Investigation of the Relationship of Nesfatin-1, Adropin Levels and Claudin-2, Renalase Immunoreactivity with Kidney Function in an Experimental Hypertension Model

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Objective: Hypertension is one of the cardiovascular diseases that causes the most mortality, and 95% of the causes are unknown. The aim of the study was to examine the possible correlation of nesfatin-1 levels, adropin levels, claudin-2 immunoreactivity (claudin-2 expression in the renal proximal tubule), and renalase immunoreactivity (renalase expression in the renal proximal tubule) with arterial blood pressure, kidney function, and kidney damage.

Methods: Adult male Sprague-Dawley rats were divided into control and hypertension groups (8 per group). Angiotensin II vehicle was given to the control group and angiotensin II (0.7 mg/kg/day) to the hypertension group, both via an osmotic mini pump for 7 days. The animals blood pressures were measured by tail cuff plethysmography on days 1, 3, 5, and 7. On day 7, 24-hour urine, blood, and tissues were collected from the rats.

Results: In the hypertension group compared with the control group, there was an increase in systolic blood pressure levels after day 1. While claudin-2 immunoreactivity was reduced in the kidneys, renalase immunoreactivity was increased. There was a decrease in creatinine clearance and an increase in fractional potassium excretion ($P < .05$).

Conclusion: Our results showed that claudin-2 and renalase are associated with renal glomerular and tubular dysfunction and may play discrete roles in the pathogenesis of hypertension. We believe that these potential roles warrant further investigation.

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Key words: Hypertension, Claudin-2, Renalase, Nesfatin-1, Adropin

An estimated 1 billion people worldwide are affected by hypertension, and it causes more than 9 million deaths, annually (1). Considering that more than 90% of people diagnosed with hypertension have essential hypertension, the importance of hypertension studies increases (2). Therefore, intensive research aimed at determining how to prevent, detect, treat, and control hypertension continues.

The renin–angiotensin–aldosterone–system is crucial to the regulation of blood pressure and fluid volume and to the maintenance of the sodium (Na+)–potassium (K+) balance (3). Angiotensin (Ang) II regulates vascular tone and blood pressure under physiological conditions, but its overproduction plays a key role in the pathophysiology of hypertension and renal failure (3).

Nesfatin-1 regulates cardiovascular function and glucose homeostasis (4). It has been reported that the direct administration of nesfatin-1 increases blood pressure (5,6). Adropin is a newly discovered peptide that is involved in the regulation of energy homeostasis. It has been reported that adropin increases nitric oxide (NO) levels by significantly increasing endothelial NO synthase activation (7). Therefore, adropin is thought to have a role in the pathophysiology of hypertension. The effects of adropin on the regulation of cardiovascular function are still unknown. The available evidence suggests that nesfatin-1 and adropin may play discrete roles in the pathophysiology of hypertension and possible renal damage caused by hypertension.

Renalase is secreted into the blood from the proximal tubules of the kidneys and is reported to play an important role in the regulation of blood pressure (8,9). This information suggests that renalase is closely related to renal damage and the regulation of systemic blood pressure.

Claudin-2 (CLDN-2) is highly present in the proximal tubule epithelium of the kidney; it forms selectively permeable paracellular channels for small cations such as Na+ and K+ (10,11). In addition, it has been reported that CLDN-2 mediates the paracellular transport of water (12).

The relationship of adropin, nesfatin-1, CLDN 2, and the renalase enzyme with the mechanisms regulating blood pressure has not yet been clarified. Despite intensive research, the mechanism of Ang II–induced hypertension is still not fully understood (3,13,14). In our current study, we aimed to investigate the relationship of nesfatin-1 and adropin levels and renal CLDN-2 and renalase immunoreactivity with arterial blood pressure, kidney function, and kidney damage.

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Materials and Methods

Animals

In our study, 16 healthy male Sprague-Dawley rats weighing 240–260 grams and reared in the Experimental Animals Unit of Trakya University were used. The rats were housed in standard laboratory conditions at 22 (±1) ºC temperature and 50%–60% humidity and under a 12-hour light/12-hour dark cycle. The groups were randomly divided into control and hypertension groups consisting of 8 rats each. The animal procedures were reviewed and approved by the Trakya University Animal Experiments Local Ethics Committee.

Experimental Design

ALZET osmotic pumps (ALZET model 2001) were implanted subcutaneously while the rats were under anesthesia. Ang II vehicle (0.01 N acetic acid in saline) was given to the control group (Fig. 1A) and angiotensin II (at a dose of 0.7 mg/kg/day) to the hypertension group (Fig. 1B), both via an osmotic mini pump for 7 days.

Blood Pressure Measurement

Blood pressure was measured in both groups on day 1, day 3, day 5, and day 7 of the experiment. The systolic blood pressure (SBP) of conscious rats was measured indirectly at the tail using tail-cuff plethysmography (NIBP250, Commat Ltd., Turkey). The largest and smallest values were excluded from the 5 blood pressure measurements that were recorded. The averages of the remaining 3 blood pressure measurements were used.

Sample Collection

After the 24-hour urine collection on the last day of the experiment and while the animals under anesthesia (50 mg/kg of ketamine and 10 mg/kg of xylazine), blood and both kidneys were taken from each animal, which then was euthanized by exsanguination. Each kidney was divided longitudinally into 2 equal parts. One half of the right kidney was placed in a 10% formalin solution for pathological examination. The other parts were washed with cold 0.01 M phosphate buffer and dried with blotting paper. Kidney pieces were taken into tubes and then placed in liquid nitrogen. The blood samples were incubated for 2 hours at room temperature and centrifuged for 15 minutes at 1000 g at +4 °C. The urine samples were also centrifuged at 1000 g at +4 °C, for 20 minutes instead of 15. At the end of the experiment, all the samples were stored at -80 °C until analysis.

Biochemical Studies

Malondialdehyde (MDA), glutathione (GSH), and NO levels were measured in the kidney tissues using the spectrophotometric method. Adropin (catalog no. E-EL-R2566), nesfatin-1 (catalog no. E-EL-R2514) and Ang II (catalog no. E-EL-R1430) levels in serum, urine, and kidney tissue were measured with commercial enzyme-linked immunosorbent assay (Elabscience, Biotechnology Co., Ltd., China) kits.

Serum urea, creatinine, Na+, K+, creatine kinase; urine creatinine, Na+, K+, and total protein levels were measured. All these results were measured with the autoanalyzer device (Konelab PRIME 60i, Thermo Scientific, Finland) in the "Trakya University Hospital Center Biochemistry Laboratory". Creatinine clearance (CrCl), fractional Na+ excretion (FENa), and fractional K+ excretion (FEK) were calculated according to the standard formulas.

Determination of Malondialdehyde

The level of MDA, which is an indicator of lipid peroxidation, was measured using the spectrophotometric method, with the pink color resulting from its reaction with thiobarbituric acid in a hot and acidic environment (15).

Measurement of Glutathione Levels

Using Ellman's reagent, the free sulfhydryl groups in the tissues were exposed. The levels of GSH were determined by measuring the color of the released free sulfhydryl groups, spectrophotometrically (16).

Determination of Nitrate and Nitrite

Nitrite and nitrate, formed after NO reacts with oxygen, are primary oxidation products. Thus, the nitrite/nitrate concentration in serum was used as a marker of NO synthesis. Nitrate and nitrite determinations were obtained using the Cortas and Wakid method (17).

Measurement of Kidney Protein Levels

We determined the tissue protein levels using the Lowry method (18). It was used to calculate tissue parameters.

Histological Studies

After the right kidney's tissue was divided into 2, longitudinally, one half was fixed in 10% buffered formalin solution for 24 hours. Five μm-thick sections of tissues that had previously been embedded in paraffin blocks were taken. A histopathological examination of the kidney sections of the rats was performed; the cast scores and degrees of kidney damage were determined by scanning 100 areas. The cast formations were scored from 0 to 4, the glomerulosclerosis and peritubular fibrosis values were calculated as the percentage of positive areas. In addition, after having undergone hematoxylin–eosin (H&E) staining, the kidney sections were examined under a light microscope. To determine the degree of kidney damage, tubular cell necrosis, cytoplasmic vacuole formation, and tubular dilatation were evaluated. The kidney damage scores were as follows: 0, normal kidney; 1, minimal damage (0%–4% involvement); 2, mild damage (5%–24% involvement); 3, moderate damage (25%–74% involvement); and 4, severe damage (75%–100% involvement).

Immunohistochemical Examination

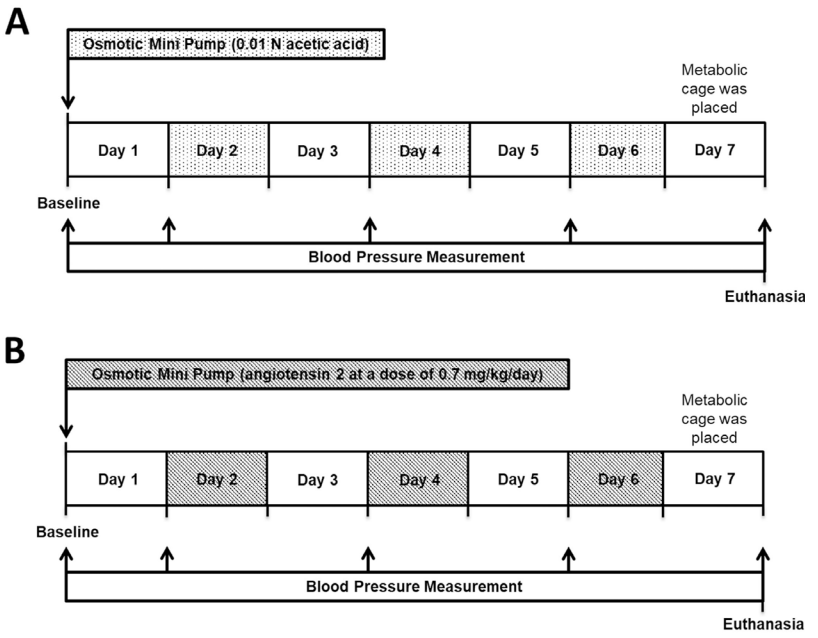
The kidney sections of the rats were stained immunohistochemically with CLDN-2 and renalase antibodies. Claudin 2 (Lifespan, catalog no. LS-C353134) and renalase (Thermo Fisher Scientific, catalog no. PA5-18376) polyclonal antibodies were used. From the samples of animals in both groups, sections 2 microns in thickness were taken from paraffin-embedded tissue blocks fixed to 10% formalin and were placed on slides coated with poly-L-lysine. The stains were evaluated under a light microscope. Immunohistochemical evaluations

were made according to the intensity of the staining of the target cells. Staining intensity reflects the chromogen staining intensity of cells showing cytoplasmic staining in tissue sections and was scored as 0 in cases with no staining, +1 in cases with light staining, +2 in cases with moderate staining, and +3 in cases with strong staining.

Statistical Analysis

All the results presented are the mean and the standard deviation of the mean. The conformity of the variables to normal distribution was determined by the Shapiro–Wilk test. Student's *t* test was used for a pairwise comparison of parameters with normal distribution. The Mann–Whitney *U* test was used for a pairwise comparison of parameters that did not fit normal distribution. Statistically, a *P* less than .05 was accepted as significant. For statistical analysis, IBM SPSS Statistics for Windows, Version 20.0, was used.

Figure 1. Experimental protocol of the groups (A. Control group; B. Hypertension group).



Results

Blood Pressure Measurement Results

There was no statistically significant difference in SBP values between the groups on baseline ($P > .05$). There was a statistically significant increase in SBP values on the first day, third day, fifth day, and seventh day measurements in the hypertension group ($P < .001$, $P < .01$, $P < .001$, and $P < .01$, respectively). A graphical representation of the SBP values appears in Figure 2.

Biochemical Results

There were statistically significant increases in serum NO and serum urea levels ($P < .05$, and $P < .001$, respectively) in the hypertension group. In addition, an increase in the kidney MDA ($P < .05$) level in the hypertension group was also statistically significant. Statistically significant decreases were observed in the urinary NO, Ang II, and CrCl levels of the hypertension group compared to the control group (all $P < .01$). There were statistically significant increases in urine volume and FEK (both $P < .01$). The results of all the previous are shown in Table 1.

Histopathological Results

In the hypertension group, statistically significant increases were found in the tubular damage scores ($P < .01$), peritubular fibrosis ($P < .01$), the glomerulosclerosis scores ($P < .001$), and the cast scores ($P < .001$) (Table 2).

When the HE-stained sections of the rat kidneys were examined under the light microscope, it was observed that the proximal tubule and glomerular structures were normal in the control group sections. There was no necrosis or cast formation in the control group (Fig. 3A). In the glomeruli of the hypertension group, capillary congestion and mild sclerosis were observed. In addition, there was peritubular fibrosis and cast deposition in the hypertension group (Fig. 3B).

Immunoreactivity in Kidney Tissue

The results are shown in Table 2. There was a statistically significant decrease in the staining intensity of CLDN-2 in the kidney sections of the hypertension group ($P < .01$). Images of

Figure 2. Comparison of systolic blood pressure values between groups (**$P < .01$; ***$P < .001$)

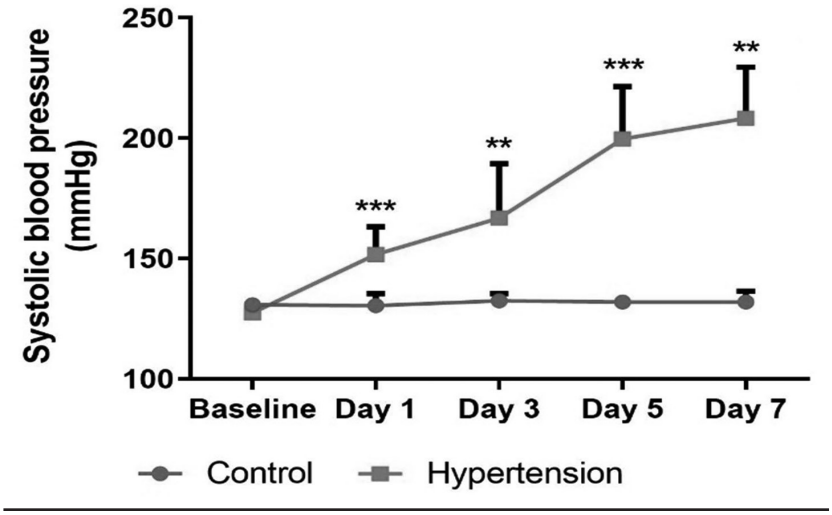


Table 1. Comparison of biochemical parameters in control and hypertension groups

Parameter	Control Group	Hypertension Group	*P* value
SERUM			
Urea (mg/dl)	38.38 ± 3.07	67.00 ± 22.37	<.001
CK (U/L)	1277.63 ± 324.36	1376.13 ± 531.81	>.05
NO (µmol/L)	6.26 ± 0.81	8.91 ± 2.50	<.05
Adropin (ng/ml)	0.13 ± 0.09	0.19 ± 0.09	>.05
Nesfatin-1 (pg/ml)	34.58 ± 23.70	33.04 ± 18.69	>.05
Ang II (pg/ml)	96.12 ± 14.95	92.29 ± 21.30	>.05
KIDNEY			
MDA (µmol/mg protein)	2.92 ± 0.60	3.79 ± 0.94	<.05
GSH (µmol/mg protein)	29.97 ± 1.96	32.29 ± 3.74	>.05
NO (µmol/mg protein)	2.34 ± 1.06	1.74 ± 0.51	>.05
Adropin (ng/mg protein)	0.54 ± 0.05	0.58 ± 0.06	>.05
Nesfatin-1 (pg/mg protein)	104.60 ± 13.65	95.75 ± 14.22	>.05
Ang II (pg/mg protein)	0.74 ± 0.26	0.72 ± 0.41	>.05
URINE			
Total Protein (mg/dl)	183.49 ± 33.15	214.51 ± 152.21	>.05
NO (µmol/L)	368.98 ± 60.95	109.75 ± 77.46	<.01
Adropin (ng/ml)	0.89 ± 0.67	1.88 ± 2.65	>.05
Nesfatin-1 (pg/ml)	36.29 ± 27.48	55.52 ± 58.76	>.05
Ang II (pg/ml)	989.19 ± 50.66	646.35 ± 236.93	<.01
FENa (%)	0.57 ± 0.15	0.76 ± 0.28	>.05
FEK (%)	25.94 ± 6.45	41.52 ± 13.39	<.01
CrCl (ml/min)	2.56 ± 0.53	1.47 ± 0.59	<.01
Urine Volume (ml/24 hours)	11.38 ± 2.07	22.50 ± 7.99	<.01

Ang II: angiotensin II; CK: creatine kinase; CrCl: creatinine clearance; FEK: fractional potassium excretion; FENa: fractional sodium excretion; GSH: glutathione; MDA: malondialdehyde; NO: nitric oxide

the CLDN-2 immunoreactivity of the groups are shown in Figure 4A–4B. The increase in renalase immunostaining was statistically significant in the hypertension group ($P < .05$). Images of the renalase immunoreactivity of the groups are shown in Figure 4C–4D.

Discussion

Our first, and main, finding was that renal CLDN-2 immunoreactivity was reduced in the animals in the hypertension group when they were given Ang II. Second was the immunoreactivity of the renalase enzyme, which is associated with hypertension, cardiovascular disease, and kidney disease; it was observed that there was a statistically significant increase in immunoreactivity in the hypertension group. Third, there was no statistically significant difference in adropin and nesfatin-1 levels. Our results suggest that the decrease in renal CLDN-2 activity in Ang II–induced hypertension may be related to the deterioration of renal tubular function. Given the important role of tubular function in blood pressure regulation, we suggest that CLDN-2 may play an important role in arterial blood pressure regulation. In addition, because of kidney damage and the deterioration of kidney function, the kidneys may have increased renalase activity as a protective mechanism.

From the first day of the experiment, SBP increased in the hypertension group. These results are similar to those of other studies in the literature that were conducted using the same dose and duration (19,20). Elevated SBP in the hypertension group caused a statistically significant decrease in CrCl level. Used to determine the glomerular filtration rate (GFR), CrCl is an indicator of glomerular dysfunction. In the study of Aizawa et al. (19), it was reported that CrCl decreased, as was the case in our study. In addition, NO has a role in the regulation of blood flow and tubular function in the kidneys. It is known that NO reduces GFR

Figure 3. Hematoxylin–eosin-stained kidney section (X400) (Fig. 3A. Control group; Fig. 3B. Hypertension group). In microscopic examination, the black arrow indicates the proximal tubule, and the white arrow indicates the glomeruli. In the control group, glomerular and proximal tubule structures were regular. In the hypertension group were observed glomerular capillary congestion, mild sclerosis, and peritubular fibrosis.

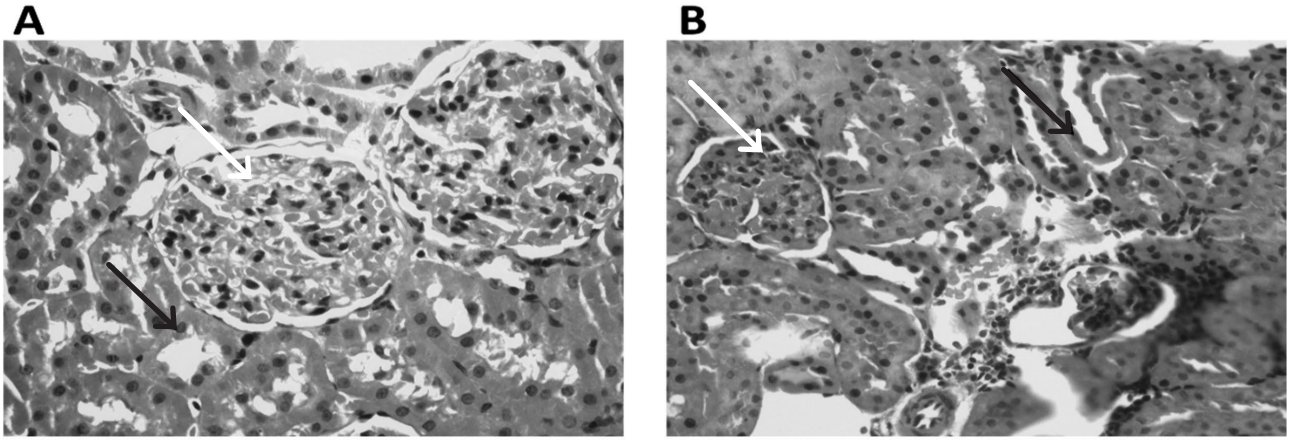


Table 2. Comparison of Histopathological Findings and Immunoreactivity Results in Control and Hypertension Groups

Parameter	Control Group	Hypertension Group	*P* value
Glomerulosclerosis Score	0.13 ± 0.35	12.50 ± 4.63	<.001
Tubular Damage Score	0.00 ± 0.00	0.75 ± 0.46	<.01
Peritubular Fibrosis Score	0.63 ± 0.92	3.50 ± 1.60	<.01
Cast Score	0.13 ± 0.35	1.50 ± 0.53	<.001
Claudin-2 Immunoreactivity	2.38 ± 0.52	1.38 ± 0.52	<.01
Renalase Immunoreactivity	1.63 ± 0.52	2.50 ± 0.53	<.05

by reducing the resistance in the afferent and efferent arterioles (21). The increase in serum NO levels in our hypertension group may be another reason for the decrease in CrCl. In addition, the decrease in urinary NO level seen in the hypertension group may also be the result of decreased GFR. In our study, the decrease in CrCl in the rats in the hypertension group shows that kidney glomerular functions were impaired.

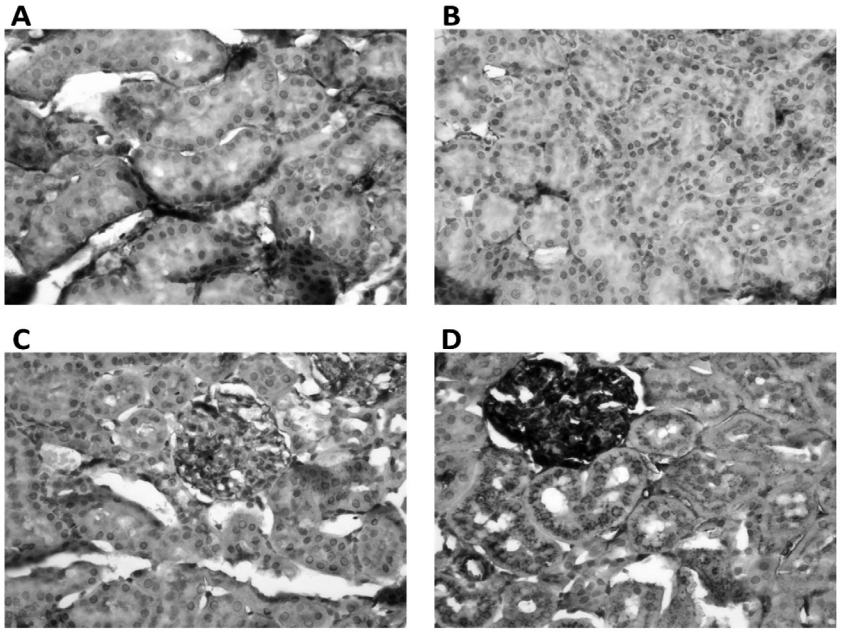
In our study, increased FENa and FEK values, indications of, which are indicators of renal tubular dysfunction, were examined. The increase in FENa was not statistically significant in the hypertension group. The lack of significant change in FENa may be the result of tubular mechanisms. However, FEK was significantly increased in the hypertension group. Gordish et al. (14) reported that there was no significant difference in FENa in their 4-week Ang II infusion study. Li et al. (13) showed that Ang II administration at a dose of 40 ng/min for 2 weeks increased FENa but did not create a significant difference in FEK. These differences in the literature may have resulted from the use of Ang II at different doses and durations. The short-term, high-dose Ang II administration in our study may have caused an increase in FEK by increasing the aldosterone level. The decrease in renal CLDN-2 activity in the hypertension group may be related to the deterioration of renal tubular function. In addition, this decrease may be a reason for the increase in FEK, which is a marker of tubular function.

Claudin-2 creates selective paracellular channels for small cations such as Na+ and K+ (11). In addition, CLDN-2 causes approximately 20%–25% of proximal water reabsorption to occur, paracellularly (11). In a study conducted in mice, it was reported that CLDN-2 inhibition was observed in conjunction with high urine volume and low urine osmolarity (22). We found that CLDN-2 immunoreactivity was significantly reduced in the hypertension group. Our results suggest that renal tubular injury may be the result of decreased CLDN-2 immunoreactivity, which is abundant in the proximal tubule. In addition, the increases in urine volume and FEK in the hypertension group may have resulted from the decrease in CLDN-2 expression.

Oxidative stress is known to play an important role in the pathology of hypertension. It has been reported that oxidative stress may cause hypertension by causing NO oxidation and inactivation (23,24). Oxidative stress is seen with decreased antioxidant capacity or increased free-radical formation in pathological conditions. Free radicals cause cell damage by causing DNA damage, lipid peroxidation, and protein modification (25). A result of lipid peroxidation MDA is also a marker of oxidative stress. The levels of MDA increased significantly in the animals in the hypertension group. However, there were no significant differences in the levels of kidney GSH, which latter is an endogenous antioxidant.

The vasoconstrictor effect caused by Ang II plays an important role in the development of fibrosis, cell proliferation, and inflammation in the kidneys (via the type 1 Ang II receptors) (26). In the histopathological evaluation of our kidney sections, significant increases were seen in the glomerulosclerosis score, tubular damage score, peritubular fibrosis, and amount of

Figure 4. Claudin-2 immunoreactivity (X400) (Fig. 4A. Control group; Fig. 4B. Hypertension group). Claudin-2 staining in the proximal tubule of the hypertension group decreased compared to the control group. Renalase immunoreactivity (X400) (Fig. 4C. Control group; Fig. 4D. Hypertension group). In the control group, mild staining was observed in the kidney glomeruli and proximal tubule. In the hypertension group, intense staining was observed in the kidney glomeruli and proximal tubule.



cast formation. These results are similar to those of the Ang II application study by Osmond et al. (27). There was no significant difference between the groups in our FENa results. However, the decrease in GFR and increase in FEK support our histopathology results. These results suggest that the administration of high dose Ang II to the rats in our sample caused glomerular and tubular damage to their kidneys.

Renalase, a newly discovered enzyme, is secreted from the proximal tubule in the kidney. It regulates blood pressure by metabolizing circulating catecholamines (9). Renalase has been associated with chronic kidney disease, diabetes, cardiovascular disease, and stroke (28,29). In addition, it was reported that renalase treatment decreased oxidative stress parameters in an experimental contrast nephropathy study (30). In our study, there was an increase in renalase immunoreactivity in the hypertension group. This result suggests that renalase may be a defense mechanism of the kidney against oxidative stress and kidney damage caused by increased blood pressure.

In an experimental study by Ayada et al., long-term administration of nesfatin-1 caused an increase in blood pressure, and it was shown that high levels of nesfatin-1 caused hypertension (5). In addition, Akcilar et al. (31), in the experimental hypertension model in which they created hypertension with deoxycorticosterone acetate–salt, reported that plasma nesfatin-1 levels increased in the hypertension group. In our study, although SBP increased significantly from the first day in the hypertension group, there was no significant difference in nesfatin-1 levels between the groups. These results may be since our experimental model of hypertension has different pathophysiological mechanisms.

Adropin, a newly discovered peptide hormone in the brain and liver, has been reported to have effects on energy homeostasis (32). In a clinical study, plasma adropin levels were found to be lower in people with hypertension compared to normotensive individuals. Decreased plasma adropin levels have been reported to be associated with increased blood pressure (33). Celik et al. (34) reported that patients with hypertension have a higher serum adropin level than normotensive people do. The relationship between adropin level and hypertension is still not clear. In our study, although serum adropin levels increased in the hypertension group, no statistical significance was observed. Similarly, no significant differences were found between the groups in renal or urinary adropin levels.

This study has some limitations. First, the duration of Ang II-1 week-may be too short to produce meaningful results. Secondly, we did not study renalase in vitro. Therefore, we do not know whether the possible protective effect of renalase is reducing blood pressure or inhibiting cardiac hypertrophy. For this reason, we think that there is a need for new studies with an in vitro component added, as well as with different experimental durations.

We aimed to examine the mechanisms of the regulation of blood pressure by renalase, nesfatin-1, and adropin as well as the relationships of the previous with kidney damage. A transmembrane protein in the renal proximal tubule, CLDN-2 has been used as an indicator of kidney damage and kidney function. The results of our study clearly show that, in rats, increased arterial blood pressure resulting from high-dose Ang II administration for 1 week impairs kidney function and causes kidney damage. There was an increase in renalase activity and a decrease in CLDN-2 activity in the kidney. We believe that adropin and nesfatin-1 levels should be examined at the molecular level by giving Ang II at different doses and for different durations. In addition, we think that there is a need for more comprehensive studies examining the parameters showing the degree of renal blood flow, kidney and oxidative stress damage, and NO metabolism by administering different doses of adropin and nesfatin-1.

Resumen

Objetivo: La hipertensión es una de las enfermedades cardiovasculares que causa más mortalidad, y el 95% de las causas son desconocidas. El objetivo del estudio fue examinar la posible correlación entre los niveles de nesfatina-1, los niveles de adropina, la inmunorreactividad de claudina-2 y la inmunorreactividad de la renalasa con la presión arterial, las funciones renales y el daño renal. Métodos: Se dividieron ratas Sprague-Dawley macho adultas en grupos de control e hipertensión (8 por grupo). Se administró angiotensina II vehículo al grupo de control y angiotensina II (0,7 mg/kg/día) al grupo de hipertensión, ambos a través de una minibomba osmótica durante 7 días. Las presiones sanguíneas se midieron mediante pletismografía del manguito de la cola los días 1, 3, 5 y 7. El día 7, se recogieron orina, sangre y tejidos de las ratas durante 24 horas. Resultados: En el grupo de hipertensión en comparación con el grupo de control, hubo un aumento en los niveles de presión arterial sistólica después del primer día. Mientras que la inmunorreactividad de claudina-2 disminuyó en los riñones, la inmunorreactividad de la renalasa aumentó. Hubo una disminución en la depuración de creatinina y un aumento en la excreción fraccional de potasio ($p<0.05$). Conclusión: Nuestros resultados mostraron que la claudina-2 y la renalasa están asociadas con la disfunción glomerular y tubular renal y pueden desempeñar un papel en la patogenia de la hipertensión. Creemos que estas posibles funciones merecen una mayor investigación.

Acknowledgments

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