THE EFFECTS OF ENALAPRIL AND IRBESARTAN IN EXPERIMENTAL DIABETIC NEPHROPATHY

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ABSTRACT
In our study we investigated the effects of angiotensin converting enzyme inhibitor and Angiotensin II receptor blocker at low-doses which do not affect blood pressure and renal hemodynamics on the experimental diabetic nephropathy. Diabetes mellitus was induced by 50 mg/kg streptozotocin on Sprague-Dawley rats. Except for the patient control group, 2.5 mg/kg Enalapril (an angiotensin converting enzyme inhibitor) and 5 mg/kg Irbesartan (an Angiotensin II receptor blocker) were given everyday via drinking water during six weeks period. After 24-hour urine collection, blood was withdrawn by cardiac puncture, and rats were sacrificed. Renal functions, histopathologic and electron microscopic alterations in renal tissues, and relative percent deposition of type IV Collagen were investigated. Diabetic nephropathy was determined with the increase of plasma glucose, HbA1C (P<0.000), urea, creatinin (P<0.005) and urinary protein, albumin, glucose (P<0.000) in patient control and Enalapril and Irbesartan treatment groups when compared to the healthy control group. Mesangiocellular proliferation, tubular basement membrane thickness, percentage of glomerular collagen accumulation reduced in treatment groups and glucose in urine in Enalapril group declined. Our findings suggest that renal protective effects of Enalapril and Irbesartan in the development of diabetic nephropathy were comparable.

KEYWORDS: Diabetic nephropathy, Diabetes mellitus, Type IV collagen deposition, Enalapril, Irbesartan.

ABBREVIATIONS:
Diabetes mellitus: DM
Diabetic nephropathy: DN
Renin-angiotensin system: RAS
Glucosidation products: GP
Radical oxygen products: ROP
Angiotensin II: Ang II
Angiotensin converting enzyme inhibitors: ACEIs
Ang II-T1 receptor blockers: ARBs
Healthy control: HC
Streptozotocin: STZ
Patient control: PC
Enalapril: EN
Irbesartan: IR
Plasma levels of glucose: Glu
Plasma levels of creatinine: pCr
Urinary levels of creatinine: uCr
Creatinine clearance: CCR
Urinary glucose: uG
Urinary protein: uP
Urinary albumine: uA
Hematoxylene-eosin: HE

Periodic acid Shiff: PAS
Transforming growth factor- β1: TGF-β1
Super oxide: O₂⁻
Nitric oxide: NO
Angiotensin T₂: AT₂
Angiotensin T₁: AT₁

Introduction
Renal failure in diabetes mellitus (DM) which is often seen in industrial societies and which is expected to increase 50% in a decade in the USA frequently occurs (17). Diabetic nephropathy (DN) develops in 30-40% of Type 1 DM, and 25% of Type 2 DM. (1). A number of factors are kept responsible for the unidentified mechanisms of DN. It is well known that enzymatic and nonenzymatic products of glucose, genetic predisposition, dislipidemia accompanying frequently DM and hypertension increase the progress of DN (11). Hyperfiltration, solute and albumin passage through the capillary walls increase as a result of hemodynamic alterations and increased renin-angiotensin system (RAS) activation in hyperglycemia, and the catabolism of the matrix proteins decreases (21). Furthermore, increased glucosidation products (GP) and radical oxygen products (ROP) due to RAS activation play an important role in development of DN (19).

Systemic and intrarenal RAS activities increase in hyperglycemia. Access production of intrarenal angiotensin II (Ang II) results in hyperfiltration by increasing intraglomerular pressure owing to its vasconstrictive effect on efferent arterioles.
Materials and Methods

Forty-five female Sprague-Dawley rats weighing 215 to 310g were utilized for this study. Except for the healthy control (HC) group, a single 50 mg/kg intraperitoneal injection of streptozotocin (Sigma) (STZ) in 0.1 mol/L citrate buffer (pH 4.5) was performed to all groups to form Type 1 DM. The ones with blood glucose levels ≥ 250mg after 48 hours were accepted DM. Except for the healthy 9 rats, three groups were formed as patient control (PC), Enalapril (EN) and Irbesartan (IR) groups consisting of 12 rats in each. Excluding the PC and the HC groups, EN was given to one group as an ACEi with a dose of 2.5mg/kg and IR was given to the other group as an ARB with a dose of 5mg/kg via their drinking water everyday during 6 weeks period. After having fed with standardized food and free water throughout the therapy, the rats were taken into a metabolic cage (Harward) at the end of this period, and were put to sleep with 50mg/kg IM ketamine injection after 24 hours urine collection. They were sacrificed and both kidneys were extirpated after obtaining blood samples by cardiac puncture. Blood counts and serum HbA1C levels were obtained at the same day. Plasma and urine samples were collected in a freezer at -70°C and analyzed at the same day. Plasma levels of glucose (Glu), urea and creatinine (pCr) and urinary levels of creatinine (uCr) were measured and then values of endogonic creatinine clearance (CCR) were calculated. Urinary glucose (uG), protein (uP) and albumine (uA) levels were measured and daily discharges were calculated in milligrams per 100 g rat weight.

Ipsilateral kidney tissues were fixed in 10% buffered formalin. The tissues were processed in an enclosed automatic tissue processor system for 16 hours. The 4μm thick sections, obtained from paraffin embedded tissues were stained with hematoxyline-eosin (HE) and periodic acid Shiff (PAS) stains and examined under a light microscope. Immunohistochemically type IV collagen (DAKO-N, 1536) was applied to one section and examined under a light microscope. Hematoxylene-eosin (HE) and periodic acid Shiff (PAS) stains and examined under a light microscope. Type IV collagen positivity per one glomerule loop was calculated. Positively stained areas were detected and percentage of average type IV collagen positivity per one glomerule loop was calculated.

Tissues of 1mm³ of renal cortex of all rats of all groups were initially fixed in 2.5% gluteraldehyde and post fixed in 1% osmium tetroxyde (OsO₄). The tissues were dehydrated in increasing dilutions of ethanol and embedded in Epon-812. The tissue blocks were sectioned with Raychart ultramicrotome, stained with uranylacetate and evaluated under Zeiss-EM-9 electron microscope. Glomerular and tubular basement

<table>
<thead>
<tr>
<th>DATA</th>
<th>HC (n:9)</th>
<th>PC (n:9)</th>
<th>EN (n:9)</th>
<th>IR (n:9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>254±9</td>
<td>258±17</td>
<td>266±07</td>
<td>258±26</td>
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<tr>
<td>Weight difference (g)</td>
<td>-2±5</td>
<td>-39±12</td>
<td>-27±20</td>
<td>-45±38</td>
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<tr>
<td>Sickness duration (day)</td>
<td>-</td>
<td>45±3,1</td>
<td>44,1±4,3</td>
<td>43,3±3,4</td>
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<tr>
<td>Htc (%)</td>
<td>39,7±0,0</td>
<td>39,0±3,6</td>
<td>38,8±2,9</td>
<td>42,6±3,1</td>
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<tr>
<td>Glu (mg/dl)</td>
<td>139±15</td>
<td>498±43</td>
<td>437±81</td>
<td>510±80</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>1,6±0,3</td>
<td>9,1±2,5</td>
<td>8,9±3,1</td>
<td>9,4±2,7</td>
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<tr>
<td>Urea (mg/dl)</td>
<td>40±8</td>
<td>72±24</td>
<td>62±34</td>
<td>66±29</td>
</tr>
<tr>
<td>pCr (mg/dl)</td>
<td>0,6±0,1</td>
<td>0,8±0,1</td>
<td>0,7±0,1</td>
<td>0,7±0,1</td>
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<tr>
<td>Urine Volume (ml/day)</td>
<td>12±4</td>
<td>109±16</td>
<td>71±45</td>
<td>73±36</td>
</tr>
<tr>
<td>CCR (μl/min/100g)</td>
<td>198±66</td>
<td>302±135</td>
<td>302±194</td>
<td>288±122</td>
</tr>
<tr>
<td>uG (mg/day/100g)</td>
<td>0,8±0,4</td>
<td>223±97</td>
<td>97±57</td>
<td>139±71</td>
</tr>
<tr>
<td>uP (mg/day/100g)</td>
<td>4±3</td>
<td>59±34</td>
<td>27±31</td>
<td>26±18</td>
</tr>
<tr>
<td>uA (mg/day/100g)</td>
<td>0,9±0,3</td>
<td>25,0±303</td>
<td>10,6±7,0</td>
<td>9,5±4,4</td>
</tr>
</tbody>
</table>

HC: Patient groups, P; a:<0.05, b:<0.01, c:<0.005, d:<0.000  
PC: Treatment groups, P; x:<0.05, y:<0.000; EN/IR groups: *; P<0.05
membrane thicknesses of five rats of each group were measured electron microscopically and their average values were calculated.

Statistical method. The data of the study groups and percentages of relative positivities for type IV collagen were loaded in SPSS 8.0 computer program and were compared by Mann-Whitney U test. The values were taken as mean ± standard deviation. Values of p < 0.05 were regarded as significant.

Results and Discussion
As three rats from each sick group died during the study, study groups were finalized by nine rats for each group. When the data of the groups were compared (Table 1), differences between sickness durations and body weights were insignificant. When compared to the healthy group, it is found that DM was formed with elevated levels of glucose and HbA1C in blood. Urea and pCr elevation in plasma and uP, uA elevation in urine with CCR increase reaching significant levels in PC group showed progress to DN.

Examination of the histochemically PAS and HE stained sections of renal tissues of HC group showed nonpathologic glomerular and tubular basement membranes, and relatively 29% type IV collagen positivity in glomerular mesangium and peritubular intersititium was noticed in immunohistochemically type IV collagen stained sections (Fig. 1a-b). The examination of the renal tissues of PC group revealed thickening, duplication and blurred appearance in glomerular basement membranes, glomerular enlargement with mesangial expansion, deposition of fibrinoid material in Bowman space, dilation of tubules and increase in the positivity of collagen type IV in both glomerular mesangium (35%) and peritubular intersitium (Fig. 2a-b). There was less mesangioacellular proliferation and tubular basement membrane thickening in therapy groups. In EN group, there were intermittent thickenings and blurred appearance in glomerular and tubular basement membranes with collagen type IV positivity in glomerular mesangium (31%) and peritubular intersititium more than the healthy group and perceptibly less than the patient group (Fig. 3a-b). There was no difference between IR group and EN group with PAS stain; however, when compared to the EN group, positivity for collagen type IV in the glomerular mesangium was slightly prominent in IR group (33%) (Fig. 4a-b).

There was no electron microscopic alteration at the filtration and resorption barriers of HC group. The basement membrane thicknesses of glomeruli and tubules were between 80 and 250nm (mean 144.5nm).

There was diffuse glomerulosclerosis at ultra structural level in the group of experimental DN. The glomerular epithelium was degenerated, large (podocyte) and small (peduncle) projections were disappeared, thus, the epithelial cells were stuck to the basement membrane and the filtration barrier was damaged. There was endothelial and mesangial proliferation, increase in the synthesis of tropocollagen, narrowed lumens and sclerosis.
in most of the capillaries. Because of the basement membrane depositions, diffuse thickening ranging from 500 to 800nm (mean 480nm) of basement membranes was detected.

Fig. 4a-b: a: Basement membranes of IR group without any difference from the EN group (PASx200). b: Positivity for glomerular mesangial type IV collagen in IR group slightly more than the EN group (Streptavidine-biotine peroxidase, X200).

In the IR group the small projections of the glomerular epithelium were well preserved in some areas. The degenerative changes were relatively scarce in the tubular epithelial cells. There were less sclerotic capillaries. The basement membranes showed focal thick areas ranging from 80 to 500nm (mean 241nm) (Fig. 5a).

Fig. 5a-b: a: Irbesartan treatment group. Basement membrane (BM) with alternating enlarged (nodular enlargement) and interposition of the mesangial cell (Me) and erythrocyte (Er) in the capillary lumina (C) can be seen. (X6000). b: Enalapril treatment group. The basement membrane (BM) with normal thickness, intact epithelial cells (P), capillaries (C) which do not show any sclerotic changes, edema (+) and endothelial vacuolization (v) can be seen. (X6000)

Most of the glomerular epithelial cells and their small projections were preserved in the EN group. The basement membrane thicknesses were measured as 80-500 nm (mean 201 nm). The activity of the mesangium was relatively decreased and the sclerotic changes in the walls of the vessels were also decreased. There were reversible changes, dystrophic changes and vacuolization in the endothelium of the capillaries (Fig. 5b).

Increase in the systemic and local RAS activation in DM plays an important role in constitution of metabolic and hemodynamic alterations that lead to DN. Blood volume and systemic/renal RAS activation increases in hyperglycemia (10). Ang II has been implicated in increasing efferent arteriolar vasoconstriction and, thus, promotes glomerular damage through hemodynamic mechanisms. On this occasion, hyperfiltration occurs and passage of solute and proteins to ultra filtrate increases (10-14). Renal tissue’s NADPH oxidase that plays a role in break down of the tissues is stimulated by hyperglycemia and augmentation of Ang II activation and production of super oxide ($O_2^-$) increases (9). $O_2^-$, reacting with nitric oxide (NO), stimulates ROP production by formation of peroxynitrite (ONOO). Ang II stimulates peroxidation of lipids and accelerates usage of antioxidants by activating synthesis of prostaglandins which causes ROP production and protein kinase C which stimulates Ang II augmentation. It facilitates thromboembolism by enhancing production of plasminogen activator inhibitor 1 with its direct influence and with intervention of transforming growth factor- β1 (TGF-β1) (4). In DM, mesangial and interstitial cells are stimulated by hyperfiltration, microalbuminuria, increased levels of GP and ROP and activation of protein C. Enhanced production of TGF-β1 provokes proliferation of the cells and increase in mesangial matrix by synthesis of matrix proteins like collagen and fibronectin. Thus, glomerulosclerosis occurs owing to vascular damage while deterioration of renal functions speeds up by increase in the renal matrix (11, 22).

Ang II shows its effects by mediation of two subgroup receptors. Angiotensin $T_2$ (AT$_2$) receptors have vasodilator, antiproliferative and apoptotic effects. However, angiotensin $T_1$ (AT$_1$) receptors speed up deterioration of organs by their proliferative, vasoconstrictor, Na$^+$ retaining effects, by increasing blood pressure and by stimulating ROP production (17, 18). It has been reported that ACEi which decreases Ang II production (7, 18), and recently ARB, which suppresses AT$_1$ receptor (5, 13), slow down progress to DN. Ang II AT$_1$ receptors speed up progress to DN by hemodynamic and local metabolic alterations via their Na retaining effects from proximal tubules. On this occasion, it is believed that, ACEi may slow down progress to DN much more by decreasing intraglomerular pressure and passage of solute and protein via their hemodynamic effects, while ARB may do the same by blocking AT$_1$ receptor and by increasing effects of AT$_2$ receptor. Ang II shows its organ damaging effects via AT$_1$ receptors. While AT$_1$ receptors suppressed by ACEi, AT$_2$, receptors, with antiproliferative effects are blocked, too. Thus, it is believed that, except for hemodynamic effects, renal protective properties of ACEi may be less than ARB (18, 20).
Electron microscopical thicknesses and mean values of glomerular and tubular basement membranes in study groups (1nm = 10Å).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Basement membrane thicknesses(nm)</th>
<th>Mean basement memb. thickness (nm)</th>
<th>Standart Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control</td>
<td>80-250 (mean. 165) 80-100 (mean. 90) 100-200 (mean. 150) 200-250 (mean. 225) 85-100 (mean. 92,5)</td>
<td>144.5 ±56.1</td>
<td></td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>500-800 (mean. 650) 300-400 (mean. 350) 500-600 (mean. 550) 400-500 (mean. 450) 300-500 (mean. 400)</td>
<td>480 ±120.4</td>
<td></td>
</tr>
<tr>
<td>Irbesartan Treatment</td>
<td>80-300 (mean. 190) 100-500 (mean. 300) 80-400 (mean. 240) 200-300 (mean. 250) 200-250 (mean. 225)</td>
<td>241 ±40.1</td>
<td></td>
</tr>
<tr>
<td>Enalapril Treatment</td>
<td>200-250 (mean. 225) 80-200 (mean. 140) 100-100 (mean. 90) 200-300 (mean. 200) 200-500 (mean. 350)</td>
<td>201 ±98.5</td>
<td></td>
</tr>
</tbody>
</table>

In our study, DN with obvious albuminuria accompanying glucosuria has developed in the rats which DM were formed by STZ. Glomerular functions were spolt with increased levels of pCr and plasma urea. Increase of CCR levels reaching significant degrees in PC group, shows that, hemodynamic alterations owing to hyperglycemia play an important role in renal deterioration (14). Especially increase of CCR observed in Type 1 DM, results from hypervolemia and increased intraglomerular pressure, and increased synthesis of NO and prostaglandin. Passage of solute and albumin, which is increased by deteriorated electrical load of capillaries owing to the increase in hydrostatic pressure, deteriorates tubules as well as glomerular capillaries by increasing the work load (11, 14). In a similar way, formation of obvious proteinuria and albuminuria in the rats in our study, results from prominent deterioration of the tubules as well as the glomeruli. The histopathologic and electron microscopic changes in the kidneys of diabetic rats in our study support the idea that diabetic nephropathy develops mostly diffuse (11, 14). It is found out that degenerative glomerulotubular changes were formed due to glomerular enlargement with prominent increase in the mesangial matrix, accumulation of fibrinoid material in Bowman’s space, tubular dilation and thickening of the basement membranes (8). In electron microscopic examination, degeneration in the glomerular epithelial cells, adhesion of the epithelial cells to the basement membrane because of the loss of big and small processes, deteriorated filtration barrier, proliferation of endothelium and mesangium, stenosis and sclerosis of the capillaries were seen. Histopathologic examination of the treatment groups revealed mesangiocellular proliferation and thickening of the basement membranes in a lesser degree. In the electron microscopic examination, when compared to PC group, treatment groups showed less sclerotic changes in the vessels, less epithelial degenerative changes (especially reversible), relatively spared foot processes, less thickening in the basement membranes. When compared to HC group there was significant type IV collagen accumulation in PC group, however, accumulation was lesser in the treatment groups (23).There was no significant difference in the comparison of treatment groups with PAS stained specimens however, type IV collagen accumulation in the EN group was lesser than the IR group. Decrease in the collagen accumulation may result from its effect on glycemia, which is the major cause of progress to DN (6, 7). When basement membrane thicknesses of all groups were compared by electron microscopic examination, it was noted that, basement membranes were fairly thick in DN and the average width of the basement membranes was lesser in EN group than IR group (Table 2).

Medication of both drugs in doses that do not have any effect on hypertension may show that drugs decelerate progression to DN (18, 20). Onozato et al. (23) declared that, treatment of rats with experimental diabetes by ACEi and ARB in doses that do not decrease blood pressure and CCR; decreases NADPH oxidase and peroxidase production and slows down progression to DN. In a similar way, in experimentaly DN formed rats, we did not observe any increase in lipid peroxidation products with low doses of those two drugs. On this occasion, partial improvements in renal functions and histopathologic alterations with treatment in our experimental rats, result from decrease of local metabolic changes and lipid peroxidation owing to the suppressor effects of ACEi and ARB on AT1 receptors (2, 7). Decrease of glomerular collagen accumulation with both drugs is consequentially related with decrease in production of mesangiocellular TGF-β1 and free oxygen radicals that play major role in stimulation of mesangial cells (24). The fact that the significant increase in blood glucose levels, which has a major role in progression to DN, is decelerated by EN but not affected by IR proves the local effects of the drug in sick group (16, 20). However, Mogyosori and Sonkodi (15) stated that, ARBs have a few side effects and may be more useful in preventing progress to DN, when compared to ACEi.

In our study we observed that, both enalapril which is an ACEi and irbesartan, which is an ARB slow down the histopathologic alterations and deterioration of renal functions in experimental diabetic nephropathy in approximate levels. Both drugs’ decelerative effect on progression to DN in low doses that do not decrease CCR, results from decrease of renal metabolic changes in diabetes. Our findings show that, enalapril’s effect on decrease of glucosuria is more prominent...
than irbesartan’s effect on decrease of proteinuria and both
drugs slow down progression to DN in a similar way.

REFERENCES
1. Andersen A.R., Christiansen J.S., Andersen J.K.,
Int., 62, 192-198.
3. Andersen S., Tarnow L., Rossing P., Hansen B.V.,
287, 2570-2581.
6. Brodsky S.V., Morrishow A.M., Dharia N., Gross S.S.,
Goligorsky M.S. (2001) Am. J. Physiol (Renal Physiol),
480-486.
Kidney Int., 57, 590-600.
1637.
246-255.
12. Lewis E.J., Hunsicker L.G., Bain R.P., Rohde R.D.
105-112.
18. Ruiz-Ortega M., Lorenzo O., Ruperez M., Egido J.
1817.
23. Zhu D., Kim Y., Steffes M.W., Groppoli T.J., Butkowski
Dis., 31, 453-463.