times ($p = 0.009$), and changes in the activity of antioxidant enzymes, such as superoxide dismutase (a decrease by 2.4 times, $p = 0.002$), catalase (an increase by 1.8 times, $p < 0.001$), and glutathione peroxidase (an increase by 1.5 times, $p < 0.001$) were observed, as opposed to the values in the controls.

Conclusion. In streptozotocin-induced experimental diabetic nephropathy in rats, eplerenone had a nephroprotective effect, but increased oxidative stress in the kidneys. The increase in FRO could be determined by the nongenomic effect of aldosterone, which accumulates under conditions of prolonged mineralocorticoid receptor (MR) blockade. The nephroprotective effect of eplerenone can be associated with the weakening of the genomic effects of aldosterone, realized with the participation of MR.

Key words: eplerenone, free radical oxidation, diabetes mellitus, diabetic nephropathy.

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Роль свободнорадикального окисления в почках в нефропротекторном действии блокатора минералокортикоидных рецепторов эплеренона при экспериментальном сахарном диабете

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РЕЗЮМЕ

Цель. Изучить влияние эплеренона на активность свободнорадикального окисления и функции почек крыс при экспериментальном сахарном диабете, вызванном стрептозотоцином.

Материалы и методы. Эксперименты проведены на 36 самцах крыс линии Вистар. Сахарный диабет моделировали однократным внутрибрюшинным введением стрептозотоцина в дозе 65 мг/кг. Эплеренон вводили в желудок в дозе 50 мг/кг.

Результаты и обсуждение. Установлено, что эплеренон при экспериментальной диабетической нефропатии (ДН) существенно ослабляет протеинурию: количество белка в моче становится меньше в 4 раза, чем при нелеченой ДН ($p < 0,001$). В почках под влиянием терапии эплереноном нормализуются структура и функции почечных клубочков, в том числе восстанавливается количество подоцитов, уменьшенное при модели ДН на 37,8%. Активность свободнорадикального окисления (СРО) в почках крыс, получавших эплеренон, усиливается: увеличивается концентрация тиобарбитуратреактивных продуктов в 1,5 раза ($p = 0,009$) и изменяется по сравнению с показателями контроля активность антиоксидантных ферментов – супероксиддисмутазы (снижается в 2,4 раза, $p = 0,002$), каталазы (увеличивается в 1,8, $p < 0,001$) и глутатионпероксидазы (увеличивается в 1,5 раза, $p < 0,001$).

Заключение. При экспериментальной ДН, вызванной у крыс введением стрептозотоцина, эплеренон оказывает нефропротекторное действие, но усиливает оксидативный стресс в почках. Усиление СРО могло быть обусловлено негеномным мембранным действием альдостерона, компенсаторно накапливающегося в условиях длительной блокады минералокортикоидных рецепторов (МКР). Нефропротекторное действие эплеренона можно связать с ослаблением геномных эффектов альдостерона, реализуемых при участии МКР.

Ключевые слова: эплеренон, нефропротекторное действие, свободнорадикальное окисление, экспериментальная диабетическая нефропатия.

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INTRODUCTION

Oxidative damage to the kidneys makes a significant contribution to the development of diabetic nephropathy (DN) [1], therefore, the study of the mechanisms of peroxidation in the kidneys, the search

for new targets for targeted antioxidant therapy, and the development of new pharmacological approaches to DN treatment on their basis are relevant.

There are four main mechanisms of oxidative stress development in the kidneys in DN: direct inhibition of cellular antioxidant systems by glucose and its metabo-

lites, accumulation of advanced glycation end products (AGEs), activation of protein kinase C, and activation of the renin-angiotensin-aldosterone system (RAAS) [2]. NADPH oxidase catalyzing the formation of reactive oxygen species (ROS) is activated in the kidneys [3]. When searching for new effective methods of drug therapy in DN, an important question remains unresolved: which mechanism contributes the most to the oxidative stress development? The answer to this question would contribute to targeted and more effective development of new pharmacological approaches to DN therapy.

We studied the effect of RAAS drug inhibition on oxidative damage to the kidneys and the course of DN in experimental diabetes mellitus (DM). For this purpose, eplerenone was chosen as a pharmacological tool, which is a non-steroidal aldosterone antagonist previously studied as a nephroprotector in DN [4].

The aim of the study was to investigate the effect of eplerenone on the activity of free radical oxidation and renal function in rats with experimental, streptozotocin-induced DM.

MATERIALS AND METHODS

Experiments were conducted on 36 male Wistar rats aged 2–3 months and weighing 300–350 grams. The study was carried out in accordance with Directive 86/609/EEC, the Declaration of Helsinki, and the “Rules for Work Using Experimental Animals”. The study was approved by the local Ethics Committee at ASMU (Protocol No. 4 of 30.04.2020). The rats were placed in individual metabolic cages adapted for urine collection. The animals were kept under natural light conditions, at room temperature and could freely consume water and “Chara” feed produced by Assortiment-Agro LLC. All manipulations with animals were carried out from 9 AM to 12 AM.

According to the design of the experiment, the animals were divided into 3 groups: a control group and an experimental group each consisting of 13 animals and a group of intact rats containing 10 animals. In the control and experimental groups, the rats were administered intraperitoneally with 1 ml of streptozotocin solution (Applichem, Germany) at a single dose of 65 mg / kg in the citrate buffer to model DM. For more accurate modeling of type 2 DM, the rats were previously injected intraperitoneally with cytoflavin solution at the nicotinamide rate of 115 mg / kg. The administration of nicotinamide weakens streptozotocin-induced damage to the islets of Langerhans and allows for moderate hyperglycemia without massive cytolysis of pancreatic cells [5].

In the experimental group for DN treatment, eplerenone (Polpharma, Poland) was injected in the stomach at a dose of 50 mg / kg once a day daily for 3 weeks, starting from the 5th week after the streptozotocin injection. The dose of eplerenone was chosen according to the results of previous studies on the nephroprotective effect of the drug in the DM model [4]. In preliminary studies, we showed that typical signs of nephropathy in rats develop only by the end of the 4th week after streptozotocin administration [6].

To assess renal functions in rats of the control and experimental groups, the concentrations of glucose, protein, and creatinine in urine were determined and their excretion with urine was calculated prior to the start of DM modeling and then weekly. The glucose, protein, and creatinine levels in the urine were measured using the DIRUICS-T240 automatic biochemical analyzer (Dirui Industrial Co., Ltd., China) with biochemical kits (DIACON-DS, Russia).

After 8 weeks of the experiment, the animals were euthanized by ether, their kidneys were extracted and washed with isotonic sodium chloride solution. One kidney was used for morphological studies, the second one was used to study the activity of FRO. According to the methods given in the manual [7], the activity of FRO was evaluated by the concentration of thiobarbituric acid reactive substances (TBARS) and the activity of antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), in the kidneys.

For morphological studies, Sakkura (Japan) devices were used. The kidneys were fixed in 10% neutral formalin solution. The material was dehydrated in isopropyl alcohol using the carousel-type machine TISSUE-TEK VIPTM6 (Japan). The material was poured into paraffin using the paraffin filling station TISSUE-TEK TEC 5 (Japan). 5–7 µm-thick histological sections were obtained using the semi-automatic rotary microtome Accu-Cut SRM (the Netherlands) and stained with hematoxylin and eosin and according to the Van Gieson’s method in the TISSUE-TEK Prisma automated slide stainer (Japan). Neutral glycosaminoglycans were determined histochemically using Schiff reagent according to McManus. The slides were placed under film in the TISSUE-TEK Film coverslipper (Japan).

Morphometric studies were performed using a computer image analysis system consisting of the Leica DME microscope (Germany), the Leica EC3 digital color camera (Leica Microsystems AG, Germany), a personal computer, and Video Test Morphology 5.2 software. The area of renal glomeruli and the area of

capillary lumina were measured, and after special computer processing of digital photos, the total area of the vascular bed, the area of the mesangium, and the number of podocytes in the glomerulus were calculated.

The results were processed statistically using the Statistica 13.3.1 software (license JPZ9061-448517FAACD-K). The results of the biochemical studies are presented by the median and the interquartile range (Me (25%; 75%)). The results of the morphometric studies were presented by the mean and the standard error of the mean ($M \pm m$). Statistical comparisons between the groups were carried out using the nonparametric Mann – Whitney U-test, comparisons within the group were performed using the nonparametric Wilcoxon test [8].

RESULTS

The experiments showed that during 8 weeks in rats of the control and experimental groups, the amount of excreted urine and renal excretion of glucose and creatinine statistically significantly exceeded the initial levels and did not differ between the groups (not shown in the tables and figures).

In the control and experimental groups during the first 28 days of DM modeling, renal excretion

of protein significantly increased (Fig. 1): in the control group – from 2.7 (1.8; 8.7) to 11.0 (5.9; 15.3) mg / day ($p = 0.004$), in the experimental group – from 3.0 (0.6; 3.8) to 10.2 (9.9; 13.4) mg / day ($p = 0.002$). Statistically significant differences between the groups were not recorded. From the 5th to the 8th week of the experiment, protein excretion in the control group continued to increase and peaked by the end of the experiment – up to 36.0 (28.6; 43.2) mg / day, which was 13.3 times higher than before the experiment ($p = 0.001$). After the start of eplerenone administration, the increase in renal protein excretion stopped, and until the end of the experiment, the excretion did not change compared to the indicator measured on the 28th day. As a consequence, at the 6–8th week of eplerenone administration, renal protein excretion was statistically significantly lower than in the control group during the specified periods of time: after 6 weeks, it decreased by 2.3 times to 5.0 (3.2; 7.5) mg / day as opposed to 11.6 (5.3; 14.8) mg / day ($p = 0.018$), after 7 weeks – by 1.8 times to 7.7 (2.6; 10.3) mg / day as opposed to 13.8 (11.6; 16.0) mg / day ($p = 0.002$), after 8 weeks – by 4 times to 9.0 (3.5; 15.0) mg / day as opposed to 36.0 (28.6; 43.2) mg / day ($p < 0.001$).

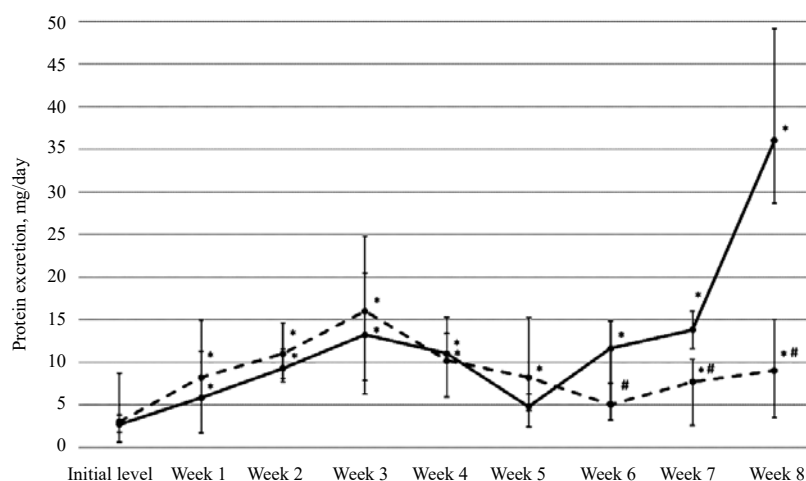


Fig. 1. Dynamics of protein excretion in urine: * indicates statistically significant differences compared to the initial level, the sign # indicates statistically significant differences between the groups

Morphological studies of the kidneys of experimental animals allowed to establish that in the control group, renal glomeruli were enlarged and the intercapillary space in the glomeruli increased due to the accumulation of periodic acid-Schiff (PAS)-positive material in the mesangium (Fig. 2). The lumina of the glomerular capillaries were narrowed, the basement membranes of the capillaries and the Bowman's

capsule were thickened. Podocytes increased in size, swelling of their nuclei occurred. In the renal interstitium, foci of nephrosclerosis were found; in these areas, the tubular basement membranes were thickened. The tubular nephrocytes were flattened, the tubular lumen was dilated. Most nephrocytes were in a state of hyaline-drop dystrophy. In some regions, lymphocytic-plasmacytic infiltration was identified.

In rats of the experimental group who received eplerenone, the area of renal glomeruli decreased (Fig. 3). A focal increase in the intercapillary space and deposition of PAS-positive material were weakly pronounced. The lumina of the glomerular capillaries were mostly wide, congestion of the capillaries was noted. Podocytes were small in size, with

rounded small nuclei. In the renal interstitium, nephrosclerosis phenomena were minimal. The morphological structure of nephrocytes approached normal one, hyaline-drop dystrophy phenomena were absent at most sites. The intergroup comparison of quantitative morphometric parameters is presented in Table 1.

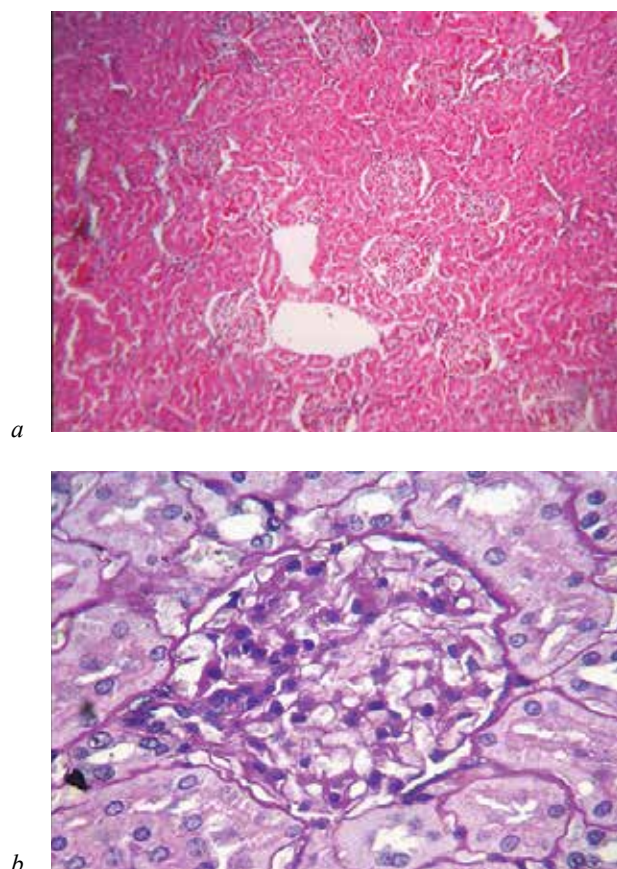


Fig. 2. Rat kidney tissue in the control group: *a* – increase in the size of glomeruli, staining with hematoxylin and eosin, $\times 100$; *b* – increase in the intercapillary space and narrowing of the capillary lumina, McManus staining, $\times 1,200$

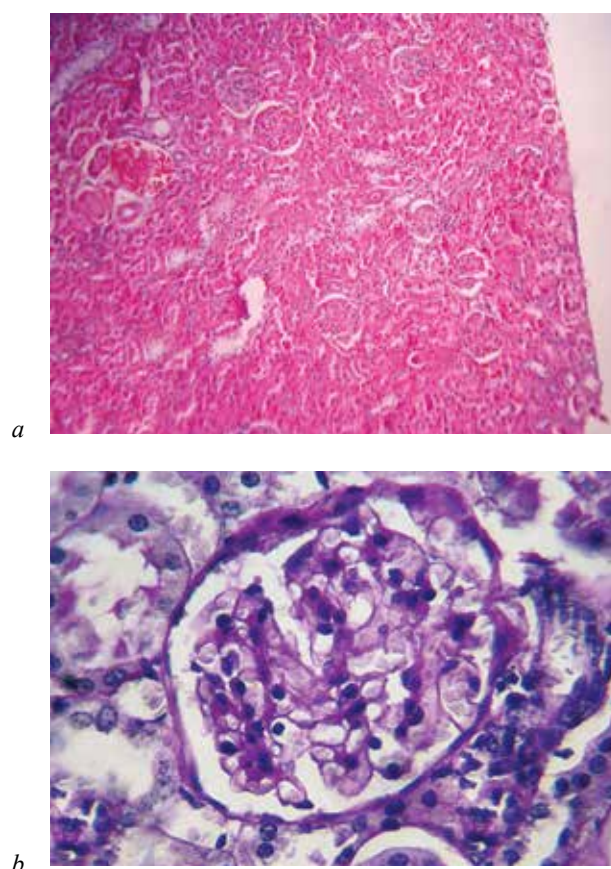


Fig. 3. Rat kidney tissue in the experimental group: *a* – decrease in the size of glomeruli, staining with hematoxylin and eosin, $\times 100$; *b* – decrease in the intercapillary space and dilation of the capillary lumina, McManus staining, $\times 1,200$

Table 1

Morphometric parameters of renal glomeruli in experimental animals, $M \pm m$			
Parameters	Intact rats	Control group	Experimental group
Area of renal glomeruli (μm^2)	$6,174.7 \pm 257.5$	$7,758.65 \pm 329.5$ $p_{\text{int}} < 0.001$	$4,810.4 \pm 202.6$ $p_e < 0.001$ $p_{\text{int}} < 0.001$
Total area of the glomerular vessels (μm^2)	$2,900 \pm 27.4$	$1,148.5 \pm 107.65$ $p_{\text{int}} < 0.001$	$1,569.65 \pm 282.05$ $p_{\text{int}} < 0.001$
Area of glomerular capillary lumen (μm^2)	47.5 ± 3.7	24.25 ± 1.65 $p_{\text{int}} < 0.001$	31.4 ± 2.4 $p_e = 0.004, p_{\text{int}} = 0.001$
Area of the glomerular mesangium (μm^2)	$4,738.7 \pm 43.3$	$5,609.9 \pm 823.1$	$3,733.3 \pm 505.9$
Podocytes (particles)	10.2 ± 0.20	7.4 ± 0.6 $p_{\text{int}} < 0.001$	11.8 ± 0.5 $p_e < 0.001$

Note. p_{int} – level of statistical significance as opposed to intact rats, p_e – level of statistical significance in the experimental group as opposed to the control group (here and in Table 2).

Table 2

Activity of free radical oxidation in the kidneys of rats in experimental groups, Me (25%; 75%)			
	Intact rats	Control group	Experimental group
TBARS concentration (μmol / mg)	6.1 (5.4; 6.9)	6.0 (4.8; 6.6)	8.5 (6.2; 9.9) $p_{\text{int}} = 0.005, p_{\text{c}} = 0.009$
CAT activity (%)	14.4 (10.2; 15.6)	32.7 (23.0; 37.7) $p_{\text{int}} < 0.001$	60.1 (50.5; 64.0) $p_{\text{int}} < 0.001$ $p_{\text{c}} < 0.001$
SOD activity (%)	18.2 (13.0; 18.5)	8.8 (7.7; 10.3) $p_{\text{int}} = 0.003$	3.7 (3.4; 5.5) $p_{\text{int}} < 0.001, p_{\text{c}} = 0.002$
GPx activity (%)	38.5 (25.2; 41.6)	58.7 (57.5; 60.3) $p_{\text{int}} < 0.001$	89.5 (88.4; 91.2) $p_{\text{int}} < 0.001$ $p_{\text{c}} < 0.001$

In the kidneys of rats in the control group, CAT activity increased by 2.3 times, GPx – by 1.5 times, SOD activity decreased by 2.1 times as opposed to values in the intact animals (Table 2). The TBARS concentration did not change.

In the experimental group of animals receiving eplerenone, contrary to expectations, FRO increased: TBARS concentrations increased by 1.4 times compared to the value in the intact and control rats; CAT activity became 4.2 times higher than in the intact rats and 1.8 times higher than in the controls. SOD activity decreased by 2.4 times compared to the value in the control group and by 4.9 times compared to the intact rats. GPx activity was 2.3 times higher than in the intact animals and exceeded the value in the control group by 1.5 times.

DISCUSSION

The rats with the DN model developed oxidative stress. A month after the streptozotocin administration against the background of FRO, the activity of the main antioxidant enzymes (GPx and SOD) increased [9]. This appears to be compensatory in response to ROS generation. Two months after the DM modeling, the SOD activity decreased, whereas the GPx and CAT activity increased. It is possible that as FRO intensity progresses, the activity of SOD is depleted, and the GPx and CAT activity undergoes substrate stimulation by a large amount of ROS. The treatment cycle with eplerenone in experimental DM increased oxidative stress in the kidneys, but had a nephroprotective effect. This allows us to conclude that RAAS inhibitors normalize the functions of the renal glomeruli and their filtration barrier, regardless of the effect on FRO activity in the kidneys in DM.

FRO enhancement in the kidneys upon eplerenone administration can be explained by a compensatory

increase in aldosterone production and a rise in its level in the kidneys [10]. Under the conditions of mineralocorticoid receptor blockade, aldosterone exhibits nongenomic activity in the form of membrane effects [11]. One of such effects is known to be activation of protein kinase C [12]. This enzyme triggers a cascade of ROS generating reactions [2].

The mineralocorticoid receptor antagonist eplerenone can weaken the genomic effects of aldosterone upon prolonged administration. The main reason for proteinuria development in DN is a decrease in the number of podocytes and weakening of their functions [13]. Numerous studies indicate a direct link between mineralocorticoid receptor activation and damage to podocytes [14]. In our study with the DN model, eplerenone normalizes the number of podocytes and their functional state and, therefore, reduces proteinuria.

CONCLUSION

In the DN model, the nephroprotective effect of eplerenone is determined by the blockade of the genomic effects of aldosterone and is not associated with the effect on FRO activity in the kidneys. Eplerenone enhances FRO in the kidneys following the accumulation of aldosterone and its nongenomic membrane effect.

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Authors contribution

Zharikov A.Yu. – conception and design, analysis and interpretation of data, final approval of the manuscript for publication. Filinova S.O. – setting up of the experimental model. Mazko O.N., Makarova O.G. – setting up of the experimental model, carrying out of the biochemical laboratory studies. Bobrov I.P. – carrying out of the morphological studies. Bryukhanov V.M. – critical revision of the manuscript for important intellectual content.

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