# DYNAMICS OF ANGIOTENSIN-CONVERTING ENZYME-2 EXPRESSION IN THE RETINA IN EXPERIMENTAL DIABETIC RETINOPATHY AND ITS MODIFICATION BY INSULIN

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**Abstract**

Activation of the renin-angiotensin system (RAS) of the eye is one of the factors in the development of diabetic retinopathy (DR). The effects of RAS are counteracted by a recently discovered axis involving angiotensin-converting enzyme type 2 (ACE2) and its product angiotensin 1-7. DR was modeled in 35 male Wistar rats. For this purpose, they were administered a single dose of streptozotocin at a dose of 50 mg/kg (Sigma-Aldrich, Co, China). Experimental rats were divided into two groups: control and with the administration of insulin (Novo-NordiskA/S, Bagsvaerd, Germany). A multiple increase in the content of ACE2 in the diabetic retina was established, first the accumulation of its free isoforms, and then binding to cellular proteins. Insulin administration reduced the content of ACE2 on the 21st day and after 2 months of treatment. The results obtained showed a possible role of increased ACE2 expression in the pathogenesis of DR and revealed its decrease during insulin treatment.

**Keywords:** diabetic retinopathy, angiotensin-converting enzyme 2, insulin.

**Background**. Diabetic retinopathy (DR) is one of the most common vascular complications of diabetes mellitus (DM), and today remains the main cause of vision loss in people of working age [1]. The renin-angiotensin system (RAS) plays an important role in the regulation of adaptive reactions of the cardiovascular system, water and electrolyte metabolism and tissue remodeling. Its main mediator is angiotensin II, which mediates the immediate physiological effects of vasoconstriction and blood pressure regulation, and is involved in the development of inflammation, endothelial dysfunction, atherosclerosis and arterial hypertension [2].

RAS is a causal factor in diabetic microvascular complications, which causes a variety of tissue reactions, including vasoconstriction, inflammation, oxidative stress, cell hypertrophy and proliferation, angiogenesis and fibrosis [3]. Both clinical and experimental models of DR and hypoxia-induced retinal angiogenesis suggest activation of the RAS. In the retina, angiotensin II induces vascular vasoconstriction, activates angiogenesis, and modulates the function of GABAergic mechanisms in amacrine cells and affects glia [4].

The discovery of angiotensin-converting enzyme 2 (ACE2) led to the establishment of a new RAS axis, which includes ACE2, its product angiotensin 1-7, and its receptor, Mas [5]. This vasoprotective axis counteracts the proliferative, fibrotic, proinflammatory, and hypertrophic effects of the classical ACE/angiotensin II/angiotensin II receptor type 1 axis. Imbalance of the vasoprotective and vasoconstrictor RAS axis in ocular tissue may lead to the development and progression of DR, and enhancement of the protective ACE2/angiotensin (1-7) axis may counteract the effects of angiotensin II [5].

Thus, the status of RAS and its regulator, ACE2, may be important in the development of DR through its influence on the processes of inflammation and damage to retinal vessels under conditions of hyperglycemia.

**The aim** of the work is to determine the content of ACE2 in the retina during the development of experimental diabetic retinopathy and to establish the effect of insulin therapy.

**Materials and methods**. The work was guided by the norms and principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), the Council of Europe Directive 86/609/EEC (1986), the Law of Ukraine No. 3447-IV "On the Protection of Animals from Cruelty to Animals", the general ethical principles of animal experiments adopted by the First National Congress of Ukraine on Bioethics (2001). The study involved 35 three-month-old male Wistar rats weighing 140-160 g. Experimental diabetes and DR were modeled by a single intraperitoneal injection of streptozotocin (50 mg/kg; Sigma-Aldrich, Co, China). 5 animals were used to obtain initial data (intact). Subsequently, every three days, the level of glycemia was monitored using a glucometer and disposable test strips (ACCU-Chek Instant, Roche, Mannheim, Germany) in blood taken from the tail vein on an empty stomach. 3 days after the injection, the blood glucose content of animals that were injected with streptozotocin was not less than 15 mmol/l. The animals were observed for 3 months.

After 7 days, animals with persistent hyperglycemia (n=30) were blindly randomly divided into 2 groups of 15 individuals. In the 1st group (control), hyperglycemia treatment was not performed. In the 2nd group, short-acting insulin (Actrapid HM Penfill, Novo Nordisk A/S, Bagsvaerd, Denmark) was administered intraperitoneally every other day to the animals at a dose of 30 U. Animals were removed from the experiment after 7, 21 days and 3 months in an amount of 5 individuals in each group by lethal injection of thiopental (75 mg/kg). Determination of ACE2 content in retinal tissue lysates was performed by immunoblotting. For morphological studies, eyes were immersed in 10% neutral formalin solution and embedded in paraffin. Serial histological sections 2–3 μm thick were made from

paraffin blocks on a rotary microtome NM 325 (Thermo Shandon, England). Statistica 10 software (StatSoft, Inc., USA) was used for statistical analysis. Descriptive statistics were performed by calculating means and their standard errors. Sample means were compared using analysis of variance (ANOVA), with a p value <0.05 considered significant.

**Results and discussion**. After streptozotocin administration, animals of all groups developed persistent hyperglycemia with maximum values of glucose content in the blood of animals of the control group after 3 months of observation (17.22±1.35 mmol/l). In animals treated with insulin, the glucose content was significantly lower (11.04±1.02 mmol/l; p<0.05). Hyperglycemia, which developed in animals after streptozotocin administration, was accompanied by early morphological manifestations of DR. A decrease in cell density in the ganglionic layer, edema of the inner plexiform layer, and dilation of capillaries along the inner surface of the retina were determined. On the 21st day, these changes progressed, edema of all retinal layers with vascular dilation and microthrombosis in the outer plexiform layer, thinning of the nuclear layers of the retina, areas of ischemia, and the formation of microaneurysms along the inner surface of the retina were detected. Later, signs of retinal dyscomplexation with loss of the structure of the layers were detected, as well as specific signs of DR – numerous microaneurysms and vascular anomalies on the inner surface, in the outer layers – the formation of cell proliferates. After insulin treatment for 3 months, a significantly better condition of the retina was morphologically noted. The retinal layers retained their normal structure, no vascular or proliferative changes were detected.

Determination of the ACE2 level in rat retinal tissues by immunoblot analysis allowed visualization of several zones of specific protein binding. The lightest of them had a molecular weight of less than 110 kDa and corresponded to isoforms of the intact ACE2 protein [6]. Higher molecular weight polypeptide zones had a mass of 150-320 kDa and corresponded to forms of ACE2 that form stable complexes with cellular ligands. Accordingly, it could be assumed that the ACE2 110 kDa fraction reflected the level of the *de novo* synthesized enzyme, while the ACE2 > 110 kDa fraction was a functionally active form of this protein in the RAS tissue.

In intact animals, the content of ACE2 110 kDa in the retina was at the level of trace concentrations, while ACE2 > 110 kDa was not detected. With the development of DR, a 10.2-fold increase in the content of ACE2 110 kDa and a 3.9-fold increase in the high molecular weight fraction was shown already on the 7th day (p<0.05 for both comparisons). After 21 days, the enzyme content remained high, but a greater increase was found for the high-molecular fraction (10.7 times compared to intact), for the ACE2 110 kDa fraction – 5.7 times (p<0.05 for both comparisons). The results obtained indicated an increase in the content of ACE2 in DR, with the expression of the enzyme itself being activated to a greater extent at the beginning of its development, while later the content of its form bound to cellular proteins increased more significantly, which indicated the activation of this RAS chain.

The use of treatment showed a decrease in the content of both ACE2 fractions on the 21st day when insulin was used (3.6 times for ACE2 110 kDa and 4.9 times for ACE2>110 kDa). After 2 months of treatment, the content of ACE2 was lower than in the untreated control (1.7 times for ACE2 110 kDa and 1.5 times for ACE2>110 kDa; p<0.05). After 3 months, the content of both ACE2 fractions compared to the corresponding control was reduced by the use of insulin (1.15 and 1.5 times, respectively; p<0.05). The greater effect was observed for the high-molecular fraction, which indicated a decrease in the binding of the enzyme to cellular proteins and, accordingly, inhibition of RAS activity.

The obtained results demonstrated important aspects of the participation of ACE2 in the pathogenesis of DR and its regulation by the applied treatment. The increase in ACE2 levels, especially the high-molecular fraction, indicated the activation of RAS in the development of DR. In the early stages of the disease, there was a predominant increase in the expression of ACE2 itself, while in the later stages, an increase in the high-molecular-weight fraction indicated the binding of the enzyme to cellular proteins, which could be a marker of disease progression. Insulin treatment reduced the levels of both forms of ACE2, indicating its positive effect on the regulation of RAS and improving metabolic status.

Thus, the results of the studies indicated the possibility of using ACE2 as a marker for monitoring pathological changes and the effectiveness of DR treatment, and also confirmed the important role of insulin therapy in the correction of RAS disorders. The significant and long-term (within 3 months) increase in ACE2 content in the retina after DR modeling that we established can be considered a reflection of the general activation of local RAS. The introduction of insulin was accompanied by a decrease in hyperglycemia, which could also explain the decrease in retinal ACE2 content. It should be noted that this reaction had a maximum degree on the 21st day, while the effect of insulin decreased in the future. Perhaps there was an accumulation of metabolic damage to the retina and the activation of RAS was mediated by mechanisms other than hyperglycemia.

Of particular importance was the establishment of the fact of ACE2 accumulation in the retina in DR for the question of the possibility of infection with coronavirus (SARS-CoV-2). It is known to damage almost all systems and organs and is a powerful comorbid factor that worsens the course of chronic human diseases, including diabetes [7]. The results of the study of the coronavirus disease (COVID-19) pandemic have shown that patients with diabetes have more adverse clinical outcomes and a high risk of developing ocular complications [8]. Since there is no doubt about the role of ACE2 as an entry gate for SARS-CoV-2, the establishment of an increase in its expression in retinal tissue in DR may explain the adverse clinical outcomes and the high risk of worsening of the course of DR in COVID-19.

**Conclusions.** In experimental DR, an increase in ACE2 expression was found in the rat retina, confirming the involvement of this important RAS regulator in the pathogenesis of diabetic retinal damage. A multiple

increase in the content of ACE2 was noted, first the accumulation of its free isoforms, and then binding to cellular proteins. The introduction of insulin reduced the content of ACE2 in the retina. The results obtained showed a possible role of increased ACE2 expression in the pathogenesis of DR and revealed its decrease during insulin treatment, which corresponded to the inhibition of the development of morphological signs of DR.

**Conflicts of interest:** author have no conflict of interest to declare.

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