The role of monoamine oxidases in diabetic nephropathy rat model

Diabetic nephropathy model

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Abstract

Aim: Diabetic nephropathy (DN) is a serious complication that can lead to renal failure in many diabetic patients. Although there are multiple mechanisms related to the pathogenesis of the disease, reactive oxygen species (ROS) play an important role in its etiology. It has been suggested that monoamine oxidases (MAOs) as an intracellularly important ROS source may cause nephropathy by contributing to redox state imbalance in the kidney of diabetics. We investigated the role of monoaminoxidase-A (MAO-A) and monoaminoxidase-B (MAO-B) in the pathogenesis of diabetic nephropathy (DN) induced by streptozotocin (STZ) in rats. We also tried to demonstrate the importance of MAO-A or MAO-B inhibition to prevent the development and progression of DN. Material and Method: Twenty-eight male Wistar albino rats were divided into four groups as normal control and three diabetic groups. A single dose of STZ was given to the peritoneal cavity of rats to induce diabetes. One of the diabetic groups was treated with MAO-A inhibitor(moclobemide, 5 mg/kg/day), another group MAO-B inhibitor, (rasagiline, 10 mg/kg/day), while those included in the DN group were not treated. After the eight-week treatment period, urine samples were collected with a metabolic cage to measure N -acetyl-b-glucosaminidase (NAG), g-glutamyltranspeptidase (GGT), creatinine, glucosuria and proteinuria, and then the animals were anesthetized and sacrificed by cardiac puncture and the kidneys were taken. Blood glucose, BUN, serum creatinine, renal MAO-A, MAO-B, superoxide dismutase (SOD) and catalase (CAT) activity and lipid peroxidation (MDA) were determined by spectrophotometric or ELISA method. Results: The untreated diabetic rats showed nephropathy symptoms such as high serum creatinine, proteinuria, glucosuria and high urinary NAG and GGT. However, renal MAO-A, MAO-B, SOD and CAT activity and MDA levels increased in DN rats. Although moclobemide or rasagiline treatment significantly reduced nephropathic findings and high renal MAO-A and MAO-B activity in diabetic rats, MAO-A inhibition showed more effect than MAO-B. Discussion: The results indicate that the renal MAO-A and MAO-B activity playsan important pathophysiological role, which is responsible for the development and progression of DN, and that MAO-A inhibition is more effective than MAO-B to prevent the formation of DN in diabetic rats.

Keywords

Diabetes; Rat; Moclobemide; Rasagiline

Introduction

DN occurs ultimately between 20-40% of patients with diabetes and it is one of the main causes of renal failure requiring dialysis and deaths because of microvascular diabetic complications [1]. Endothelial dysfunction, tubular fibrosis, glomerular hypertrophy, podocyte injury, and progressive proteinuria are the hallmarks of the disease [2,3]. DN has five different phases, as early hypertrophy-hyper function stage, glomerular lesions without clinical disease stage, microalbuminuria stage, overt DN stage and terminal stage of renal failure [4]. Despite treatment, the number of people with diabetic kidney disease continues to increase. Physiopathological mechanisms of DN is not fully understood and the reason why not all diabetic patients develop this complication is unknown.

The underlying mechanisms which contribute to the evolution of DN are extremely complex, and metabolic, genetic, and several hemodynamic factors have been identified [5]. Among the metabolic factors, ROS are thought to play an important role in the development of DN [6]. ROS are cytotoxic to renal cells and lead to activation of various pathways leading to glomerular hypertrophy, decline in glomerular filtration rate resulting in nephropathy [7,8]. Studies in rat models have shown that the increase in free oxygen radical production due to oxidative stress may be responsible for the development of DN [9,10]. The ROS producing sources contribute to diabetic complications remains fully understood. Mitochondrial oxidative enzymes are major intracellular ROS generating sources [11]. MAOs are a family of enzymes that catalyze the oxidation of monoamines and located bound to the outer membrane of mitochondria in most cell types in the body. Oxidative deamination of monoamines by MAOs result in formation of ROS which might cause diabetic nephropathy contributing to unbalanced kidney redox state [12-17].

Recently, it has been suggested that the increase in angiotensin II activity is responsible for the pathogenesis of DN. Angiotensin-II mediates the constriction of the efferent artery, enhances glomerular pressures, induces hypertrophy and increases tissue oxidative stress. In addition, Angiotensin II increases MAO activity in rat kidneys by activating AT1 receptor. Diabetic animal models, clinical trials, and meta-analysis have clearly demonstrated the effectiveness of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers to improve glomerular and tubulointerstitial damage, reduce proteinuria, and decrease progression of chronic kidney disease [18-21]. According to the above description, MAO levels might be found increased in DN and this state might contribute to reducing the natriuretic effect of dopamine and to unbalanced cell redox state. However, the pathophysiological mechanisms associated with angiotensin II in DN are not fully understood. Studies show that the pathological process of DN is irreversible and resistant to clinical treatment. Therefore, it is necessary to identify new therapeutic drugs to delay the development and progression of DN. Is the increased renal MAO-A and/or MAO-B activity can be responsible for the pathogenesis of DN? One of the aims of this study is to shed light on the answer to this question with current knowledge about the pathogenesis of DN, and the other aim is to help develop new treatment strategies to prevent the genesis and progression of nephropathy in diabetics.

Material and Method

Animals

Male Wistar albino rats with body weights ranging from 220-250 g were fed with commercial rodent food, water, and ad libitum diet, under standard laboratory conditions with seven animals per cage. Animals were acclimated to the laboratory for 1 week prior to the start of theexperiment. The temperature in the cages was 22 ± 2°C, and the relative humidity was 50-60%. The ethical approval for the study was obtained from Dicle University Institute of Animal Experiments of Ethics Committee (Diyarbakir, Turkey), which is engaged in animal protection (Approval no: 2017/18).

Diabetic rat model

STZ dissolved in citrate buffer was injected to induce experimental diabetes in a single dose of 60 mg/kg into the peritoneal cavity of rats that were fasted for 12 hours. In order to prevent fatal hypoglycemia, which occurred 6 hours later in STZ injected rats, the animals were fed orally with 10% glucose solution for one day. Tail blood samples were obtained 72 hours after streptozotocin injection and the blood glucose concentration was determined by the glucometer (Abbott Optium Xceed) to make sure the formation of diabetes. It was decided that animals with a blood glucose of 300 mg/dl or higher were diabetic and were included in the study.

Study design

Rats were placed in their cages as four different groups, each containing seven animals. Group 1 served as control rats. Group 2 served as diabetic rats. Group 3 served as diabetic rats with daily treatment of moclobmide (5 mg/kg/day) orally for 56 days. Group 4 served as diabetic rats with daily administration of rasagiline (10 mg/kg/day) orally for 56 days. Feed and water consumption, body weight, blood glucose, and physical conditions of the animals were monitored weekly from the beginning to the end of the experiment. The dose of moclobemide and rasagiline was adjusted throughout the experiment period according to body weight changes each week to maintain a similar dose per kg body weight of the animal. Animals were housed in metabolic cages a day before the end of the study to collect samples of 24-h urine for measuring protein, NAG and GGT levels. On the last day of the study, animals were anesthetized with ketamine (80 mg/kg; i.p.) after 12 hours fasting and sacrificed by cardiac puncture. Kidneys were removed, washed immediately with ice-cold saline, and stored at -80 °C until the parameters were measured.

Kidney homogenate

The kidneys were homogenized in ice-cold Tris-HCL buffer (0,1 M; pH:7,4) using a teflon homogenizer and centrifuged at 4°C (12000g x 30 min.) to collect the supernatant. Supernatants were used to determine intrarenal dopamine levels, oxidant enzymes, MAO-A and MAO-B activity, and antioxidant enzymes, SOD and CAT activities. The protein content in renal homogenate samples was determined by relevant extra sensitive protein Assay Kit.

Antioxidant and oxidant enzyme activities and lipid peroxidation
CAT activity of the kidney was measured in the supernatants of homogenates by an enzymatic method as described by Aebi [22]. Renal SOD activity was determined in the supernatant via a photometric method described by Sun et al. is based on the capacity of SOD to inhibit reduction of nitro blue tetrazolium [23]. Lipid peroxidation was determined by measuring the level of MDA in kidney homogenate using an assay kit provided by Sigma–Aldrich. MAO-A and MAO-B activities in kidney homogenates as intracellular oxidant enzymes responsible for dopamine catabolism were measured using the rats-specific ELISA Kit provided by LifeSpan BioSciences, Inc. North America.

Kidney functions
The levels of creatinine in serum and urine samples were determined by using an available colorimetric kit from Cayman Chemical Company (Michigan, USA). Serum and urine glucose levels were measured by the Rat Glucose Test Kit produced by the Crystal Chemical Company. Urinary pathologic enzymes activities in control and diabetic groups such as renal damage markers, GGT and NAG, were evaluated by Colorimetric Assay Kits and the results of the test were expressed as units.

Statistical analysis
The results obtained from seven rats in each group of this study were expressed as mean ± SEM. For statistical examination, SPSS version 20.0 for Windows statistical package program was used. The Kruskal Wallis Analysis of Variance test was used for statistical evaluation. The paired comparison of the differences between the parameters was evaluated with the non-parametric Mann-Whitney U test. In addition, Spearman correlation test was used to clarify possible relationships between parameters. Values were regarded as statistically significant when $p < 0.05$.

Results
Metabolic characteristics of the animals
Three days after STZ injection, rats with a significant increase in fasting blood glucose levels, water, and food consumption, and urine volume were considered to be diabetic animals and included in the study. In DN-group rats, body weight significantly reduced with respect to the initial body weight three weeks after STZ injection, and gradually decreased further in the following weeks till the end of study (Table 1). Treatment with moclobemide or rasagiline of diabetic rats prevented significant loss of weight compared to DN group.

Parameters related to kidney function
The level of serum creatinine and BUN in DN group rats showed a significant increase compared to the normal control because of reduced greatly excretion of urinary creatinine and urea. Treatment with moclobemide or rasagiline led to increased urinary creatinine and urea excretion in diabetic rats, thus resulting in normalization of serum creatinine and BUN concentration (Table 2). As shown in Table 2, urinary glucose loss and proteinuria showed a significant increase in diabetic rats with respect to the normal control group. The increase in blood glucose level, urine volum, urinary glucose loss and proteinuria due to diabetes, was significantly prevented by treatment with mo-

Table 1. Metabolic properties of the animals: Three weeks after the onset of diabetes, water and food consumption, changes in body weight and at the end of the study fasting blood glucose levels. The effect of MAO inhibitors, moclobemide and rasagiline.

Parameters	Groups			
	Control	DN	DN+Moclobemide	DN+Rasagiline
Fasting blood glucose (mg/dl)	97,2 ± 4,8	415,3 ± 13,2[a]	294,1 ± 9,7[b, c]	318,5 ± 8,9[a, b]
Daily water consumption (ml)	28,3 ± 3	80,3 ± 6[a]	40,1 ± 3[a, b]	43,4 ± 4[a, b]
Daily food intake (g)	20,3 ± 1,2	186 ± 8,4	59 ± 5,3[a, b]	71 ± 5,9[a, b]
Body weight (g), first week	225,8 ± 7,3	230,2± 9,1	221,3 ± 7,4	228,7± 8,1
Body weight (g) third weeks	285,4 ±10,6[d]	168,2 ± 8,3[d a]	201,4 ± 8,7[e,b]	198,9 ± 9,2[e, b]

DN: Diabetic Nephropathy.
The data are presented as the mean ± SEM of indicated number of rats (n = 7).
a: compared with the control group; p<0.001. b: compared with the DN group; p<0.01.
c: compared with DN group; p<0.05. d: compared with first week; p<0.01 e: compared with first week p<0.05.

clobemide or rasagiline, although the effect of rasagiline was less according to moclobemide. Urinary excretion of GGT and NAG has been used for a long time as a valid indicator of renal damage in nephropathy studies on human beings, laboratory rodents, and dogs. GGT and NAG levels were significantly higher in urine samples of untreated diabetic rats than in normal control values. MAO inhibitors, moclobemide, and rasagiline significantly reduced GGT and NAG urinary levels of diabetic rats. However, the effect of moclobemide was more than rasagiline (Table 2)

Renal antioxidant enzyme activities and lipid peroxidation
Renal SOD and CAT activity in DN rats was found significantly higher than normal control rats. Treatment with moclobemide or rasagiline for 8 weeks significantly reduced both SOD and CAT activity, thusboth antioxidant enzyme levels were close to normal control values with MAO inhibitors. MDA level increased significantly as an indicator of oxidative stress and lipid peroxidation in the kidney of diabetic rats. MAO-A or MAO-B inhibitors, moclobemide or rasagiline, significantly prevented the increase in lipid peroxidation in kidneys induced by diabetes (Table 3).

Renal MAO-A and MAO-B activity
Both MAO-A and MAO-B activity in diabetic rats were found to be significantly higher than the normal control group. The high MAO-A activity in diabetic rats kidney was reduced by moclobemide treatment, and similarly, high MAO-B activity decreased to normal levels with rasagiline treatment (Table 3).

Correlation Between Renal Function Parameters and Oxidant and Antioxidant Enzyme Activity and Lipid Peroxidation
As shown in Figures 1, 2, 3 and 4, there is a relationship between oxidant and antioxidant enzyme activities and renal function in diabetic rats. We found a positive correlation between SOD and urine GGT, CAT and urinary GGT, MAO-A and urinary GGT in kidneys of DN rats (Figure 1,2,3). In addition, renal MAO-A activity showed a linear correlation with proteinuria (Figure 4).

Table 2. Parameters related to kidney function; glycosuria, proteinuria, creatinine clearance and urinary GGT and NAG levels. The effect of MAO inhibitors, moclobemide and rasagiline.

| | Groups | | | |
Parameters	Control	DN	DN+Moclobemide	DN+Rasagiline
Proteinüria (mg protein/mg creatinine)	1,9 ± 0,2	3,9 ± 0,28[a]	2,2 ± 0,18[b]	2,8 ± 0,25[c,d,e]
Glucosuria (mg/ml)	0 ± 0	34,6 ± 2,7[a]	11,2 ± 2,4[a, b]	18,7 ± 2,8[a, c,e]
Serum creatinine (mg/dl)	0,45 ± 0,09	1,5 ± 0,2[a]	0,78 ± 0,3[b]	0,83 ± 0,2[c,d]
BUN (mg/dl)	43,6 ± 1,3	72,4 ± 2,8[a]	51,8 ± 3,1[b, d]	59,1 ± 2,9[a,b,c]
Urinary GGT (U/mg protein/30 min.)	0,06 ± 0,01	0,39 ± 0,03[a]	0,065 ± 0,0[b]	0,25 ± 0,02[a, c]
Urinary NAG (U/mg protein/30 min.)	0,013 ± 0,006	0,091±0,012[a]	0,012 ± 0,0005[b]	0,06 ± 0,01[c,d]

DN: Diabetic Nephropathy.
The data are presented as the mean ± SEM of indicated number of rats (n = 7).
a: compared with the control group; p<0.001. b: compared with the DN group; p<0.01.
c: compared with DN group; p<0.05. d: compared with the control group; p<0.05.
e: compared with DN+Moclobemide group; p<0.05.

Table 3. Renal monaminoxidases and antioxidant enzyme activities, lipid peroxidation (MDA) and dopamine levels. The effect of MAO inhibitors, moclobemide and rasagiline.

| | Groups | | | |
Parameters	Control	DN	DN+Moclobemide	DN+Rasagiline
MAO-A (pg/mg protein)	15,7 ± 0,9	24,6 ± 2,1[a]	14,8 ± 2,4[b]	22,1 ± 1,7[a,e]
MAO-B (pg/mg protein)	12,4 ± 1,1	18,4 ± 1,8[a]	17,6 ± 2,2	13,2 ± 0,8[b,e]
SOD (U/mg protein)	4,6 ± 0,3	6,8 ± 0,5[d]	4,8 ± 0,2[c]	5,7 ± 0,4[a,e]
CAT (U/mg protein)	2,1 ± 0,2	5,2 ± 0,4[a]	3,1 ± 0,3[b]	3,9 ± 0,2[a,e]
MDA(µM)	2,5 ± 0,1	4,8 ± 0,3[a]	2,9 ± 0,1[b,d]	3,7 ± 0,3[b,d,e]
Dopamine (ng/mg protein)	112,4 ± 10,3	81,6 ± 7,1[a]	157,4 ± 13,5[a,b,d]	138,1 ± 13,3[a,b,d]

DN: Diabetic Nephropathy.
The data are presented as the mean ± SEM of indicated number of rats (n = 7).
a: compared with the control group; p<0.001. b: compared with the DN group; p<0.01.
c: compared with DN group; p<0.05. d: compared with the control group; p<0.05.
e: compared with DN+Moclobemide group; p<0.05.

Discussion

DN is the main cause of morbidity and mortality as one of the serious complications of diabetes leading to end-stage renal failure. Multiple pathophysiological mechanisms that contribute to the development of DN are quite complex. In recent years, experimental evidences have shown that reactive free oxygen radicals may be responsible for the pathogenesis of DN [1-7]. Free reactive oxygen species are produced continuously under physiological and pathological conditions as a result of metabolism and are balanced by the body's antioxidant defense systems. If the amount of oxygen radicals produced exceeds the capacity of the defense system or the strength of antioxidant mechanisms decreases, oxidative stress can lead to tissue damage. It is believed that mitochondrial free oxygen radical production is increased in diabetics and this condition plays an important role in the formation of nephropathy [8-10]. In the present study, we determined that oxidative stress indicators such as MAO-A, MAO-B, SOD and CAT activities and MDA level were significantly increased in kidney tissues of DN rats. In addition, we found a positive correlation between nephropathy symptoms and renal MAO-A and antioxidant enzyme activities. Treatment with MAO inhibitors, moclobemide or rasagiline significantly prevented nephropathy symptoms in diabetic rats. Therefore, it was concluded that oxidative stress increased in kidney tissues of diabetic rats and this adverse condition may cause nephropathy. However, antioxidant agents were not ef-

fective sufficiently to meet the expectations for the prevention or treatment of nephropathy in diabetic patients.

One of the most investigated subjects related to the pathophysiology of DN is the renin-angiotensin system. Regardless of their hemodynamic effects, it has been reported that Ang II contributes to the development and progression of renal damage through the AT1 receptor in diabetic patients [17-20]. The activity of monoamine oxidases, especially MAO-A, is increased by Angiotensin II through the AT1 receptor in kidneys [21]. On the other hand, MAO activity also reduces the availability of dopamine. Therefore, the reduction of intrarenal dopamine activity by the renin-angiotensin system suggests that it may be one of the pathophysiological mechanisms of DN in diabetics. In fact, increased renal MAO-A activity and nephropathy findings in diabetic rats treated with AT1 receptor antagonist losartan have returned to normal [17].

Dopamine is mainly produced by proximal tubule cells in the kidneys independently of the nerves and regulates renal functions such as sodium excretion and glomerular filtration [24]. Another important effect of dopamine is activation of antioxidant enzymes while inhibiting pro-oxidant enzymes and thus to reduce oxidative stress [25]. Studies based on the renoprotective effects of intrarenal dopamine have shown that when dopamine levels in the kidneys are raised, nephropathy can be prevented very effectively. According to Mikusic et al., the renal dopaminergic system prevents the development of hypertension and renal inflammation in the experimental metabolic syndrome [26]. However, the studies have shown that intrarenal dopamine activity is low and this state may be an important physiopathological mechanism responsible for abnormal renal functions in STZ-
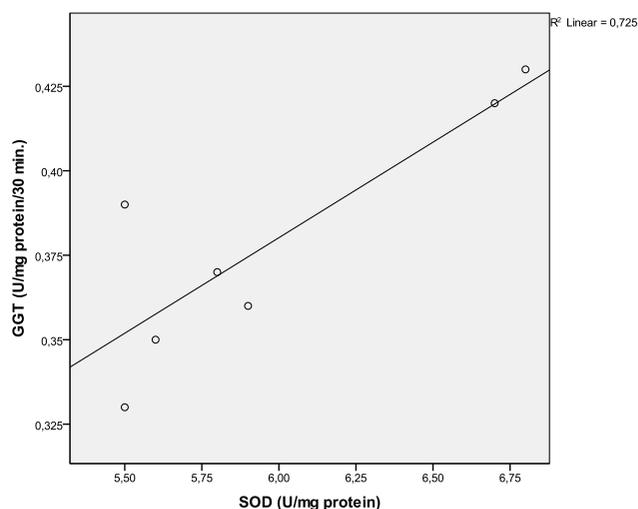

Figure 1. Correlation between renal SOD activity and urinary g-Glutamyl transpeptidase levels in DN rats:
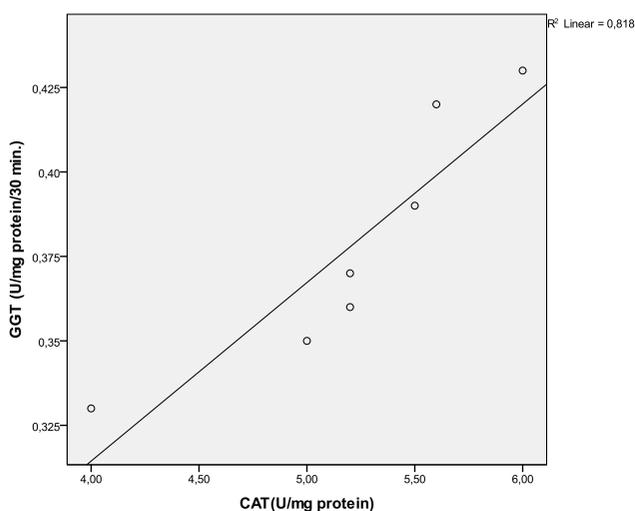r = 0.852, n = 7, p <0.05

Figure 2. Correlation between renal CAT activity and urinary g-Glutamyl transpeptidase levels in DN rats:
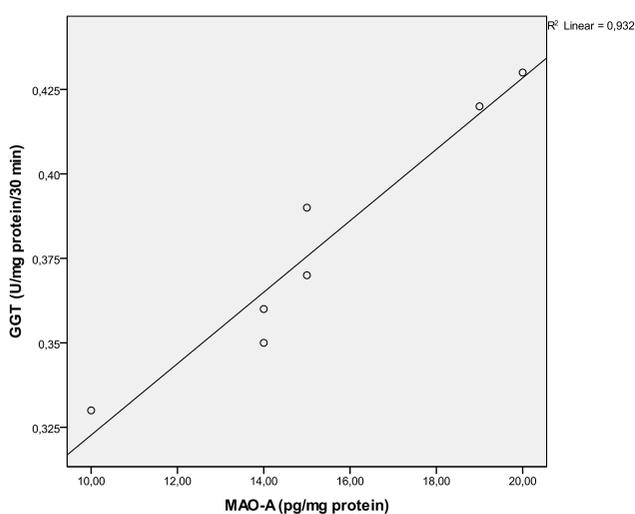r = 0.904, n= 7, p <0.01

Figure 3. Correlation between kidney MAO-A activity and urinary g-Glutamyl transpeptidase levels in DN rats:
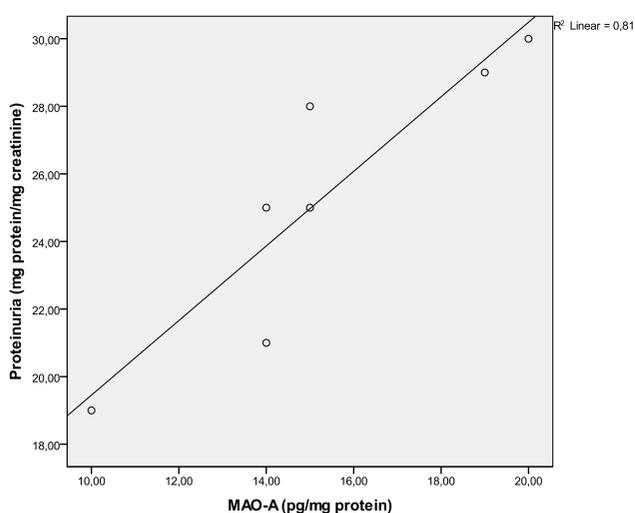r = 0.953, n = 7, p <0.01

Figure 4. Correlation between kidney MAO-A activity and proteinuria in DN rats:
r=0,90, n=7, p<0.01.

induced diabetic animals [27,28,29]. We found renal dopamine levels in diabetic rats were significantly lower than the control values. In diabetic rats treated with MAO inhibitors, intrarenal dopamine levels were elevated, nephropathy findings were significantly eliminated and renal functions improved significantly. In addition, treatment with MOA inhibitors diminished lipid peroxidation in kidney tissue of diabetic rats. However, we have found that to prevent the development or progression of nephropathy in diabetic rats by MAO-A inhibition is more effective than MAO-B inhibition.

The mitochondrial MAOs responsible for the catabolism of monamines produce reactive oxygen species in the kidneys. Experimental evidences have suggested that MAOs may be one of the important factors in many chronic pathological conditions such as DN. In this study, we found that the activity of MAO-A and MAO-B was significantly increased in the kidney of DN rats and there was a correlation between MAO-A and nephropathy findings such as high urinary GGT and proteinuria. We also demonstrated that nephropathy findings reduced significantly in diabetic rats treated with MAO-inhibitors, moclobemide or rasagiline. These results support data from studies that directly or indirectly show that long-term high levels of renal monoamine oxidases are associated with the pathogenesis of DN. [15,17,19,20,27,28,29].

In conclusion, antioxidant enzyme activities are increased as a compensatory mechanism, against to lipid peroxidation and increasing MAOs activity in kidney tissue of DN rats. However, increased antioxidant enzymes SOD and CAT in the kidney of diabetic rats were not sufficient to prevent the development and progression of DN. MAO inhibitors such as moclobemide and rasagiline can significantly reduce the severity of nephropathy symptoms in diabetic rats. Inhibition of MAO-A in diabetic rats shows more renoprotective effect than MAO-B inhibition. Therefore, MAO-A inhibition may be added to new treatment protocols to prevent nephropathy in diabetic patients.

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