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Experimental Research

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The Investigation of the Effects of Enalapril and Losartan on Ghrelin Immunoreactivity in Kidney of Streptozotocin-Induced Diabetic Rats

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ABSTRACT

Objective: The aim of this study was to examine the effects of enalapril and losartan on ghrelin immunoreactivity in the kidney tissues of rats with streptozotocin (STZ) induced diabetes.

Materials and Methods: The study involved 28 Wistar albino rats. The rats were allocated to four groups: control group (n=7), diabetes (DM) group (n=7), DM + enalapril group (n=7) and DM + losartan group (n=7). DM, DM + enalapril and DM + losartan groups were administered a single dose of 50 mg/kg of STZ, intraperitoneally. The rats in the treatment groups were orally administered 5 mg/kg/day of enalapril and 10 mg/kg/day of losartan, starting with the onset of diabetes. At the end of the fourth week of the experiment, rats were decapitated. Kidney tissues collected from the animals were processed by using routine histological techniques. Ghrelin immunoreactivity was determined by avidin-biotin-peroxidase method.

Results: Ghrelin immunoreactivity in the distal tubules was moderate (++) in the control group and severe (+++) in the diabetic group. In the distal tubules of the treatment groups, ghrelin immunoreactivity was observed to be moderate (++), similar to the control group.

Conclusion: It was determined that enalapril and losartan were effective against ghrelin immunoreactivity in the diabetic rat kidney tissues.

Key Words: *Diabetes mellitus, Enalapril, Losartan, Ghrelin*

ÖZET

Deneysel Diyabetik Sıçan Böbrek Dokusunda Enalapril ve Losartan'ın Ghrelin İmmunreaktivitesi Üzerine Etkilerinin İncelenmesi

Amaç: Bu çalışmada, streptozotosin (STZ) ile deneysel olarak oluşturulan diyabetik sıçanların böbrek dokusunda, enalapril ve losartan'ın ghrelin immunreaktivitesi üzerine etkilerinin incelenmesi amaçlanmıştır.

Gereç ve Yöntem: Çalışmada 28 adet erişkin, dişi Wistar albino cinsi sıçan kullanıldı. Deney hayvanları Kontrol grubu (n=7), diyabetik (DM) grup (n=7), DM + Enalapril (n=7) ve DM +Losartan (n=7) olmak üzere dört gruba ayrıldı. Diyabetik, DM + Enalapril ve DM +Losartan gruplarına 50 mg/kg olacak şekilde tek doz STZ (Sigma Chemical Co Louis Missouri) 0,1 M Fosfat-sitrat tamponunda (pH: 4,5) çözdürülerek intraperitoneal olarak uygulandı. Tedavi gruplarındaki sıçanlara diyabetin başlangıcından itibaren 5 mg/kg/gün Enalapril ve 10 mg/kg/gün losartan oral olarak verildi. Deneyin 4. haftasının sonunda tüm gruptaki ratlar dekapite edildiler. Deney hayvanlarından alınan böbrek dokularına rutin histolojik teknikler uygulandı. Avidin-biotin-peroksidaz yöntemi ile ghrelin immünreaktivitesi belirlendi.

Bulgular: Böbrek dokusunda distal tübüllerde kontrol grubunda orta şiddette (++), diyabetik grupta ise şiddetli (+++) ghrelin immünreaktivitesi gözlendi. Tedavi gruplarında ise distal tübüllerde her iki grupta da kontrol grubuna benzer şekilde orta şiddette (++) ghrelin immünreaktivitesi izlendi.

Sonuç: Diyabetik sıçanların böbrek dokusunda enalapril ve losartanın ghrelin immunreaktivitesi üzerine etkili olduğu belirlendi.

Anahtar Kelimeler: *Diabetes mellitus, Enalapril, Losartan, Ghrelin*

Diabetes mellitus is a disease marked by acute and chronic complications (1). Chronic degenerative complications constitute one of the major health problems. Patients who have had long-term diabetes suffer from impairments in all vessels. Changes involve vascular cells and their basal membranes. Although all microvascular structures are involved, clinical pathologies arise only in retina, renal glomerules and major nerves (2).

Diabetic nephropathy develops in about one-third of insulin-dependent diabetic patients. It, by itself, leads to laststage renal disease, which requires chronic dialysis and transplantation (3). Diabetic nephropathy develops as a result of the interaction of hemodynamic and metabolic factors (4). Among the major factors causing diabetic nephropathy are hormonal factors. Previous studies have demonstrated that renin angiotensin aldosterone system and growth hormones (GH)

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have an important part in the development of diabetic nephropathy. The effects of angiotensin converting enzyme inhibitors (AECI) and angiotensin receptor blockers (ARB), which are most commonly used for chronic kidney disease, on oxidative stress and renal protection have been extensively researched. ACEI and ARB were shown to relieve the oxidative burden, both systemic and solely renal (11), by decreasing the superoxide level (9) through NADPH oxidase inhibition (5–8) and by reducing the advanced glycolization end products (AGE) and oxidized LDL level (10). One of the pathogenetic factors of diabetic nephropathy is elevated GH levels.

Ghrelin, which strongly stimulates the release of GH, is a recently discovered hormone that physiologically regulates the appetite and body weight. It was established that it plays an important role in insulin and glucose metabolism (12–14). Ghrelin is found in many organs, including gastrointestinal organs, and the kidneys (15). The present study aims to examine the effects of enalapril and losartan, which are used as treatment agents in diabetic kidney tissue, on ghrelin immunoreactivity.

MATERIALS AND METHODS

Animal Procedure

The study included 28 adult female Wistar albino-type rats obtained from the Experimental Research Center of Fırat University (FUDAM). The rats were sheltered under the conditions of 12 hour (7.00 a.m.–7.00 p.m.) light/12 hour (7.00 p.m.–7.00 a.m.) dark at a room temperature of 21oC. They were kept in cages, the floors of which were cleaned daily. Their feed was provided in steel bowls and their water (tap water) in glass feeding bottles. All of the rats were kept under surveillance at the same place and fed ad-libitum with standard rat pellet and water. Blood samples were collected from the tail vein of all animals after 12 hours fasting to determine basal blood glucose levels. The animals were divided into four groups: control group (n=7), diabetes (DM) group (n=7), DM + enalapril group (n=7) and DM + losartan group (n=7). Diabetes, DM + enalapril and DM + losartan groups were administered a 50 mg/kg single dose of STZ (Sigma Chemical Co., St. Louis, Missouri) through an intraperitoneal route, after STZ was dissolved in 0,1 M phosphate-citrate buffer (pH: 4.5). Blood samples were collected 72 hours after the injection from the tail vein following 12 hours of fasting and measured using a glucose meter. Rats whose fasting blood glucose level was over 250 mg/dl were accepted as diabetic. The rats in the treatment groups were orally administered 5 mg/kg/day of enalapril (Vasolapril 10 mg, DEVA, Istanbul, Turkey) and 10 mg/kg/day of losartan (Eklips 50 mg, Sanovel Drug Co., Istanbul, Turkey) starting with the onset of diabetes. At the end of the fourth week of the experiment, the rats in all of the groups were decapitated after being anesthetized with ketamine. Kidney tissues collected from the animals were fixed with 10% neutral formalin for light microscopy examination and were buried in paraffin blocks after routine histological analysis procedures.

Immunohistochemistry

Cross-sections of 5–6 μm were obtained from the blocks and placed on poly-L-lysinecoated slides. After being deparaffinized, the tissues were dehydrated in a graded alcohol series and treated with a hydrogen peroxide block solution (Thermo Scientific, TA–060-HP, Fremont, USA) for seven minutes to prevent endogenous peroxidase activity. They were also treated with an Ultra V Block solution (Thermo Scientific, TA–060-UB, Fremont, USA) for five minutes to prevent floor staining and then were incubated with a primary antibody, ghrelin goat polyclonal IgG (Santa Cruz Biotechnology, California, USA) at +4oC in a humid environment for one night. The following day, they were subjected to biotin secondary antibody, a donkey antigoat IgG, (Santa Cruz Biotechnology, California, USA) for 30 minutes and then to streptavidin horseradish peroxidase enzyme (Thermo Scientific, TS–060-HR, Fremont, USA) for another 30 minutes. Finally the tissues were treated with 3,3'-Diaminobenzidine (DAB) chromogen (DAB Plus Substrate System, Thermo Scientific, TS–060-HDX, Fremont, USA) and were counterstained with Harris hematoxylin. Tissues intended as the negative control were prepared using a phosphate buffer saline (PBS) instead of a primary antibody, but other procedures were applied in the same way. Stomach tissue was used as the positive control. Tissues that were passed through PBS and distilled water were closed using the appropriate closing solution. The preparations were examined, evaluated and photographed using a research microscope (Olympus BH–2).

Semi-quantitative analysis

Evaluation of the immunohistochemical staining was based on the severity of staining. Severity of cytoplasmic immune staining was semi-quantitatively scored from (-) to (+++). The intensity of ghrelin expression was graded as follows: (-), no staining; (+), mild staining, (++), moderate staining; and (+++), severe staining.

RESULTS

When cross-sections from the control group were evaluated, moderate (++) ghrelin immunoreactivity was observed in the renal cortex (Figure 1a) and medulla (Figure 1b). No ghrelin immunoreactivity was observed in the glomerules and proximal tubule (Figures 1a and 1b).

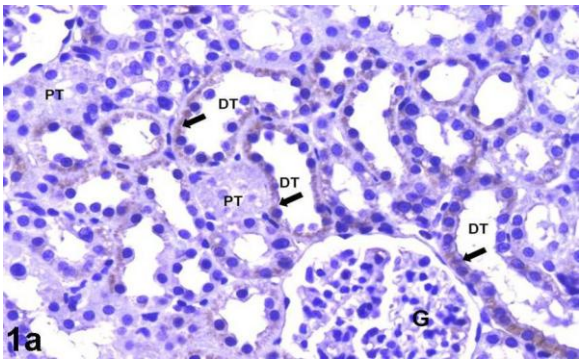

Figure 1a. *(++) Ghrelin immunoreactivity in the distal tubules (DT) of the renal cortex in the control group. Glomerule (G), Proximal tubule (PT), Immunoreactive area (→) x 20.*

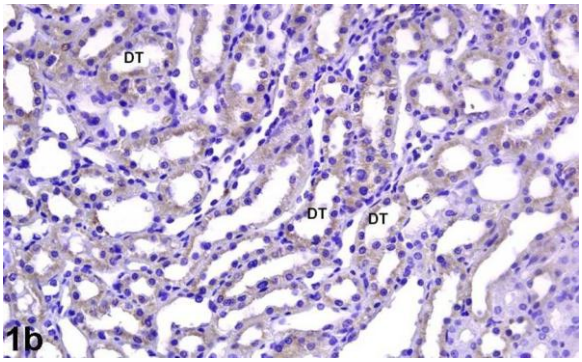

Figure 1b. *(++) Ghrelin immunoreactivity in the renal medulla in the control group. Distal tubule (DT) x 20.*

Significant differences were found in the kidneys of the diabetic group in terms of ghrelin immunoreactivity. Severe (+++) ghrelin immunoreactivity was observed in the renal cortex (Figure 2a) and medulla (Figure 2b) of this group. There was no ghrelin immunoreactivity in the glomerules and proximal tubule (Figures 2a and 2b). Ghrelin immunoreactivity in the renal cortex and medulla of the DM + Enalapril and DM + losartan groups looked similar to that in the control group (Figures 3a, 3b, 4a and 4b). Likewise, moderate (++) ghrelin immunoreactivity was seen in the distal tubules of the renal cortex (Figures 3a, 4a) and medulla (Figures 3b, 4b).

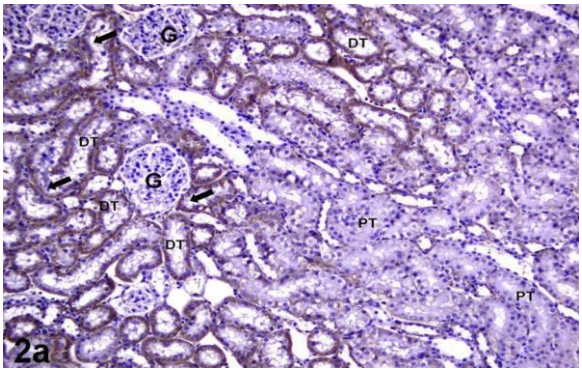

Figure 2a. *(+++) Ghrelin immunoreactivity in distal tubules (DT) of the renal cortex in the diabetic group. Glomerule (G), Proximal tubule (PT), Immunoreactive area (→) x 10.*

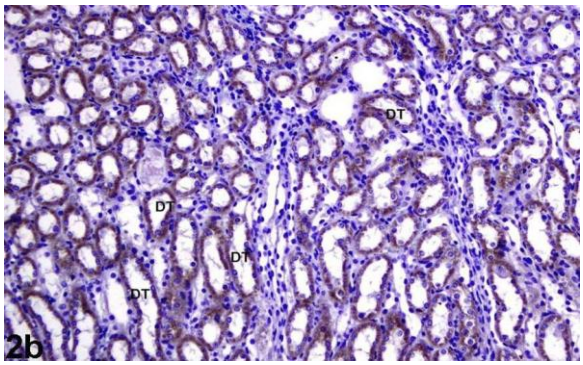

Figure 2b.*(+++) Ghrelin immunoreactivity in the renal medulla of the diabetic group. Distal tubule (DT) x 20.*

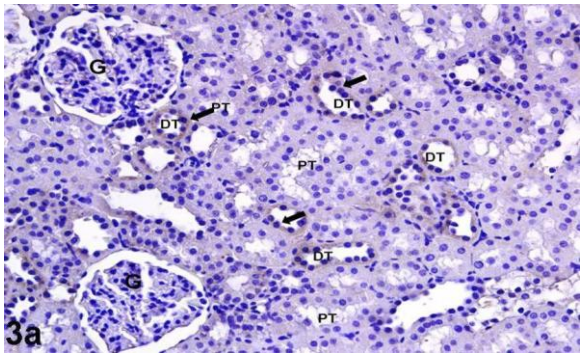

Figure 3a. *(++) Ghrelin immunoreactivity in the distal tubules (DT) of the renal cortex in DM + enalapril group. Glomerule (G), Proximal tubule (PT), Immunoreactive area (→) x 20.*

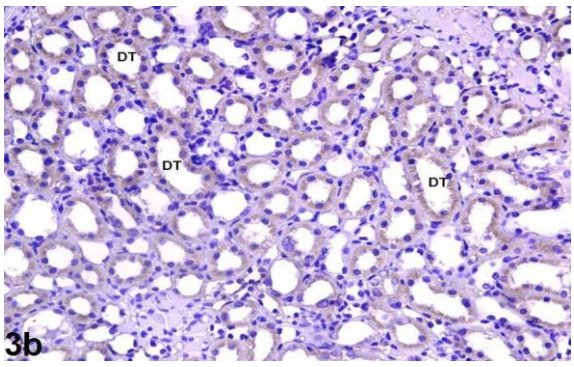

Figure 3b. *(++) Ghrelin immunoreactivity in the renal medulla of DM + enalapril group. Distal tubule (DT) x 20.*

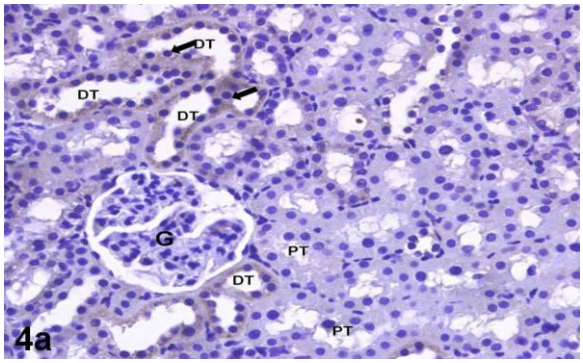

Figure 4a. *(++) Ghrelin immunoreactivity in the distal tubules (DT) of the renal cortex in DM + losartan group. Glomerule (G), Proximal tubule (PT), Immunoreactive area (→) x 20.*

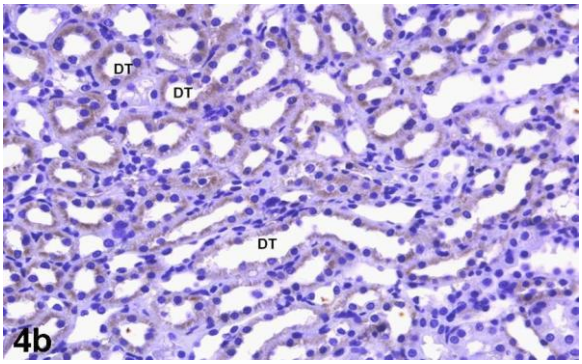

Figure 4b. *(++) Ghrelin immunoreactivity in the renal medulla of DM + losartan group. Distal tubule (DT) x 20.*

Glomerules and proximal tubule did not show any ghrelin immunoreactivity (Figures 3a and 3b, 4a and 4b). Staining performed in the negative control did not reveal any immunoreactivity in the kidney tissue (Figure 5a). However, positive control showed ghrelin immunoreactive cells in the stomach tissue (Figure 5b).

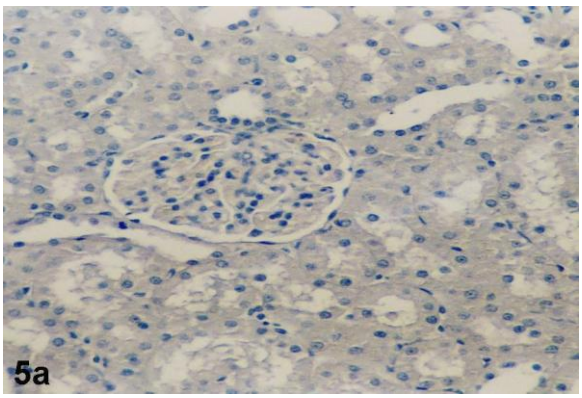

Figure 5a. *Negative control kidney tissue x 40.*

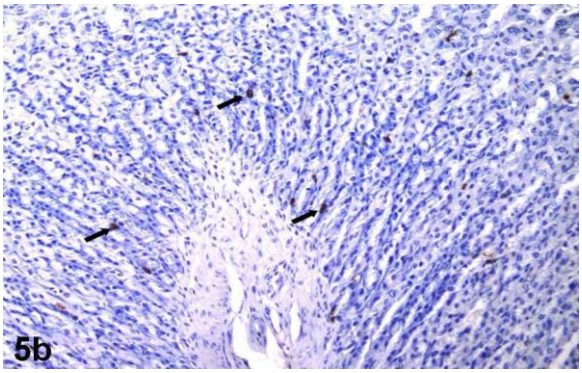

Figure 5b. *Positive control. Ghrelin immunoreactive cells in the stomach (→) x 10.*

DISCUSSION

Diabetes mellitus, which is characterized by elevated blood sugar and proceeds with impaired carbohydrate, protein and lipid metabolisms, results from absolute or relative deficiency or ineffectiveness of the insulin hormone which is secreted from the pancreas or structural defects of the insulin molecule (26).

Cases who have had diabetes for a long time suffer from impairments in all vessels. Changes affect both the vascular cells that make up capillaries and arterioles and their basal membranes. Although all microvascular structures are involved, clinically, the pathology occurs only in the retina, renal glomeruli, and major nerves (2).

Diabetic nephropathy, in which etiology and pathogenesis has not been clarified yet, is a major cause of end-stage renal failure and the incidence of nephropathy increases with prolonged duration of diabetes (27, 28). Renal failure is the second most common cause of mortality associated with diabetes after myocardial infarction (16).

Although diabetic nephropathy involves structural changes that affect all parts of the kidney, the most characteristic changes have been identified in the glomeruli (17).

Oxidative stress refers to a variety of molecular changes resulting from the disturbance of the balance between oxidants and antioxidants in favor of the oxidants in the body (18, 19). The significance of oxidative stress has been shown particularly in conditions like aging, diabetes, uremia, cardiovascular diseases, malnutrition and cancer (20). The balance between prooxidants and antioxidants shifts towards oxidative stress in end-stage renal failure (ESRF). Studies about oxidative stress and antioxidants in ESRF patients have recently received increasing attention (29).

Growth factors also play a considerable role in the pathogenesis of diabetic nephropathy due to their contributions to functional and structural changes in the development of diabetic kidney disease, as well as their growth-accelerating and proliferative effects. The major growth factors that take a place in diabetic nephropathy include GH, IGF-1, vascular endothelial growth factor, transforming growth factor, epidermal growth factor and platelet-derived growth factor. Of these, the molecules that constitute the GH/IGF system are found in the circulation, extracellular distance and most of the tissues. They serve important functions related to growth (27). Research suggests that this system may play a significant role in diabetic nephropathy (30).

GH has a place in the development of diabetic microangiopathy. Besides, GH and IGF are believed to play a pathogenic role in diabetic nephropathy (21, 22). GH was shown to be markedly elevated in the serum of non-obese diabetic mice with induced type-1 diabetes model and rats which had diabetes induced by STZ (15, 31).

It has also been shown that renal and glomerular hypertrophy and albuminuria could be prevented by GH receptor antagonists in non-obese diabetic mice and mice which had diabetes induced by STZ (31, 32).

Ghrelin is a peptide-hormone with 28 amino acids, isolated as an endogenous ligand for the Growth Hormone Stimulating Receptor (GHS-R) which stimulated GH secretion both in vivo and in vitro (12).

In our study, enalapril and losartan, both of which inhibit oxidation pathways and provide renal protection by reducing proteinuria, were administered to rats which had experimental diabetes induced by STZ, and their ghrelin immunoreactivity was observed (5-8). Ghrelin immunoreactivity in the renal cortex and medulla of both groups which were administered enalapril and losartan was found moderate (++) similar to the immunoreactivity found in the controls. Likewise, moderate (++) ghrelin immunoreactivity was observed in the distal tubules. However, the diabetic group had severe (+++) ghrelin immunoreactivity in the renal cortex, medulla and distal tubules.

Masaoka et al (15) induced diabetes with STZ in Wistar rats. They established a significant decrease in serum insulin and IGF-1 levels and a marked increase in serum GH, serum total and active ghrelin levels. They attributed the elevated plasma ghrelin concentration to ghrelin immunoreactive stomach cells, but they also suggested as a possibility that ghrelin synthesis might have increased in an organ other than the stomach in diabetes.

Mice which had been subjected to bilateral nephrectomy and partial nephrectomy were found to have increased levels of plasma total ghrelin, but were not found to have any increase in either the ghrelin mRNA levels or ghrelin content in the stomach. Therefore, the concerned increase may be due to a decrease in the renal clearance or destruction of ghrelin (25).

Mori et. al. (23) who were the first to demonstrate the prepro-ghrelin gene expression in the mouse kidney, besides the gastrointestinal system and the brain, argued that ghrelin could be locally produced in the kidney and also that ghrelin could play endocrine and/or paracrine roles in this organ. Kuloğlu et. al. (24) showed in their study that ghrelin immunoreactivity increased in proportion to the length of diabetes in the distal tubules of renal cells of rats with experimentally induced diabetes.

Ghrelin is filtered through the distal tubule epithelium and blood. It is actively secreted through the urine. The urine was found to contain more ghrelin than the amount of ghrelin in the circulation (25).

Enalapril which acts upon the reactive oxygen system reduces free radicals. In the same way, ghrelin may decrease the levels of free radicals as it eliminates free radicals (33). Küçüksu (33) argued in his study that, as both ghrelin and enalapril sweep away oxidative stress through similar pathways, enalapril use reduced the level of ghrelin by blocking the balancing mechanism that ghrelin offers. In our study, we also think that enalapril and losartan molecules may be interacting with ghrelin in the same way. Therefore, we are of the opinion that use of enalapril and losartan in diabetes may affect the ghrelin expression of distal tubules in a manner that is similar to the one in the control group.

In conclusion, we believe that enalapril and losartan administration is effective in the expression of ghrelin in the renal tissue of diabetic rats and that more extensive studies are needed on this topic.

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