Pharmacological blockade of B2-kinin receptor reduces renal protective effect of angiotensin-converting enzyme inhibition in \( {db/db} \) mice model

Marie Buléon,1,2,3 Julien Allard,1,2,3 Acil Jaafar,1,2,3 François Praddaude,1,2,3 Zara Dickson,1,3 Marie-Thérèse Ranera,1,2,3 Christiane Pecher,2,3 Jean-Pierre Girolami,2,3 and Ivan Tack1,2,3

1Laboratoire de Physiologie, Faculté de Médecine Rangueil, Toulouse; 2Institut National de la Santé et de la Recherche Médicale, Unité 858, Toulouse; and 3Université Toulouse III Paul-Sabatier, Institut de Médecine Moléculaire de Rangueil, Equipe 5, IFR31, Toulouse, France

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nephropathy. To achieve this goal, diabetic mice received treatment with ramipril, a molecule that has shown its efficacy in this circumstance (2, 29), either alone or in association with HOE-140, a specific and poorly reversible B2R antagonist (43). The consequences of this treatment on the development of DN (i.e., albuminuria, renal failure, and glomerulosclerosis) and on some of the main pathogenic mechanisms of diabetic glomerulopathy were studied after 20 wk of treatment. We found that the pharmacological blockade of the B2R markedly reduces the renal protective action of ACEi in this model of diabetic nephropathy. Furthermore, some of the crosstalk signaling pathways between B2R and ACEi renal protective effects were identified.

METHODS

Animal model. Obese diabetic C57BLKS db/db mice and heterozygote C57BLKS db/m mice were purchased from the Jackson Laboratories and kept in a temperature-controlled room on a 12:12-h light-dark cycle. To avoid a sex-related discrepancy in the results, only female mice were used. Animals were allowed free access to standard diet and tap water. Experiments were performed using 7-wk-old mice randomly assigned to four groups: either 1) untreated, or treated for 20 wk with 2) ACEi (ramipril, Aventis Pharma Germany; 1 mg·kg⁻¹·day⁻¹ in drinking water), 3) ACEi and B2R selective antagonist HOE-140 (Icatibant HOE-140, Aventis Pharma Germany; 250 μg·kg⁻¹·48 h⁻¹ subcutaneously), or 4) HOE-140 alone (250 μg·kg⁻¹·48 h⁻¹ subcutaneously). This dose was able to prevent the hypertensive effect of a single 100-μl intravenous bolus of 2 × 10⁻⁵ M BK (see RESULTS). Based on pilot experiments, this protocol of administration avoided the implantation of minipumps in these diabetic and obese mice, which are sensitive to infections and healing complications. In each group, age-matched heterozygote C57BLKS db/m mice were used as nondiabetic controls. Every other week, the mice were weighed and blood samples were collected from the tail in 12-h fasted mice to measure glucose concentration using Glucose RTU (BioMèrieux, France). The mice were placed in metabolic cages to collect 24-h urine, and urinary albumin excretion was determined with a specific ELISA (Bethyl). Renal function, histology, and glomerular protein extraction were performed 20 wk later. Serum fructosamine, an index of glucose exposure, was dosed by a colorimetric method based on the reduction of nitrotetrazolium blue by ketoamines. All procedures involving experimental animals were approved by the French Accreditation of Laboratory Animal Care and performed in accordance with the guiding principles for animal research.

Renal function studies. For surgical procedures, mice were anesthetized with an intraperitoneal injection of 30 mg/kg of Etmidate Lipuro (Braun Medical). After tracheotomy, the left jugular vein was cannulated to perfuse insulin, and the left femoral artery was cannulated to monitor arterial blood pressure and to obtain blood samples. Urine was collected through an intravesical catheter. After surgery, mice were allowed to recover for 30 min. Renal function was evaluated for a 60-min clearance period. Glomerular filtration rate (GFR) was assessed by inulin clearance and expressed per gram of kidney weight. At the end of the study, a catheter was introduced into the abdominal aorta. The left kidney was excised and weighed. The right kidney was washed with 10 ml of PBS, fixed by an infusion of 10 ml of 10% formalin (pH 7.40), removed, and stored in formalin.

Glomerular histology and morphology. Formalin-fixed right kidneys were embedded in methyl methacrylate. Sections of 3-μm thickness were stained with periodic acid Schiff (PAS). Pictures were captured using a digital camera Nikon D1 (Nikon, Japan) connected to a light microscope. For each animal, pictures from 50 randomly chosen glomeruli were taken at magnification ×400 and analyzed using Photoshop software (Adobe System, San Jose, CA) and a public domain NIH Image program (http://rsb.info.nih.gov/nih-image/).

Briefly, the total cortical area was examined. A total of 50 glomerular images were randomly recorded by moving the slide from the outer to the inner cortex to obtain noncrossing sample fields. Images were processed using Photoshop software. Glomerular tufts (including all the cellular and interstitial components of the glomerulus, including podocytes and capillaries) were encircled, and the enclosed area was copied to create a new picture (5). The number of pixels (independent of their individual density) of this picture gave the surface area density of the tuft. According to DeHoff’s equation for the measurement of spheroids of differing size, the harmonic mean of glomerular areas (Svm) was used to calculate the mean glomerular volume (GlmVm) for each animal, using the formula GlmVm = 4/3(Svm)³/2. From the glomerular picture, the entire amount of PAS-positive material, except for the peripheral basement membranes, was selected automatically using the properties of color recognition of the software and was manually completed by the inclusion of nuclei. The number of pixels in this area was considered to represent the mesangial area (i.e., mesangial cells and extracellular matrix areas) and was expressed as a fraction of the tuft surface area (5).

Glomerular isolation by magnetic sorting. Colloidal iron oxide suspension was prepared as described by Cook and Pickering (9) for rabbits and modified in our laboratory. Briefly, mice from the different experimental groups described above were anesthetized by intraperitoneal injection of 150 mg/kg Inactin. Ten milliliters of colloidal iron oxide suspension were injected through the abdominal aorta following a 10-ml PBS rinse. Kidneys were removed, and renal cortex was sliced and pressed through a 125-μm² meshed sieve. The glomeruli were then magnetized and washed three times in PBS to obtain a 93% purity suspension. Proteins were extracted using a lysis buffer [10% glycerol, 20 mM Tris, 140 mM NaCl, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM sodium orthovanadate, 3 mM EDTA, 10 μg/ml aprotinin, 1.5 μM benzamidine, 50 μM PMSF, 10 μg/ml leupeptin, 1% phosphatase inhibitor cocktail 2 (Sigma), and 1% Triton X-100] added to the last sediment of glomeruli. All procedures were performed at 4°C. The suspension of glomeruli was sonicated for 1 min and centrifuged (15,000 rpm for 45 min at 4°C). The supernatant was stored at −80°C until Western blot analysis was performed. Protein concentration was determined using a colorimetric method (Bio-Rad DC protein assay).

Western blot analysis. Proteins (20–25 μg) were separated by electrophoresis in polyacrylamide gels (NuPage 4–12% Bis-Tris precast gels; Invitrogen) and electrophoretically transferred to nitrocellulose membranes (Hybond-ECL; Amersham). After 1-h incubation at room temperature in Tris-buffered saline, 5% milk, or 1% milk plus 1% BSA and 0.05% Tween-20, the blots were exposed to antibodies recognizing either insulin-like growth factor-1 receptor (IGF-1R), insulin receptor substrate-1 (IRS-1), ERK1/2, phospho-ERK1/2, AGE receptor (RAGE), TGF-β type II receptor (TGF-βRII), B2R, and 4-hydroxy-2-nonenal (4-HNE) for 1 night at 4°C. The primary antibodies were revealed using the corresponding rabbit or donkey peroxidase-conjugated secondary antibodies for 1 h at room temperature. Peroxidase activity was detected using a chemiluminescence substrate (Super Signal West Pico chemiluminescence substrate; Pierce) and Kodak Biomax Light film. Primary and secondary antibodies were all purchased from Santa Cruz Biotechnology, except for anti-RAGE (Chemicon International), anti-B2R (BD Transduction Laboratory), and anti-β-actin (Sigma).

Statistical analysis. Since the number of animals did not allow us to assume a Gaussian distribution, comparisons among experimental groups were performed using the nonparametric Kruskal-Wallis test. When this test indicated a significant difference, a post hoc test (Dunn’s multiple comparison test) was performed using GraphPad Prism 4.0 (GraphPad Software). P < 0.05 was considered statistically significant.
RESULTS

Efficiency of B2R blockade in vivo. The first step was to determine the time course of HOE-140 administration that allowed in vivo blockade of B2 receptors. The efficiency of this treatment with the B2R antagonist was studied by blood pressure responsiveness to an intravenous bolus of BK (2 × 10⁻⁵ M, 100 µl). In db/db mice treated with HOE-140 alone at the dose of 250 µg·kg⁻¹·48 h⁻¹ for 1 wk, the hypotensive response to BK was almost completely blunted (Fig. 1). The maximum decrease of mean arterial blood pressure (MABP) following the bolus of BK was 36.0 ± 13.4 mmHg (n = 6) for control db/db mice and 13.3 ± 7.0 mmHg for db/db mice treated with HOE-140 (n = 5, P < 0.05). In HOE-treated mice, BK induced a transient hypertensive effect that may result from the consequences of prolonged B2R blockade of vasoconstrictor tone.

General characteristics. The characteristics of the five groups of mice at the end of the experimental period are presented in Table 1. The body and kidneys of db/db mice showed substantial hyperglycemia (P < 0.001) and a significant increase in the fructosamine level (P < 0.05) compared with db/m mice. Treatment with ACEi had no effect on the increases in body and kidney weight or on blood glucose and fructosamine levels at the end of the experimental period. MABP in db/db mice was significantly (P < 0.05) decreased compared with nondiabetic db/m mice. MABP was slightly but significantly (P < 0.05) lower in ACEi-treated mice than in untreated diabetic mice. This effect of ACEi was completely reversed in the presence of HOE-140.

Urinary albumin excretion. As shown in Fig. 2A, urinary albumin excretion at the end of the study was significantly higher in db/db mice than in db/m mice. ACEi treatment prevented this increase, and coadministration of HOE-140 blunted the benefit of ACEi on urinary albumin excretion. Unexpectedly, db/db mice that received HOE-140 alone exhibited a significant increase in urinary albumin excretion compared with untreated db/db mice.

Glomerular filtration. In diabetic db/db untreated mice, GFR was significantly (P < 0.05) lower than in the nondiabetic db/m mice (Fig. 2B). GFR was not significantly different among the three groups of db/db mice despite a tendency to be slightly higher in ACEi + HOE-140-treated mice.

Glomerular histology and morphology. As illustrated in Fig. 3, all db/db mice presented a significant increase in glomerular volume (P < 0.001) compared with the nondiabetic db/m mice. Treatment with ACEi, the B2R antagonist alone, or both drugs failed to induce any significant changes in glomerular volume. Glomerulosclerosis was assessed by the determination of mesangial volume. In db/db control mice, mesangial volume was significantly higher (P < 0.05) than in the nondiabetic db/m mice. Interestingly, treatment with ACEi significantly prevented (P < 0.05) this increase in mesangial volume, leading to a volume similar to that of the nondiabetic mice. Concomitant treatment with HOE-140 abolished (P < 0.01) the effect of ACEi treatment, whereas HOE-140 alone had no significant effect on mesangial expansion.

Glomerular protein expression. The B2R was spontaneously expressed in the glomeruli of both db/m and db/db mice (Fig. 4A). Although the statistical difference was not significant, B2R tended to increase in diabetic mice and remained closer to that of the db/m group in the glomeruli of animals treated with ACEi alone or with HOE-140 (Fig. 4A). Glomerular protein expression of IGF-1R, IRS-1, RAGE, and TGF-βRII was increased in diabetic db/db mice compared with nondiabetic db/m mice (Fig. 4, B, C, E, and F). Treatment with ACEi reduced overexpression of all these signaling proteins, particularly for TGF-βRII. The changes associated with ACEi treatment on IGF-1Rβ expression were attenuated by coadministration of HOE-140. Although diabetes had no significant impact on total ERK1/2 glomerular expression, activated forms of these MAP kinases, assessed by the expression of their tyrosine phosphorylated forms, were strongly increased in diabetes (Fig. 4D). The band corresponding in particular to phospho-ERK1 was reduced when db/db mice were treated with ACEi alone or in combination with HOE-140. Figure 4G shows glomerular expression of 4-HNE adducts, an index of protein peroxidation. Formation of 4-HNE occurs endogenously during lipid peroxidation of polyunsaturated fatty acids that can act as a "toxic second mediator" exhibiting a wide range of biological activities. Compared with the pattern observed in db/m mice, 4-HNE-protein conjugates were significantly increased in db/db C mice, showing major bands at 250, 100, 75, 70, and 45 kDa. In the diabetic ACEi-treated group, the 4-HNE-protein profile was noticeably modified, especially the 100- and 70-kDa bands. These bands were suppressed, exhibiting a pattern very close to that of nondiabetic db/m mice, except for a more pronounced accumulation of 4-HNE on the same proteins. Interestingly, this restoration of a near-normal glomerular pattern of 4-HNE-protein conjugates was partly prevented when HOE-140 was coadministered with ACEi, thus going back to a profile close to that of diabetic mice untreated or treated with HOE-140 alone (not shown).

Fig. 1. Effect of B2-kinin receptor (B2R) blockade by HOE-140 on the acute hypotensive effect of bradykinin (BK). Db/db mice were treated with a subcutaneous injection of HOE-140 (250 µg·kg⁻¹·48 h⁻¹), mean arterial blood pressure was measured 48 h after the last injection with HOE-40. Control db/db mice (n = 6) and db/db mice treated by HOE-140 (n = 5) received an intravenous bolus of 100 µl of saline followed by 2 boluses of BK (2 × 10⁻⁵ M, 100 µl), and mean arterial blood pressure was measured intrafemorally.
### DISCUSSION

Until now, the most efficient approach delaying the progression of DN relied on the blockade of the renin angiotensin system, particularly with ACE inhibitors. This pharmacological strategy is multifaceted, since it includes control of blood pressure, decrease in proteinuria, inhibition of fibrosing cytokines, vasopeptidase inhibition, and oxidative stress modulation. Which of these events occurs first and is the most important remains an unanswered question. It also creates a space for optimization of the therapeutic approach of blocking or even reversing diabetic nephropathy. The dual action of ACEi, namely, angiotensin II synthesis inhibition and BK degradation prevention, already leads to unanswered questions about the mechanism(s) of their renal protective effect. As recently reviewed (11, 14), BK by itself exerts some renal protective effects that involve activation of the type 2 receptor (B2R). Two of the most convincing arguments are that 1) B2R knockout in mice is associated with the worsening of renal lesions in various models of nephropathy, including DN (23, 33), and 2) direct B2R activation using kinin infusion prevents renal inflammation and glomerulosclerosis via inhibition of oxidative stress in Dahl salt-sensitive rat (7). In a recent study (2), we observed that the blockade of the B2R in a model of STZ-induced diabetes in rats blunted the beneficial effects of ACEi treatment on urinary albumin excretion. However, another recent publication (40) provides a contradictory conclusion regarding the protective action of B2R activation in diabetes, thus reopening the debate on this critical point. In this study, B2R−/− knockout mice were protected against the increase in urinary albumin excretion and glomerular lesions resulting from STZ-induced diabetes. This discrepancy could be explained by the fact that, compared with B2R+/+ animals, B2R−/− mice also exhibit spontaneous upregulation of the B1R (15) that was even reinforced by diabetes. This is the reason why we chose the pharmacological blockade of the B2R rather than its genetic knockout model. Furthermore, we focused on the renal effects of direct inhibition of the B2R in db/db mice, a model of spontaneous type 2 diabetes that exhibits delayed glomerulosclerosis far closer to the human disease than the rapidly evolving nephropathy observed in the acutely catabolic STZ-induced type 1 diabetes. Finally, in the report by Tan et al. (40), the lack of the B2R during diabetes was rather protective. Such a result is in contradiction with other recent publications (11) providing a contradictory conclusion. In a recent study (2), we observed that the blockade of the B2R in a model of STZ-induced diabetes in rats blunted the beneficial effects of ACEi treatment on urinary albumin excretion. However, another recent publication (40) provides a contradictory conclusion regarding the protective action of B2R activation in diabetes, thus reopening the debate on this critical point.

### Table 1. Characteristics of experimental groups of mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight, g</th>
<th>Kidney weight, mg</th>
<th>Fructosamine, µmol/l</th>
<th>Blood pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>db/m C</td>
<td>29.1 ± 1.5</td>
<td>305 ± 21</td>
<td>2.05 ± 0.11</td>
<td>89.9 ± 3.3</td>
</tr>
<tr>
<td>db/db C</td>
<td>39.0 ± 2.9</td>
<td>401 ± 26</td>
<td>9.6 ± 0.8</td>
<td>78.0 ± 2.0</td>
</tr>
<tr>
<td>db/db ACEi</td>
<td>37.7 ± 3.8</td>
<td>389 ± 8</td>
<td>8.55 ± 0.71</td>
<td>64.8 ± 2.9</td>
</tr>
<tr>
<td>db/db ACEi+HOE</td>
<td>41.9 ± 2.1</td>
<td>412 ± 16</td>
<td>8.10 ± 0.44</td>
<td>78.8 ± 3.4</td>
</tr>
<tr>
<td>db/db HOE</td>
<td>50.8 ± 3.6</td>
<td>416 ± 20</td>
<td>7.69 ± 0.62</td>
<td>74.7 ± 2.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. db/m C, nondiabetic control; db/db mice; db/db C, diabetic control mice; db/db ACEi, diabetic db/db mice treated with angiotensin-converting enzyme inhibitors; db/db ACEi+HOE, diabetic db/db mice treated with ACEi and B2R antagonist HOE-140; db/db HOE, diabetic db/db mice treated with HOE-140 alone. *P < 0.05; bP < 0.01; cP < 0.001 vs. db/m C. dP < 0.05 vs. db/db C. eP < 0.05 vs. db/db ACEi. When results were not significant for any comparison, no symbol was added.
which is consistent with some of our preliminary results (Buleón M, personnel communication; not shown). However, because B2R blockade demonstrated very significant effects compared with those of ACEi, we focused in this study on the role of the B2R only.

The reduction of glomerulosclerosis by ACEi has been well demonstrated by several independent groups (4, 37). However, until now, most of the conclusions suggested that ACEi acts via suppression of angiotensin II generation, tending to disprove any noticeable role for BK. Using a model of STZ-induced diabetes in rats, Allen et al. (3) showed that HOE-140 treatment had no effect on albuminuria or glomerulosclerosis in ACEi-treated animals. In contrast, we found that direct B2R blockade severely reduced the protective effect of ACEi on morphological renal changes, which strongly supports the critical involvement of the BK pathway. One difference could well be reduced intrarenal ACE activity associated with high ACE2 expression in \( \text{db/db} \) mice (45). Another possible difference is that B2R glomerular expression tends to increase in \( \text{db/db} \) mice (Fig. 4), leading to an increased sensitivity to BK. ACEi exert important systemic and renal hemodynamic effects. The decrease in systemic blood pressure (Table 1), which is believed to partly mediate the renal protective effect of ACEi (10), is completely prevented by concomitant HOE-140 treatment in our model. A critical difference between the \( \text{db/db} \) model and human type 2 diabetes is that the former develops a decrease in blood pressure, whereas the latter is commonly associated with hypertension. The low blood pressure observed in \( \text{db/db} \) mice could be explained by the low ACE activity and increased ACE2 expression (45). Such a pattern results in low angiotensin II availability, possibly associated with angiotensin 1–7 generation and thereby with B2R activation (35, 41). Although this increased sensitivity of \( \text{db/db} \) mice to BK has not been investigated per se, this point is consistent with 1) the lower MABP of \( \text{db/db} \) mice, 2) the correction of the ACEi hypotensive effect by HOE, and 3) the effect of HOE alone, especially on microalbuminuria. This difference could explain the prominent impact of BK during ACEi treatment in our model, whereas the role of angiotensin II in its human counterpart is probably more prominent.

Another difference between humans and \( \text{db/db} \) mice is the apparent lack of improvement of the GFR in anesthetized animals under the influence of ACEi. It is likely that the hypersensitivity of \( \text{db/db} \) mice to BK accounts for such a result, comparable in a way to the temporary worsening of chronic renal failure in some patients after the introduction of ACEi (19).

Our study provides more information regarding the mechanisms of the renoprotective effect of B2R activation during ACEi treatment. In addition to its beneficial effect on the diabetic kidney structure, certain critical signaling pathways involved in the pathophysiology of DN are also affected. On the basis of previous results (2, 6, 26, 27, 36), B2R activation could contribute to the reduction of growth factor/cytokine glomerular pathways recruitment and a reduction of oxidative stress indexes. It is beyond the scope of this report to review the involvement of these numerous factors in the pathogenesis of DN. On the basis of a previous study (2), we chose to focus on IGF-1, TGF-β, and RAGE pathways, whose contributions to DN appear to be modulated by B2R activation. The role of the renal IGF-1 pathway during the early phase of DN has been suggested (16, 21, 39). More recently, TGF-β has emerged as a major deleterious cytokine that largely contributes to the progression of DN, since long-term prevention of mesangial
matrix expansion and renal insufficiency can be achieved by the administration of a monoclonal anti-TGF-β antibody in db/db diabetic mice (46). Not surprisingly, protein glomerular expression of IGF-1R, IRS-1, and TGF-βRII as well as ERK1/2 activation were significantly increased in untreated diabetic mice. ACEi treatment partly prevented the increase in TGF-βRII, IGF-1R, and the activation of ERK1/2 MAP kinases. The decrease in IRS-1 overexpression could result not only from a decreased recruitment of IGF-1 pathway but also from the improvement of insulin sensitivity. In this respect, it is well documented that ACEi improve insulin resistance (11, 17, 25, 30, 31, 36, 37). As expected, ACEi-treated mice showed a reduced expression of both IGF-1Rβ and IRS-1. Furthermore, cotreatment with HOE-140 blunted the beneficial effect of ACEi treatment regarding IGF-1 receptor expression.

Oxidative stress is a major contributor to the development of DN. Among the mechanisms that induce an increase in cellular oxidative stress, the involvement of AGE/RAGE pathway plays a pivotal role in diabetes, since it is highly stimulated (32). RAGE-overexpressing mice are partly protected against DN (44), but conversely, the administration of anti-RAGE antibodies was able to prevent upregulation of the TGF-β pathway and to attenuate DN (13). It has been shown in vitro that ACEi stimulate the expression of soluble RAGE and thereby prevent AGE accumulation in experimental diabetes (17, 18). In our work, ACEi treatment largely prevented the upregulation of RAGE glomerular expression, an effect that appears to be independent of B2 receptor activation. However, AGE/RAGE is not the only pathway involved in increase oxidative stress in diabetes. Since one of the final effects of oxidation can be biochemical modification of proteins, we chose to focus on the glomerular accumulation of HNE-protein complexes that reflect lipid peroxidation. HNE-protein complexes are relatively stable molecules that can pass among subcellular compartments (42) and have the capacity to interact with many cell proteins and thereby impair their functions. It is now widely recognized that 4-HNE adducts are major cytotoxic products playing a role in the progression of atherosclerosis (24), ischemic acute renal failure (28), and human diabetic nephropathy (38). Therefore, controlling 4-HNE adduct accumulation could contribute to renal protection in diabetes. Our results indicate that in the db/db model as well, 4-HNE adducts were increased at the glomerular level. Most of these changes were prevented by ACEi treatment. This benefit was mostly...
dependent on B2R activation, since the 4-HNE profile almost returned to that of diabetic untreated mice when HOE-140 was coadministered with ACEi. This strongly suggests that BK contributes to protecting against cellular oxidative stress in diabetes, an observation that fits with previous studies by ourselves and others (2, 6, 23). Further investigation into the mechanisms of BK-mediated reduction of oxidative stress in diabetes is needed.

In conclusion, ACEi (i.e., ramipril) are able to attenuate the development of diabetic nephropathy in this model of spontaneous type 2 diabetes in mice and are associated with a beneficial impact on several critical glomerular signaling pathways such as those involving IGF-1, TGF-β, and RAGE but also oxidative stress. This effect is partly mediated by B2R activation. Through in vivo B2R blockade, the present study finally provide a critical but not exclusive contradiction to the common belief that ACEi renal protective effect is mostly mediated by their ability to decrease angiotensin II production. This now provides the rationale to examine whether B2R activation by itself could represent a new and complementary therapeutic approach for diabetic nephropathy.

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