

Effects of pravastatin or 12/15 lipoxygenase pathway inhibitors on indices of diabetic nephropathy in an experimental model of diabetic renal disease

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ABSTRACT

الأهداف: توضيح تأثير مرض السكري المستحدث في الفئران على وظائف الكلى وتقليل تلف الكلى الناتج عن هذا المرض وذلك باستخدام إما البرافاستاتين أو مثبطات 15/12 ليبوأكسجينيس (nordihydroguaiaretic acid (NDGA)).

الطريقة: أُجريت هذه الدراسة في مستشفى الملك خالد الجامعي، جامعة الملك سعود، الرياض، المملكة العربية السعودية وذلك خلال الفترة من نوفمبر 2010م إلى نوفمبر 2011م. وقد شمل البحث ٨٨ فأراً تم تقسيمهم إلى ١١ مجموعة بالتساوي كالآتي: المجموعة الأولى مجموعة الشاهد. والمجموعة الثانية وشملت الفئران المصابة بداء السكري ولديها نسبة إخراج الألبومين إلى الكرياتينين أقل من ٣٠ ميكروجرام/ ميلليجرام. وتم تقسيم هذه المجموعة إلى ٥ مجموعات فرعية: مجموعة لم يتم علاجها (أ)، والمجموعة التي عولجت بمثبطات NDGA (ب)، أو NDGA + أنسولين (ج)، أو البرافاستين (د)، أو البرافاستين + أنسولين (هـ). أما المجموعة الثالثة فهي الفئران المصابة بداء السكري ولديها نسبة إخراج الألبومين إلى الكرياتينين ما بين ٣٠-٢٩٩ ميكروجرام/ ميلليجرام. وتم أيضاً تقسيم هذه المجموعة إلى ٥ مجموعات فرعية: مجموعة لم يتم علاجها (أ)، أو تم علاجها بمثبطات NDGA (ب)، أو NDGA + أنسولين (ج)، أو البرافاستين (د)، أو البرافاستين + أنسولين (هـ). وبعد نهاية فترة العلاج والتي استمرت ٤ أشهر تم سحب عينات الدم من أجل تحليلها.

النتائج: أوضحت الدراسة قدرة كل من NDGA و البرافاستاتين على تقليل حدوث التلف الكلوي الناتج عن السكري وبالأخص عند التحكم بمستوى السكر بالدم باستخدام الأنسولين. وكان هذا واضحاً بنقص نسبة الألبومين إلى الكرياتينين بالبول عند الفئران التي أعطيت (NDGA) أو برافاستاتين مع عدم فرق ملحوظ بين هاتين الطريقتين في العلاج. كما حدث نقص ملحوظ في مستوى بيروكسيد الدهون، وعامل نمو الغشاء المخاطي المبطن للأوعية الدموية والهيموسيتين عند الفئران المصابة بمرض السكري سواء كان مصاحباً أو غير مصاحباً بالتلف الكلوي بعد إعطائهم (NDGA) أو برافاستاتين أيضاً مع عدم وجود فرق ملحوظ بين هاتين الطريقتين للعلاج.

خاتمة: تفيد الدراسة إمكانية استخدام (NDGA) أو البرافاستاتين لعلاج أو تقليل حدوث التلف الكلوي الناتج عن السكري خاصة في المراحل الأولى من تلف الكلى وعند التحكم في مستوى السكر بالدم.

Objectives: To attenuate the effects of early streptozotocin-induced diabetes on renal functions through supplementation with either pravastatin or 12/15-lipoxygenase pathway inhibitors.

Methods: The study was carried out at King Khalid University Hospital, Riyadh, Saudi Arabia from November 2010 to November 2011. Rats were assigned to control rats (group I) receiving vehicle; normoalbuminuric diabetic rats receiving vehicle (group IIa), nordihydroguaiaretic acid (NDGA) (group IIb), NDGA + insulin (group IIc), pravastatin (group IId) or pravastatin + insulin (group IId); and microalbuminuric diabetic rats receiving vehicle (group IIIa), NDGA (group IIIb), NDGA + insulin (group IIIc), pravastatin (group IIId) or pravastatin + insulin (group IIIe). The NDGA and pravastatin were administered for 4 months. At the end of the experiment, renal function tests were measured and blood samples were analyzed.

Results: Both NDGA and pravastatin had favorable effects on renal function to the same extent, and more favorable effects when diabetes was controlled. Indices of diabetic nephropathy (DN) and oxidative stress were reduced by NDGA or pravastatin therapy with no statistical difference between the 2 lines of therapy.

Conclusion: Pravastatin and 12/15-lipoxygenase pathway inhibitor (NDGA) have beneficial effects on streptozotocin-induced DN. The findings may provide insight into the feasibility of their clinical use as a complementary therapy for the prevention/treatment of DN.

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Diabetic nephropathy (DN) is defined as the appearance of persistent 'clinical' albuminuria in an individual with diabetes for more than 5 years and concomitant retinopathy, in the absence of urinary tract infection, other renal diseases and heart failure. People with both type 1 and type 2 diabetes are at risk. The risk is higher if blood-glucose levels are poorly controlled. Significant structural changes, particularly thickening of the glomerular basement membrane and mesangial expansion occur only after several years of diabetes.¹ At this stage, the kidney may start allowing more serum albumin than normal in the urine (microalbuminuria). As DN progresses, increasing numbers of glomeruli are destroyed by nodular glomerulosclerosis. Now the amounts of albumin being excreted in the urine increases, and may be detected by ordinary urine analysis techniques. Therefore, DN is categorized into stages: microalbuminuria (urinary albumin excretion [UAE] >20 µg/min and ≤199 µg/min) and macroalbuminuria (UAE ≥200 µg/min).

Vascular endothelial growth factor (VEGF) is a potent naturally occurring vascular permeabilizing factor and an important stimulus of physiological and pathological angiogenesis.² It has been demonstrated that VEGF controls physiological and pathophysiological conditions, such as the early stages of DN.³ The VEGF mRNA and protein are located mainly in podocytes, and VEGF receptor-2 mRNA is located in glomerular endothelial cells. The VEGF has been proposed to play a role in the development of diabetic renal changes in animal models of type 1 and 2 DM through enhancing the permeability of the glomeruli to macromolecules leading to albuminuria.²

Several studies have indicated that even moderately raised plasma homocysteine (Hcy) levels are associated with macrovascular disease in non diabetic subjects as well as in patients with diabetes, and the presence of DN is a major determinant of elevated plasma Hcy concentrations.⁴ However, the association between hyperhomocysteinemia and diabetic microangiopathy is still a controversial subject. It is increasingly evident that changes in cellular function resulting in oxidative stress play a key role in the development and progression of DN.⁵ Lipoxygenases (LOs) are a family of nonheme

iron containing enzymes that insert molecular oxygen into polyunsaturated fatty acids. The LO are classified as 5-, 8-, 12-, and 15-LOs according to the carbon atom of arachidonic acid at which oxygen is inserted. Three major isoforms of 12-LO are platelet-type 12-LO, macrophage- or leukocyte-type 12-LO (12/15-LO), and epidermal-type 12-LO. Human and rabbit 15-LOs, as well as leukocyte-type 12-LO have high homology, and are classified as 12/15.⁶ Studies have indicated the presence of leukocyte-type 12/15-LO in various cells, including vascular smooth muscle cells, brain, and kidney. In rats, both leukocyte 12/15-LO and platelet 12-LO are expressed.⁷ Glomerular 12/15-LO is found to increase in streptozotocin (STZ)-induced diabetic rats associated with an early increase in glomerular activated nuclear transcription factor cyclic-adenosine monophosphate-responsive binding protein, thereby implicating the 12/15-LO pathway in the pathogenesis of the expanded mesangial matrix characteristic of DN. However, the effect of 12/15-LO inhibition *in vivo* on key indices of DN and its relation to inflammatory mediators of renal injury (as VEGF) have not yet been studied.⁷

Nordihydroguaiaretic acid (NDGA) is a lignan found in large amounts in the "Larrea tridentate" plant. Research on this compound and its natural and synthetic derivatives has shown them to be potentially useful in the treatment of cancer, diabetes, viral, and bacterial infections.⁸ The NDGA is a potent LO inhibitor that specifically inhibits the leukocyte 12/15-LO pathway.⁷ Three-hydroxy 3-methylglutaryl (HMG)-Co-enzyme A (CoA) reductase inhibition also has been shown to provide protection against a variety of renal diseases characterized by inflammation or enhanced cellular proliferation.⁹ Many of the beneficial effects occur independently of cholesterol lowering effects because they can alter cellular proliferation/apoptosis, reduce reactive oxygen species generation, and inhibit neutrophil and macrophage recruitment. More important is their action increasing expression and activity of endothelial nitric oxide synthase (eNOS), even under hypoxic conditions.¹⁰ In light of these findings, the aim of this study is to evaluate the effects of early STZ-induced diabetes on renal functions, and to attenuate the diabetic renal inflammatory and vasomotor injury through supplementation with either pravastatin or NDGA, which specifically inhibits the leukocyte 12/15-LO pathway. We also hope to gain insight into the possible beneficial effects of pravastatin and NDGA in regulation of renal function, and so their use as therapeutics for DN.

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Methods. The current study was carried out on 88 male Wistar rats supplied by the Medical College animal house at King Khalid University Hospital, Riyadh, Saudi Arabia from November, 2010 to November, 2011. The rats were 13-18 weeks old, and their average weight was 250-300 g. They were housed in a controlled environment with free access to water ad libitum. To induce diabetes, rats were injected with STZ 65 mg/kg intraperitoneally.¹¹ The development of diabetes was confirmed by tail-vein blood glucose levels (glucometer) on the third day after STZ injection. Rats whose blood glucose levels were between 300-500 mg/dl were selected.

Over 3 consecutive days, 24-hour urine samples were collected and centrifuged at 3000 g for 10 minutes. Urinary albumin excretion was measured. Urinary creatinine was measured in the same sample. To adjust for the variability of urine collection, the urinary albumin to creatinine ratio (ACR) was measured in each sample.² Another urine sample was taken after 4 months. Urinary albumin and creatinine were measured in each sample for selection of normoalbuminuric and microalbuminuric rats that were divided according to the following categories: 1. Non-diabetic control rats with normal ACR. Those rats received neither STZ nor any medication. 2. Normoalbuminuric diabetic rats with urinary ACR <30 µg/mg. 3. Microalbuminuric diabetic rats with urinary ACR in the range of 30-299 µg/mg in at least 2 urine samples. The microalbuminuric group was defined as a DN group.³ Male rats were assigned to 3 groups: group I: included 8 control rats receiving vehicle. Group II (8 rats/subgroup): included normoalbuminuric diabetic rats receiving vehicle (IIa), NDGA (IIb), both NDGA and insulin (IIc), pravastatin (IIId), or both pravastatin and insulin (IIe). Group III (8 rats/subgroup): included microalbuminuric diabetic rats receiving vehicle (IIIa), NDGA (IIIb), both NDGA and insulin (IIIc), pravastatin (IIId), or both pravastatin and insulin (IIIe). All the drugs and chemicals were supplied from Sigma (St. Louis, MO, USA).

Long acting insulin (ultralente) was administered subcutaneously at a dose of 3-4 U/day immediately after induction of diabetes and continued up to the end of the study (for 4 months), namely until sacrificed.¹² The dosage of insulin was adjusted on the basis of daily blood glucose determination in an effort to maintain blood glucose concentration below 13 mmol/l during the course of the experiment. Blood samples were taken from the rat-tail vein and glucose level was measured using a glucometer. The NDGA was injected subcutaneously 5 mg/kg, daily for 4 months.¹³ Pravastatin was gavaged at a dose of 0.4 mg/kg in dilution of normal saline

daily for 4 months.¹⁴ Some renal function tests were measured including urine volume, creatinine clearance as a measure of GFR,¹⁵ and urinary ACR.¹⁶

Blood samples were analyzed for glycosylated hemoglobin (HbA1c). Serum samples were used to measure the levels of cholesterol and triglycerides using the colorimetric method,¹⁷ total nitric oxide (NO) products as the sum of nitrite and nitrate (R&D System Inc. Minneapolis, Minnesota, USA),¹⁸ and lipid peroxide (Oxford Biomedical Research, Rochester Hills, Michigan, USA).¹⁹ Plasma samples were used for measuring the levels of VEGF (R&D Systems, Minneapolis, Minnesota USA),²⁰ and homocysteine (Hcy) (Axis-Shield Diagnostics Ltd., Dundee, United Kingdom).²¹

Statistical analysis. Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) program for windows version 18. Data were expressed as mean±SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) with 95% confidence intervals. Differences were considered to be statistically significant at $p<0.05$.

Results. A summary of the results of the present study is shown in Tables 1 and 2, and in Figures 1-4. Non treated, NDGA, and pravastatin treated normo- and microalbuminuric diabetic rats showed a significant increase in urine volume as compared to controls, NDGA+insulin, and pravastatin+insulin treated normo-, and microalbuminuric diabetic rats. Moreover, a significant increase was found in non treated, NDGA, and pravastatin treated microalbuminuric rats as compared to non treated, NDGA, and pravastatin treated normoalbuminuric rats.

The creatinine clearance significantly decreased in all non treated, and treated normo- and microalbuminuric diabetic rats as compared with controls. Insulin treatment in combination with either NDGA, or pravastatin in normo- and microalbuminuric rats (groups IIc, IId, IIe, IIIc, IIId, IIIe) increased creatinine clearance as compared with treatment by NDGA or pravastatin alone both in normo- and microalbuminuric rats (groups IIb, IIId, IIId, IIId). While no significant changes were found between NDGA+insulin and pravastatin+insulin treated diabetic rats. In addition, urinary ACR was significantly increased in all microalbuminuric rats as compared with controls and normoalbuminuric rats. Treatment of microalbuminuric rats with either NDGA, NDGA+insulin, pravastatin, or pravastatin+insulin significantly decreased urinary ACR compared with non treated microalbuminuric rats.

Table 1 - Mean values±SD of the measured parameters in controls (group I), normoalbuminuric rats: non treated (groups IIa), NDGA treated (group IIb), NDGA+insulin treated (group IIc), pravastatin treated (group IId) and pravastatin+insulin treated rats (group IId) and microalbuminuric rats: non treated (groups IIIa), NDGA treated (group IIIb), NDGA+insulin treated (group IIIc), pravastatin treated (group IIId), and pravastatin+insulin treated rats (group IIId).

Groups	Urine volume (ml/hour)	Creatinine clearance (ml/min)	Urinary albumin to creatinine ratio (µg/mg)	HbA1c (%)	Cholesterol (mg/dl)
Group I (controls)	0.693±0.047	96.13±5.22	10.13±2.696	3.90±0.43	207.8±15.15
Group IIa (non treated)	2.245±0.158*	56.63±6.30*	12.63±3.378	5.33±0.40*	259.5±18.02*
Group IIb (NDGA treated)	2.228±0.130*	63.50±8.05*	11.75±2.915	5.33±0.31*	260.4±19.27*
Group IIc (NDGA+insulin)	0.681±0.063	81.38±6.32*	11.75±2.435	3.98±0.62	261.3±19.75*
Group IId (statin treated)	2.276±0.136*	67.63±7.35*	11.75±2.435	5.33±0.50*	212.6±12.44
Group IId (statin+insulin)	0.680±0.074	81.50±6.91*	11.13±2.232	4.03±0.51	210.9±13.11
Group IIIa (non treated)	2.596±0.159	45.25±6.45*	238.3±30.64*	6.08±0.40*	260.0±19.71*
Group IIIb (NDGA+insulin)	2.599±0.168*	55.88±10.22*	169.3±11.95*	5.88±0.59*	261.0±19.58*
Group IIIc (NDGA+insulin)	0.690±0.052	70.63±2.93*	172.6±11.89*	3.95±0.57	262.0±19.25*
Group IIId (statin treated)	2.603±0.150*	61.00±6.41*	173.4±9.456*	6.01±0.57*	210.6±13.56
Group IIId (statin+insulin)	0.686±0.049	74.13±3.44*	173.4±9.456*	4.01±0.54	209.4±13.95
F-value	484.21	37.627	508.11	26.518	19.352
P-value	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*

* Significance was considered at $p<0.05$, 95% confidence interval. NDGA - nordihydroguaiaretic acid, HbA1c - glycosylated hemoglobin

Table 2 - Mean values ± SD of the measured parameters in controls (group I), normoalbuminuric rats: non treated (groups IIa), NDGA treated (group IIb), NDGA+insulin treated (group IIc), pravastatin treated (group IId), and pravastatin+insulin treated rats (group IId) and microalbuminuric rats: non treated (groups IIIa), NDGA treated (group IIIb), NDGA+insulin treated (group IIIc), pravastatin treated (group IIId), and pravastatin+insulin treated rats (group IIId).

Groups	Triglycerides (mg/dl)	NO (µmol/l)	Lipid peroxide (nmol/l)	VEGF (pg/ml)	Hcy (µmol/l)	TM (ng/ml)
Group I (controls)	105.6±5.476	13.81±2.635	2.214±0.430	116.1±7.568	9.675±1.557	16.98±2.168
Group IIa (Non treated)	123.3±7.324*	30.76±6.238*	5.330±0.484*	153.5±6.969*	13.938±2.755*	18.66±2.063
Group IIb (NDGA treated)	123.1±5.515*	20.59±3.132	2.293±0.434	114.6±7.130	9.450±1.759	18.78±2.222
Group IIc (NDGA+insulin)	123.8±6.563*	14.46±2.646	2.426±0.378	117.1±7.140	9.950±1.759	18.30±2.214
Group IId (Statin treated)	106.1±7.434	20.61±3.048	2.301±0.468	116.3±4.862	9.175±1.843	18.74±1.380
Group IId (Statin+insulin)	105.5±8.000	14.65±2.505	2.401±0.434	117.8±6.065	9.775±1.843	18.58±1.789
Group IIIa (Non treated)	125.0±7.309*	53.11±6.559*	7.154±0.374*	173.3±7.066*	15.363±2.319*	25.80±2.126*
Group IIIb (NDGA treated)	124.1±6.379*	30.33±4.323*	2.373±0.437	115.6±7.501	9.550±1.462	18.46±2.327
Group IIIc (NDGA+insulin)	124.3±5.898*	15.92±2.842	2.529±0.373	117.5±7.690	10.05±1.462	18.91±2.093
Group IIId (Statin treated)	106.1±9.062	30.86±4.853*	2.381±0.454	118.5±4.567	9.388±1.860	18.85±2.171
Group IIId (Statin+insulin)	105.0±11.588	16.17±3.135	2.500±0.408	120.5±4.781	9.975±1.849	18.26±2.187
F-value	12.893	68.474	115.05	68.636	9.437	9.581
P-value	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*

* Significance was considered at $p<0.05$, 95% confidence interval, NDGA - nordihydroguaiaretic acid, NO - nitric oxide, VEGF - vascular endothelial growth factor, Hcy - homocysteine, TM - thrombomodulin

Concerning blood HbA1c, a significant increase was detected in non treated, NDGA, and pravastatin treated normo- and microalbuminuric diabetic rats as compared with controls, and those rats treated by insulin concomitantly with either NDGA or pravastatin both normo- and microalbuminuric. Also, serum cholesterol

and triglycerides showed significant increases in normo- and microalbuminuric (non treated, NDGA treated, NDGA+insulin treated) diabetic rats as compared with controls, normo- and microalbuminuric (pravastatin treated and pravastatin+insulin treated) diabetic rats with no statistical difference between normo and microalbuminuric rats.

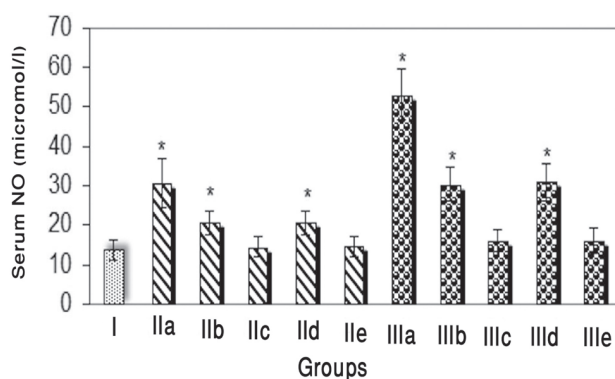


Figure 1 - Mean values \pm SD of serum total nitric oxide (NO) products (μ mol/l) in controls (group I), normoalbuminuric rats: non treated (groups IIa), NDGA treated (group IIb), NDGA+insulin treated (group IIc), pravastatin treated (group IId) and pravastatin+insulin treated rats (group IIe) and microalbuminuric rats: (non treated (groups IIIa), NDGA treated (group IIIb), NDGA+insulin treated (group IIIc), pravastatin treated (group IIId) and pravastatin+insulin treated rats (group IIle).

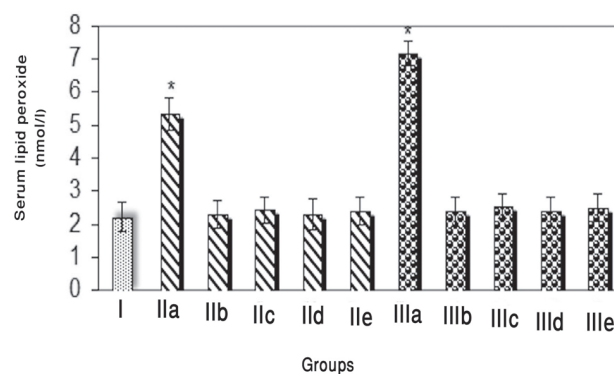


Figure 2 - Mean values \pm SD of serum total nitric oxide (NO) products (μ mol/l) in controls (group I), normoalbuminuric rats: non treated (groups IIa), NDGA treated (group IIb), NDGA+insulin treated (group IIc), pravastatin treated (group IId) and pravastatin+insulin treated rats (group IIe) and microalbuminuric rats: (non treated (groups IIIa), NDGA treated (group IIIb), NDGA+insulin treated (group IIIc), pravastatin treated (group IIId) and pravastatin+insulin treated rats (group IIle).

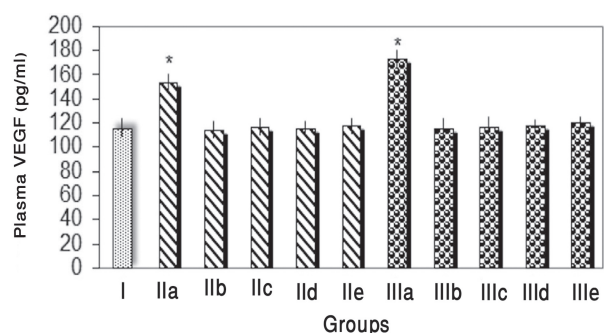


Figure 3 - Mean values \pm SD of serum total nitric oxide (NO) products (μ mol/l) in controls (group I), normoalbuminuric rats: non treated (groups IIa), NDGA treated (group IIb), NDGA+insulin treated (group IIc), pravastatin treated (group IId) and pravastatin+insulin treated rats (group IIe) and microalbuminuric rats: (non treated (groups IIIa), NDGA treated (group IIIb), NDGA+insulin treated (group IIIc), pravastatin treated (group IIId) and pravastatin+insulin treated rats (group IIle).

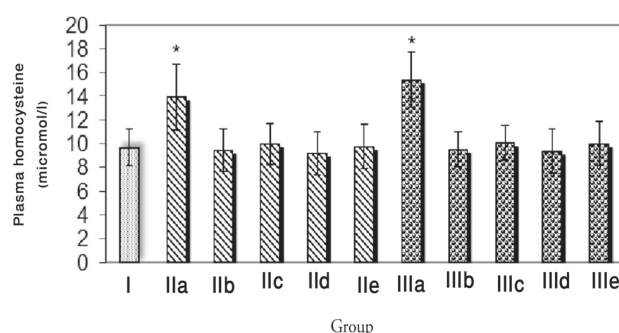


Figure 4 - Mean values \pm SD of plasma homocysteine (μ mol/l) in controls (group I), normoalbuminuric rats: non treated (groups IIa), NDGA treated (group IIb), NDGA+insulin treated (group IIc), pravastatin treated (group IId) and pravastatin+insulin treated rats (group IIe) and microalbuminuric rats: (non treated (groups IIIa), NDGA treated (group IIIb), NDGA+insulin treated (group IIIc), pravastatin treated (group IIId) and pravastatin+insulin treated rats (group IIle).

Regarding serum NO, this was significantly increased in non treated microalbuminuric rats (groups IIIa) as compared with controls, non treated normoalbuminuric rats, as well as NDGA, NDGA+insulin, pravastatin, pravastatin+insulin treated normo- and microalbuminuric rats. Moreover, insulin treatment concomitantly with either NDGA or pravastatin in both normo- and microalbuminuric rats significantly decreased serum NO as compared with microalbuminuric rats treated only by either NDGA or pravastatin.

Concerning serum lipid peroxide, plasma VEGF, and Hcy, the results are similar in all groups. Their levels were significantly increased in non treated rats, whether normo- or microalbuminuric (groups IIa, IIIa) as compared with controls, NDGA, NDGA+insulin, pravastatin, pravastatin+insulin treated normo- and microalbuminuric rats. However, no significant changes could be detected between those rats treated by NDGA or pravastatin separately or concomitantly with insulin and whether normo- or microalbuminuric.

Group IIIa showed a significant positive correlation between UAE and each of Hcy ($p=0.004$) and TM

($p=0.006$). The NDGA and pravastatin treated rats (group IIIb, IIIc) showed similar correlation results where there were significant positive correlations between UAE and each of HbA1c, triglycerides, lipid peroxide, VEGF, Hcy, and TM ($p=0.024, 0.000, 0.000, 0.035, 0.002$ and 0.004 for group IIIb, $p=0.007, 0.006, 0.001, 0.018, 0.006$ and 0.001 for group IIIc). In group IIIc (NDGA+insulin treated rats), UAE correlated positively with each of cholesterol ($p=0.012$), triglycerides ($p=0.039$), and lipid peroxide ($p=0.002$). Moreover, pravastatin+insulin treated rats showed significant positive correlations between UAE and each of triglycerides ($p=0.007$), lipid peroxide ($p=0.001$), VEGF ($p=0.000$), and Hcy ($p=0.001$).

Discussion. Diabetic nephropathy is characterized clinically by the development of albuminuria (a marker of glomerular injury) and proteinuria.²² Substantial evidence suggests that 12/15-LO and its products of arachidonic acid metabolism play an important role in systemic homeostasis and renal-cardiovascular pathology,²³ through mediating growth factor effects in vascular smooth muscle cells, fibroblasts, and mesangial cells, as well as responses to vascular injury.¹¹ Deletion of the 12/15-LO gene is associated with reduced atherosclerosis in animal models. High glucose levels have been shown to directly increase 12/15-LO expression in cultured mesangial cells.

The 12/15-LO pathway has been shown to be a critical mediator of angiotensin II-induced mesangial cell hypertrophy and extracellular matrix accumulation.²⁴ According to the aforementioned information, many studies contribute LO-derived products to the pathogenesis of diabetic complications including DN.²⁵ Therefore, 12/15-LO inhibition by NDGA may be useful as a therapeutic strategy for prevention or amelioration of DN as shown by improvement of renal function, decreased urinary ACR, and indices of DN in microalbuminuric rats treated by NDGA separately or concomitantly with insulin.

Patel et al²⁶ reported a reduction of renal ischemic reperfusion injury in mice by 5-LO inhibitor. In addition, Anjaneyulu et al,¹³ observed that treatment with NDGA in diabetic rats significantly prevented renal dysfunction, oxidative stress, and renal morphological alterations as compared with vehicle-treated diabetic rats. In addition to 12/15 LO inhibitors, statins may have beneficial effects on STZ-induced DN. Administration of pravastatin to STZ-induced diabetic rats used in this study improved renal function, decreased urinary ACR, and indices of DN in microalbuminuric rats. Moreover, it decreased cholesterol and triglyceride levels. This is in agreement with the study of Gojo and Fried et al.^{27,28} In

fact, dyslipidemia has been found to be an important predictor of renal function loss and progression of albuminuria. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase by statins leads to multiple proposed 'pleiotropic' mechanisms of action that may affect progression of renal disease independent of their lipid-lowering effect. First, statins inhibit mesangial cell and leukocyte expression of inflammatory chemokines. Second, statins can inhibit the proliferation of mesangial cells, renal tubular cells, and vascular smooth muscle cells.²⁹ Third, they have an important effect on intracellular signaling and the key mediators of multiple biological processes, including the regulation of cell proliferation, oxidative stress, and production of endothelial-derived vasoactive mediators. Fourth, they can improve vascular hemodynamic responses through their effect on endothelial function. Another potential benefit of statins is their antioxidant effect. Given the important role of reactive oxygen species in mediating the microvascular responses to hyperglycemia, this may be another relevant effect of statins.

In a study, which included patients with diabetes, increases in plasma creatinine levels were significantly smaller in patients treated with statins compared with placebo.³⁰ Another experimental study on rats suggested that statins ameliorate DN by its pleiotropic effects. Although the precise mechanism involved in the statin effect on extracellular matrix proteins at the cellular and molecular level is not known, it appears that statins lead to an increase in extracellular matrix-degrading enzymes and reduce accumulation of extracellular matrix proteins.³¹

In this study, HbA1c levels increased in non treated microalbuminuric rats as compared with controls. Neither NDGA nor pravastatin could affect HbA1c levels, while combined insulin+NDGA, or insulin+pravastatin treatment of diabetic rats could normalize HbA1c levels. The study of Araszkievicz et al³² showed that subjects with positive microalbuminuria had higher values of HbA1c in addition to triglycerides, and VEGF than patients without microalbuminuria. In the advanced stage of renal disease, better glycemic control has been suggested to be associated with reducing the risk of death from diabetic complications.³³

The field of VEGF has recently witnessed a surge of research into its role in diabetic kidney disease, and in development of DN. In the early diabetic subjects with microalbuminuria, up-regulation of VEGF, and the expression of its receptor can be markedly increased. Podocyte-derived VEGF is believed to participate in the glomerular capillary hyperpermeability of macromolecules that potentially underlies the pathogenesis of diabetic albuminuria.³⁴ Elevated VEGF

levels also cause renal hypertrophy, increase glomerular permeability, and GFR.³⁵ Our results revealed that treatment of diabetic rats with NDGA or NDGA+insulin normalized plasma VEGF level to the same extent as pravastatin or pravastatin+insulin treatment.

Several reports suggest the enhanced generation or actions of NO in the pathogenesis of preferential afferent arteriolar dilatation, glomerular hyperfiltration and hyperperfusion, and glomerular enlargement that occurs in the early stages of DN. It is reasonable to assume that the higher blood levels and urinary NO excretions are the results of a generalized increase in the synthesis of NO throughout the body. Furthermore, it is suggested that the endothelial dysfunction observed in diabetic subjects results mainly from impaired action of NO accompanying up-regulation of NO production secondary to enhanced inactivation of NO. The significant increase in serum NO among non treated microalbuminuric diabetic rats, compared with control rats, and non-treated normoalbuminuric rats, strongly suggested that high levels of NO might play a role in increasing albumin excretion. Thus, NO overproduction, which is thought to be due to an increased filtration fraction, providing a driving force for albumin infusion into the Bowman's space, is thought to play a prominent part in the pathogenesis of microalbuminuria.³⁶ However, a high level of NO may reflect tissue attempt to overcome vascular dysfunction. We support the suggestion that hyperglycemia may be responsible for the increased NO levels in diabetic rats as evidenced by the normalized serum NO level in microalbuminuric rats treated with NDGA+insulin or pravastatin+insulin.

Although hyperhomocysteinemia confers its association with diabetic vascular complications, studies concerning the total Hcy levels in various types of diabetics have revealed conflicting results. Furthermore, its etiology and relationship with nephropathy is equivocal. Homocysteine is not usually present as a direct result of type 2 DM unless there is an associated development of impaired renal function. As nephropathy develops, there is an associated elevation of total Hcy associated with a decline in GFR.³⁷ The study of Agulló-Ortuño et al³⁸ demonstrated that patients with type 1 DM with complications had higher plasmatic Hcy concentrations than those without complications, and that a relationship between high Hcy levels and prevalence of nephropathy was found. The precise kinetic mechanism(s) leading to the hyperhomocysteinemia of DN is unknown. It may be caused by increased production, decreased removal, or both.³⁹ The degradation in renal tissue following tubular reabsorption of Hcy is a major fraction of

total plasma Hcy in the rats. It was reported that the reduced metabolic capacity of kidney tissue might be rate limiting for renal total Hcy clearance, and might cause elevation of total Hcy seen in human end stage renal disease.⁴⁰ Our results of decreased plasma Hcy level in microalbuminuric rats are in agreement with other studies.⁴¹

Lipid peroxidation, owing to free-radical activity, plays an important role in the development of complications of diabetes.⁴² Statins have been proven to have antioxidant effects via elimination of free radicals directly, blockage of synthesis of mediators important for post-translational modifications of proteins, or promotion of synthesis of nuclear factor that upregulates paraoxonase promoter activity to protect low density proteins from oxidative stress.⁴³

The present study showed that not only pravastatin and pravastatin+insulin treatment of diabetic rats could improve serum lipid peroxide level, but also NDGA and NDGA+insulin could normalize serum lipid peroxide in diabetic rats. This proves that both NDGA and statins have antioxidant properties that can improve DN.

Study limitation. The sample size is small. The starting number was more than the number mentioned in the method sections, due to the prolonged period of diabetes (4 months before and 4 months after treatment), some of the rats died before the completion of treatment). Moreover, we have only limited budget.

In conclusion, pravastatin and the 12/15 LO inhibitor, NDGA, have beneficial effects on STZ-induced DN by improvement of renal function, decreasing urinary ACR, and indices of DN in microalbuminuric rats. The findings may provide insight into the feasibility of clinical use of pravastatin or 12/15-LO pathway inhibitors as a complementary therapy for the prevention/treatment of DN. Despite the possible theoretic benefit, long-term, prospective clinical trials are needed before clarifying the medical and, perhaps, economic benefits of statins or 12/15-LO inhibitors for DN.

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