Effects of Advanced Glycation End-Product Inhibition and Cross-Link Breakage in Diabetic Rats

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The accelerated formation of advanced glycation end-products (AGEs) due to elevated glycemia has repeatedly been reported as a central pathogenic factor in the development of diabetic microvascular complications. The effects of a novel inhibitor of AGE formation, NNC39-0028 (2,3-diaminophenazine), and a breaker of already formed AGE cross-links, *N*-phenacylthiazolium bromide (PTB), were investigated in streptozotocin-diabetic female Wistar rats. Diabetes for 24 weeks resulted in decreased tail collagen pepsin solubility, reflecting the formation of AGE cross-linking. Collagen solubility was significantly ameliorated by treatment with NNC39-0028, whereas PTB had no effect. Increased urinary albumin excretion (UAE) in diabetic rats was observed in serial measurements throughout the study period, and was not reduced by any treatment. Vascular dysfunction in the eye, measured as increased clearance of ^{125}I-albumin, was induced by diabetes. NNC39-0028 did not affect this abnormality. This study demonstrated a pharmacological inhibition of collagen solubility alterations in diabetic rats without affecting diabetes-induced pathophysiology such as the increase in UAE or albumin clearance. Treatment with PTB, a specific breaker of AGE cross-links, had no effects in this study.

NONENZYMATIC GLYCATION and subsequent advanced glycation end-product (AGE) formation inevitably occur during chronic diabetes and have been suggested as a primary cause of diabetic late complications.[1] Increased amounts of AGE-modified molecules, measured by immunochemical methods or methods based on the fluorescence properties of some AGEs, have been demonstrated in diabetic patients.[2-5]

Different pathways for the involvement of AGE formation in the pathogenesis of diabetic late complications have been suggested. Activation of cell surface receptors specific for AGE-modified proteins leads to cytokine activation, and AGE formation on extracellular matrix collagen results in protein cross-linking.[6,7] Thus, AGE formation may contribute to the altered biochemical and functional properties of the vessel wall such as thickening of the capillary basement membrane, trapping of serum proteins, and increasing vessel wall stiffness and permeability.[8-11] Collagen solubility as assayed by pepsin digestion, which serves as an indirect measure of cross-linking, is decreased in both experimental and human diabetes.[12-14] Pharmacological inhibition of AGE formation until now has been obtained primarily by treatment with the hydrazine compound aminoguanidine.[13,15] Aminoguanidine restores the decreased pepsin digestibility of collagen in diabetic rats.[13,14] In experimental diabetes, aminoguanidine treatment has demonstrated beneficial effects on retinopathy, nephropathy, and neuropathy.[15-17] However, the interpretation of data from intervention studies with aminoguanidine has been complicated by the fact that aminoguanidine, besides being an AGE inhibitor, is an inhibitor of nitric oxide synthase (NOS).[18,19] Alterations in nitric oxide activity may also be involved in the pathogenesis of diabetic vascular complications through its effects on blood flow and vasodilatation.[20]

The breaking of already formed AGE cross-links as a pharmacological approach directed against a further worsening of the microvascular complications of diabetes has recently been suggested. The thiazolium derivative, *N*-phenacylthiazolium bromide (PTB), cleaved collagen AGE cross-links in vitro in tail fiber material isolated from diabetic rats, and in vivo treatment with PTB decreased immunoglobulin G binding to red blood cells.[21]

In this study, we aimed to link the in vivo inhibition of AGE formation measured as collagen solubility with diabetic pathophysiology monitored as albuminuria and regional vascular dysfunction. We achieved this by investigating the effects of the cross-link breaker, PTB, and a novel selective inhibitor of AGE formation, NNC39-0028 (2,3-diaminophenazine), in streptozotocin-diabetic rats.

MATERIALS AND METHODS

Animal Model

The study was performed according to the European Community guidelines for the use of experimental animals and was approved by the National Authorities for Animal Experiments. Female Wistar rats aged 6-weeks were purchased from Møllegaard Breeding & Research Centre (Skensved, Denmark). After a 7-day acclimatization period, diabetes was induced by a single intravenous injection of streptozotocin (Sigma Chemical, St. Louis, MO) 65 mg/kg after an overnight fast. Rats demonstrating a body weight loss and a blood glucose (BG) level above 18 mmol/L after 2-days were included in the study. The diabetic animals were randomly allocated to a control group and 2 groups treated with either NNC39-0028 or PTB. Rats that did not receive streptozotocin served as normal controls. The rats were housed in groups of 4 per cage with a 12-hour artificial light cycle and free access to drinking water and standard rat chow (Altromin 1320; Altromin, Lage, Germany). No insulin was administered to the animals. In the NNC39-0028-treated group, the active compound was added to the drinking tap water at a concentration of 0.06 mg/mL. The animals had unlimited access to the drinking water, which was prepared once every week. The retrospectively calculated mean dose of NNC39-0028 was 28 mg/kg/d (range, 20 to 33). This dose is comparable to dose regimens resulting in pharmacological effects of NNC39-0028 in the retina and mesenteric vasculature of diabetic rats.[22,23] PTB dissolved at a concentration of 10 mg/mL in phosphate-buffered saline was administered as a single daily subcutane-

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ous injection of 10 mg/kg. The PTB solution was prepared every week in 7 containers, one for every day, and kept at room temperature until use. Normal and diabetic control rats and NNC39-0028-dosed rats were treated once daily with placebo, isotonic saline 2 mL/kg subcutaneously. The metabolic state was monitored by biweekly weighing and BG measurements on tail vein blood. Twenty-four-hour water intake was determined in control and orally dosed animals after 12 weeks and in all rats after 21 weeks. At 4- to 6-week intervals, the rats were placed in metabolic cages and urine was collected for 24 hours. The urine volume was determined, and urine samples were kept at −80°C until further analysis. After 24 weeks of diabetes, regional albumin clearance was measured in randomly selected subgroups of animals as described later. The remaining rats were killed and the kidneys were removed, decapsulated, and weighed. The most proximal 3-cm piece of the tail was cut and frozen on dry ice and kept at −80°C until further analysis.

Laboratory Analyses

Glucose was determined by the glucose oxidase method on an EBIO 6666 autoanalyzer (Eppendorf, Hamburg, Germany). Urine albumin concentrations were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) using goat anti-rat antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) as the catching antibody and the same antibody conjugated with peroxidase as the detecting antibody. Rat albumin was used as the standard. The detection limit of the assay was 10 ng/mL. The intraassay and interassay coefficient of variation was 7% and 14%, respectively. All analyses were performed in duplicate.

Regional Albumin Clearance

Albumin clearance in eye and skin was measured in rats from the 2 control groups and the NNC39-0028-treated group using a double-isotope dilution technique.[19,24,25] ^{125}I- and ^{131}I-human serum albumin ([HSA] Isotopapoteket, Copenhagen, Denmark) were used as markers for the tissue uptake and blood vessel content of albumin after intravenous injection of the tracers. Prior to the clearance measurements, the animals were anesthetized with halothane inhalation and Tygon catheters (Norton Performance Plastics, Akron, OH) were implanted in the right carotid artery and jugular vein. The catheters were exteriorized in the neck and filled with 30% PVP K6 (polyvinylpyrrolidone; Fluka, Buchs, Switzerland) and 3.75% trisodium citrate dihydrate (Merck, Darmstadt, Germany) in saline. Three days after surgery, the animals were placed in restraining cages. After drawing an initial arterial blood sample (plasma 0), 0.1 MBq ^{125}I-HSA was injected into the venous catheter (time 0). Additional blood samples were taken at 4 minutes and 10 minutes (plasma 4 and 10) followed by injection of 0.1 MBq ^{131}I-HSA at 10.5 minutes. At 12 minutes, a final arterial blood sample was taken (plasma 12) and the rat was killed by intravenous injection of 100 mg/kg pentobarbital. Tissue samples were immediately excised and weighed. Plasma and tissue sample tracer activities were determined in a gamma counter (Packard, Meriden, CT) and normalized to plasma and tissue weight. Assuming that ^{125}I tissue activity represents extravasated in addition to intravascular albumin and ^{131}I activity represents intravascular tracer content alone, the regional clearance of albumin was calculated as clearance $= {}^{125}I_{extravascular}/AUC - {}^{125}I_{plasma}$, where ${}^{125}I_{extravascular} = {}^{125}I_{tissue} - ({}^{131}I_{tissue} \times [{}^{125}I_{plasma\text{-}12}/{}^{131}I_{plasma\text{-}12}])$ and $AUC - {}^{125}I_{plasma} \approx 12\ min \times {}^{125}I_{plasma\text{-}average(4,10,and12)}$.[19,24] Tissue clearance is given as nanoliters of plasma per gram of tissue per minute.

Tail Tendon Collagen Pepsin Solubility Assay

A previously published method was used with various modifications.[14] After thawing, all visible tail tendon material was removed and washed 3 times on a Millipore HAWP 0.45-µm filter (Millipore, Bedford, MA) with 0.9% NaCl (total 100 mL). The material was dried on the filter and cut into small pieces. These procedures were performed at 20°C as quickly as possible. The material was then kept at −20°C until further analysis.

Five-milligram aliquots of the dried material, all in duplicate, were preincubated in 1 mol/L acetic acid for 1 hour at 4°C by gently shaking. A limited digestion with pepsin was performed for 1 hour at 4°C by the addition of a pepsin solution (20 mg pepsin/mL, 1 mmol/L acetic acid), resulting in 5 µg pepsin/mg tail fibers. After centrifugation at 40,000 × *g* for 1 hour at 4°C, the collagen concentration was determined in the supernatant (digestible collagen). After vacuum-drying to remove acetic acid, the pellet was placed in a second digest buffer (20 mmol/L *N*-ethylmorpholin and 10 mmol/L $CaCl_2$, pH 7.5) for 1 hour at 4°C followed by the addition of collagenase (1 µg/mL final concentration). A pH adjustment to pH 7.5 by the use of 100 mmol/L *N*-ethylmorpholin and 10 mmol/L $CaCl_2$ was necessary. The digest was continued overnight, and only a small amount (<0.1% wt/wt of aliquot) of nonsolubilized material was removed after centrifugation as described before. The pellet had no collagen content, and thus the collagen content of the supernatant represented the nondigestible part. Quantification of collagen was performed by total hydrolysis with 6 mol/L HCl and determination of the total amount of amino acids. The measurement of fluorescence (excitation at 420 nm and emission at 490 nm, LS 50 fluorometer; Perkin-Elmer, Norwalk, CT) was made after reaction of the liberated amino acids with naphthalenedialdehyde and KCN.[26] A HCl-hydrolyzed sample of commercial rat tail collagen was used as standard for the fluorometric method. As an additional control, several of the hydrolyzed samples were analyzed by normal amino acid analysis on a 121 MB microcolumn analyzer (Beckman Instruments, Fullerton, CA) to verify the quantification and amino acid composition of the collagen. Collagen pepsin solubility is expressed as the amount of collagen in the supernatant after the first pepsin digestion step relative to the total collagen content including collagen liberated from the second digest with collagenase.

Statistical Methods

Data are presented as the mean ± SD or as the median (interquartile range) for non–normally distributed data. Due to the skewed distribution of the data for urinary albumin excretion (UAE), the data were logarithmically transformed to obtain a normal distribution before statistical analysis. UAE data are presented as the geometric mean ×/÷ antilog SEM. Group comparisons were performed with the *t* test for independent samples or the Mann-Whitney rank-sum test. The Spearman correlation coefficient was used to test the correlation between different parameters.

RESULTS

Metabolic Control and Water Intake

The mean BG, body weight gain, and 24-hour water intake are shown in Table 1. All diabetic groups were equally hyperglycemic at about 25 mmol/L, apart from the PTB-treated group, which had a slightly but significantly lower BG compared with the diabetic control group ($P < .05$). In accordance with this, diabetic rats gained less weight compared with the normal rats. NNC39-0028-dosed rats had a significantly smaller weight gain than the diabetic control rats. All diabetic groups drank more water than the normal rats. At 12 weeks, NNC39-0028-dosed rats had a lower water intake compared with the diabetic controls; at 21 weeks, the intake was equal among diabetic groups (Table 1).

Albuminuria and Kidney Weight

UAE is shown in Fig 1. UAE was significantly elevated in diabetic rats compared with normal rats throughout the study period. After 24 weeks of diabetes, UAE was significantly increased in NNC39-0028-treated rats compared with the diabetic control group. At all other time points, UAE did not differ between diabetic groups. UAE after 24 weeks was

Table 1. Characteristics and 24-Hour Water Intake of Rats That Were Diabetic for 24 Weeks

Parameter	Normal (n = 16)	Diabetic Control (n = 14)	Diabetic + NNC39- 0028 (n = 13)	Diabetic + PTB (n = 14)
BG (mmol/L)*	5.5 ± 0.3†	26.4 ± 2.6	25.7 ± 2.3	24.5 ± 2‡
Body weight gain (%)	45 (42-51)†	14 (7-20)	2 (−4-6)§	10 (4-20)
Water intake (mL/d)				
12 weeks	22 ± 4†	81 ± 8	62 ± 9†	—
21 weeks	20 ± 2†	78 ± 9	79 ± 10	73 ± 10
Kidney weight (g)	0.72 ± 0.07†	0.94 ± 0.12	0.98 ± 0.11	0.92 ± 0.10

NOTE. Data are the mean ± SD or the median (interquartile range).
*Average of biweekly measurements.
†$P < .001$, ‡$P < .05$, §$P < .01$ *v* diabetic control rats.

0.17 ×/÷ 1.14, 0.44 ×/÷ 1.3, 2.44 ×/÷ 1.55, and 0.4 ×/÷ 1.32 mg/24 h in normal rats and diabetic rats treated with placebo, NNC39-0028, or PTB, respectively. Wet kidney weight was significantly increased in diabetic rats and did not differ among diabetic groups (Table 1).

Regional Albumin Clearance

^{125}I-albumin clearance was measured in the normal and diabetic control groups and the NNC39-0028-treated rats. Due to the clogging of catheters or postsurgical mortality, only a few of the operated rats completed the clearance measurement. Albumin clearance in the whole eye increased by 41% in diabetic control rats (n = 3) compared with normal rats (n = 7), 120 ± 23 and 71 ± 23 nL/g/min, respectively ($P < .05$) (Fig 2). NNC39-0028 treatment (n = 4) did not significantly influence this increase (165 ± 68 nL/g/min, $P = .24$). In skin, the clearance was insignificantly increased in both diabetic groups (Fig 2).

Tail Collagen Solubility

Tail tendon collagen solubility was significantly decreased in diabetic control rats compared with normal rats (30% ± 14% and 58% ± 11%, $P < .01$) (Fig 3). Treatment with NNC39-0028 partly prevented this decrease (48% ± 3%, $P < .05$ *v* diabetic controls), whereas PTB treatment had no significant effect (36% ± 10%, $P = .41$). When including normal and diabetic control rats, mean BG levels (week 0 to 24) correlated inversely with collagen solubility ($r = -.93$, $P < .001$). When analyzing only diabetic rats, the control group and the PTB-treated group (in which collagen solubility was unaffected by treatment) together, a significant inverse correlation was still present ($r = -.61$, $P < .05$) (Fig 4).

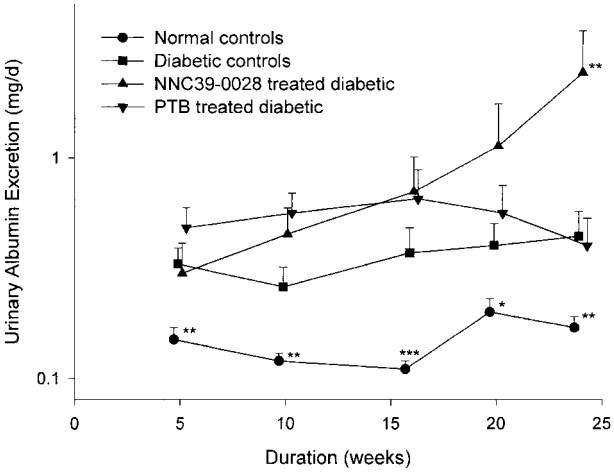

Fig 1. Urinary albumin excretion in rats measured on 24-hour urine collections during 24 weeks of diabetes. The geometric group mean × antilog SEM are shown. *$P < .05$, **$P < .01$, ***$P < .001$ *v* diabetic controls.

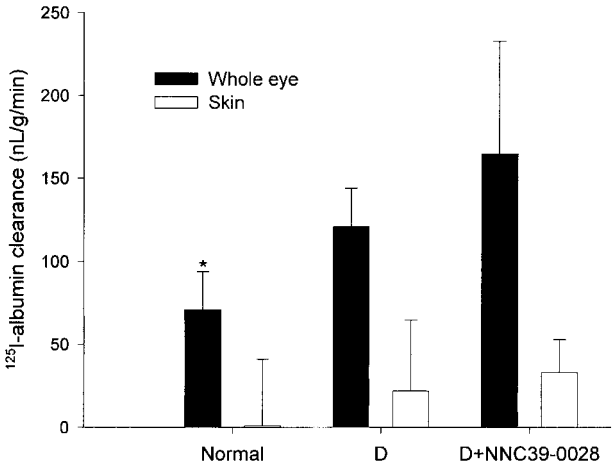

Fig 2. ^{125}I-albumin clearance in rat eye and skin after 24 weeks of diabetes. Data are the mean + SD; n = 3-7 per group. D, diabetic rats. *$P < .05$ *v* diabetic controls.

DISCUSSION

Diabetes throughout 24 weeks led to a significant decrease in tail collagen pepsin solubility in rats. This is in accordance with previous studies demonstrating a diabetes-induced decrease in the pepsin solubility of both aortic and tail tendon collagen in rats.[7,13,14] The observed biochemical alterations of collagen are believed to reflect the formation of AGE cross-links.[11] With a

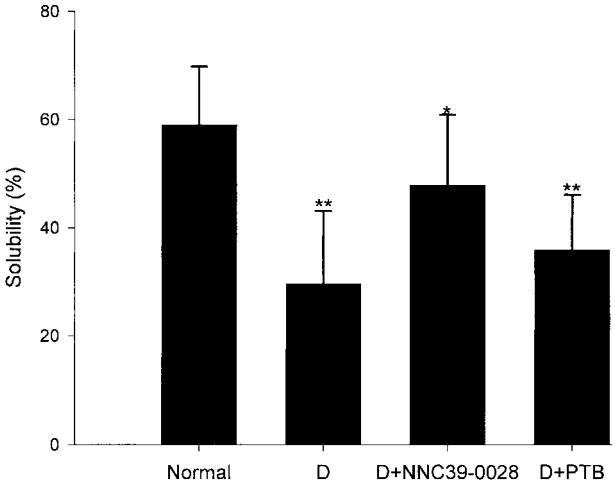

Fig 3. Tail tendon collagen solubility by pepsin digestion in rats after 24 weeks of diabetes. Data are the mean + SD; n = 5-6 per group. D, diabetic rats. *$P < .05$ *v* diabetic controls, **$P < .01$ *v* normal controls.

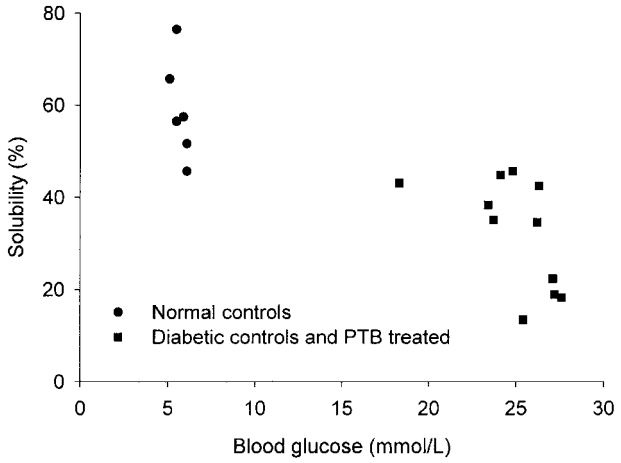

Fig 4. Rat tail tendon collagen solubility by pepsin digestion after 24 weeks of diabetes against mean blood glucose (0-24 weeks). Spearman correlation coefficient: $r = -.93$, $P < .001$ for all 3 groups; $r = -.61$, $P < 0.05$ for only diabetic groups.

newly developed ELISA using monoclonal antibodies raised against AGE albumin, it was shown that an increasing fraction of pepsin-insoluble collagen in diabetic rats was associated with increasing AGE content.[7] As AGE formation is a direct result of the increase in glucose concentration, a relationship between the level of glycemia and collagen pepsin solubility may be expected. Our data confirm such a relationship, because a significant inverse correlation between the 24-week mean BG level and the collagen pepsin solubility was found, when including diabetic control rats and PTB-treated diabetic rats (pepsin solubility was not affected by this intervention). In the present study, selective AGE inhibition with NNC39-0028 significantly reduced the effect of diabetes on collagen pepsin solubility without affecting glycemia. Recently, Kochakian et al[14] found similar effects from treatment with aminoguanidine and other novel more potent AGE inhibitors. That the effects on collagen solubility reflect the inhibition of AGE formation is supported by recently published data showing that NNC39-0028 reduces the formation of AGEs in diabetic rats as measured by radioimmunoassay.[23]

We found that diabetes led to both elevated UAE and increased regional albumin clearance. These are well-known characteristics of the streptozotocin-diabetic rat model. Studies in an in vitro model for AGE-modified basement membranes have suggested that cross-linking of basement membrane proteins increases the permeability to macromolecules.[10] However, despite the beneficial effects on collagen pepsin solubility, NNC39-0028 did not affect diabetes-induced alterations in albuminuria or vascular dysfunction. Beneficial effects of aminoguanidine treatment on diabetes-induced vascular dysfunction in various tissues have been reported,[19,24] whereas reports on diabetic nephropathy have not presented a consistent picture of treatment effects. Aminoguanidine has been shown to prevent albuminuria, mesangial expansion, and thickening of the glomerular basement membrane in some studies,[15,27] but in other studies, it has shown no effects on glomerular morphology in diabetic rats.[28,29] The NOS-inhibitory effect of aminoguanidine has been suggested to be responsible for the beneficial effects in diabetic microvascular complications.[19] If the AGE-inhibiting effect does not influence diabetes-induced vascular dysfunction, this may explain the lack of NNC39-0028 effects on albuminuria and albumin clearance in our study. NNC39-0028 is primarily an inhibitor of AGE formation and does not inhibit NOS.[23] Furthermore, discordant effects of aminoguanidine on vascular function versus AGE formation have recently been described.[29] However, recent studies in diabetic rats to test the effects of aminoguanidine, methylguanidine, nitro-L-arginine methyl ester (L-NAME), and NNC39-0028 have suggested that the beneficial effects of aminoguanidine in diabetic microvascular complications are primarily due to the AGE-inhibiting effect.[22,23,30] Especially during the last part of the study period, NNC39-0028 treatment resulted in an additional increase in diabetes-induced albuminuria. Macroscopic evaluation of the kidneys from this treatment group did not reveal any gross abnormalities. Whether NNC39-0028 per se induces increased UAE is not clear, since an NNC39-0028-treated normal group of rats was not included in the present study. The inhibition of body growth was more pronounced in the NNC39-0028-treated group compared with the other diabetic groups such that some toxicity of the compound cannot be excluded. However, mortality did not differ among the diabetic groups, and in other recently published rat studies of shorter duration, signs of toxicity from NNC39-0028 were not reported.[22,23] This important issue deserves further exploration in future long-term studies.

PTB treatment did not affect the elevated UAE or the decreased collagen pepsin solubility observed in diabetic rats. Effects on diabetes-induced albuminuria have not yet been reported for this new class of compounds. However, the lack of effect on collagen cross-linking is not in agreement with previously published data. Vasan et al[21] demonstrated a reduction of collagen cross-linking when treating tail tendon collagen from diabetic rats with PTB in vitro. Furthermore, the amount of IgG cross-linked to red blood cells was reduced in diabetic rats treated with PTB for 4 weeks. In vivo effects of PTB on diabetes-induced decreased collagen solubility were not reported. PTB was administered subcutaneously in our study. It is not clear from the previous study which mode of administration was used, oral or subcutaneous dosing.[21] Thus, even though we have used the same dose, a different mode of administration may explain the lack of effect in the present study. Recently, it has been reported that PTB under physiological conditions rapidly degrades and therefore probably exerts only limited pharmacological action.[31]

In conclusion, the selective AGE inhibitor NNC39-0028 partly normalized diabetes-induced alterations in tail tendon collagen cross-linking without affecting albuminuria or vascular permeability. These findings may question the role of AGE cross-linking in the pathogenesis of diabetic late complications and emphasize the complexity of the pathogenic processes involved in the development of such complications.

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