The Continuous Erythropoietin Receptor Activator Affects Different Pathways of Diabetic Renal Injury

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ABSTRACT

This study explored the tissue-protective properties of the continuous erythropoietin receptor activator (CERA) in an experimental model of (nonischemic) diabetic kidney injury (i.e., the db/db mouse). Mice were randomly treated with placebo (n/H11005/25), low-dosage CERA (n/H11005/25), and high-dosage CERA (n/H11005/25). Also studied were 25 nondiabetic db/m mice. Hematocrit was comparable in placebo and low-dosage CERA–treated mice but increased significantly with high-dosage CERA (P < 0.01 versus both). Significantly reduced expression of TGF-β, vascular endothelial growth factor, and collagen IV was found in glomeruli and the tubulointerstitial area with CERA treatment, and these beneficial molecular effects were clearly dosage dependent (both P < 0.05 versus placebo). Similarly, CERA treatment caused a dosage-dependent increase in p-Akt, nephrin, and perlecan tissue expression (all P < 0.05 versus placebo). However, the accelerated mesangial expansion that was observed in placebo-treated db/db mice (versus db/m controls) was significantly reduced only in low-dosage CERA–treated mice (P < 0.01). Moreover, albuminuria was significantly reduced in low- but not high-dosage CERA–treated mice compared with placebo treatment (P < 0.05). In an ancillary study, phlebotomy was performed in high-dosage CERA–treated db/db mice to keep hematocrit within normal (baseline) levels. This procedure resulted in significantly (P < 0.05) less albuminuria as compared with high-dosage CERA–treated mice without phlebotomy, thus preserving the tissue-protective potential of CERA. Long-term CERA treatment has beneficial dosage-dependent effects on molecular pathways of diabetic kidney damage. Low-dosage CERA does not affect hematocrit and therefore may be a feasible method of tissue protection in this setting.


Recent experimental studies revealed that erythropoietin (EPO) has numerous tissue-protective effects apart from its action on erythropoiesis and that it prevents vascular and tissue damage as a result of ischemia in the heart, the brain, and also the kidney.1,2 EPO maintains normal red blood cell mass by exerting a continuous antiapoptotic activity in erythrocyte precursors via stimulation of crucial surviving intracellular signaling cascades such as JAK2/STAT5 and phosphatidylinositol 3-kinase/Akt pathways.2 This signaling subsequently leads to phosphorylation of the proapoptotic factor Bad, which in turn dissociates from a cell survival factor, Bcl-XL, resulting in protection from programmed cell death. We recently demonstrated that long-term treatment with the recombinant human EPO (rHuEPO) analogue darbepoetin α conferred renal vascular and tissue protection, preserved renal function, and significantly improved survival in the remnant rat kidney model (five-sixths nephrectomy).3 Importantly, we used a hematologically non-effective dosage of darbepoetin to obviate potential adverse effects of rHuEPO therapy. In this experimental setting of chronic renal ischemia, darbe-
etin persistently activated the Akt pathway and reduced apoptotic cell death in renal tissue.

In this study, we set out to explore the therapeutic potential of another erythropoiesis-stimulating agent—the continuous erythropoietin receptor activator (CERA)—for tissue protection in an animal model of nonischemic chronic kidney injury (i.e., the leptin receptor knockout db/db mouse). In this animal model for type 2 diabetes, the mice develop characteristic molecular and clinical features of diabetic nephropathy. We administered a hematologically noneffective (low) and effective (high) dosage of CERA and studied several molecular pathways of diabetic kidney damage. CERA is chemically synthesized through integration of amide bonds between amino groups of rHuEPO and methoxy polyethylene glycol-succinimidyl butanoic acid. This modification results in a substantial prolongation of duration of action.

RESULTS

Clinical and Laboratory Data
The db/db mice became obese and developed frank hyperglycemia during the observation period when compared with their nondiabetic db/m littermates (Table 1). Blood glucose levels were already slightly higher in db/db mice in comparison with db/m mice at the start of the observation period, and in the next 2 wk, blood glucose increased significantly and remained consistently higher in db/db mice than in db/m controls. We observed no significant difference in blood glucose levels between untreated and CERA-treated db/db mice. Similarly, body weight was not affected by CERA treatment. Hematocrit increased in all four groups during the study period (Table 1), but this increase was more pronounced in the db/db mice. During the study period, we observed no significant difference in hematocrit levels between placebo- and low-dosage CERA–treated db/db mice. However, hematocrit increased significantly in high-dosage CERA–treated mice in comparison with all other treatment groups. At the end of the treatment period, systolic BP (SBP) was significantly higher in placeto- and low-dosage CERA–treated mice in comparison with db/m mice at the start of the observation period, and in the next 6 wk of treatment, whereas later, it returned to the level that was observed in placebo-treated db/db mice (Table 1). We observed no significant differences in serum creatinine levels between placebo-treated and low-dosage and high-dosage CERA–treated mice (data not shown).

Renal Histology and Immunohistochemistry
The glomerular appearance in placebo-treated db/db mice showed accelerated mesangial expansion characterized by an increase in periodic acid-Schiff–positive mesangial matrix area relative to that observed in db/m mice at the end of the study period (Figure 1). In mice that were treated with low-dosage CERA, the score was significantly reduced ($P < 0.01$). In contrast, in mice that were treated with high-dosage CERA, we observed an even higher score than in placebo-treated mice. Furthermore, we analyzed small arteries for evidence of arteriolar hyalinosis as a marker for diabetic vascular damage (Figure 1). We found no arteriolar hyalinosis in db/m control mice, whereas in placebo-treated db/db mice, a mild degree of arteriolar hyalinosis was present. Arteriolar hyalinosis was less frequent in mice that were treated with low-dosage CERA, but similar to the previous finding on mesangial expansion, we observed a higher degree of arteriolar hyalinosis in mice that were treated with high-dosage CERA.

To examine the underlying molecular mechanism of the clinical and laboratory observation, we first analyzed the expression of TGF-$\beta_1$ as an important mediator of diabetic kidney damage (Figure 2, A through D). Not unexpected, we found significantly higher TGF-$\beta_1$ expression in db/db mice as

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Serum Glucose (mmol/L)</th>
<th>Body Weight (g)</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 14</td>
<td>Baseline</td>
</tr>
<tr>
<td>db/m control</td>
<td>6.6 ± 0.3</td>
<td>6.0 ± 0.3</td>
<td>22.2 ± 0.3</td>
</tr>
<tr>
<td>db/db placebo</td>
<td>9.1 ± 0.9</td>
<td>19.4 ± 1.1$^b$</td>
<td>27.7 ± 0.6$^b$</td>
</tr>
<tr>
<td>db/db low-dosage CERA</td>
<td>8.8 ± 0.7</td>
<td>21.2 ± 1.2$^b$</td>
<td>29.7 ± 0.4$^b$</td>
</tr>
<tr>
<td>db/db high-dosage CERA</td>
<td>9.7 ± 0.8$^d$</td>
<td>18.1 ± 1.2$^b$</td>
<td>28.8 ± 0.5$^b$</td>
</tr>
</tbody>
</table>

$^a$CERA, continuous erythropoietin receptor activator.

$^bP < 0.001$ versus db/m control.

$^cP < 0.001$ versus db/db high-dosage CERA.

$^dP < 0.05$ versus db/m control.
compared with db/m controls. It is interesting that we observed a dosage-dependent and significant reduction of TGF-β1 expression in mice that were treated with CERA. When we analyzed the collagen IV expression in glomeruli and the tubulointerstitial area, we found a similar pattern in the different treatment groups (Figure 2, E through H). Next, we analyzed the expression of nephrin (Figure 3, A through D). We found a significant reduction of the nephrin expression in db/db mice in comparison with db/m mice, whereas in tissue of CERA-treated mice, higher nephrin levels were detectable. The effect was dosage dependent. We found similar results when analyzing glomerular perlecan expression (Figure 3, E

Table 2. Parameters of kidney function in control mice (db/m) and in db/db mice treated with saline (placebo) and low- and high-dosage CERA

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Renal Weight (mg)</th>
<th>Renal/Body Weight (%)</th>
<th>Albuminuria (µg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 6</td>
<td>Week 13</td>
</tr>
<tr>
<td>db/m control</td>
<td>184 ± 3</td>
<td>6.2 ± 0.1</td>
<td>33.2 ± 5.6</td>
</tr>
<tr>
<td>db/db placebo</td>
<td>262 ± 13b</td>
<td>4.8 ± 0.3b</td>
<td>121 ± 14b</td>
</tr>
<tr>
<td>db/db low-dosage CERA</td>
<td>216 ± 16</td>
<td>4.1 ± 0.3b</td>
<td>143 ± 27b</td>
</tr>
<tr>
<td>db/db high-dosage CERA</td>
<td>217 ± 13c</td>
<td>4.1 ± 0.1bc</td>
<td>162 ± 15b</td>
</tr>
<tr>
<td>db/db placebo</td>
<td>262 ± 13b</td>
<td>4.8 ± 0.3b</td>
<td>121 ± 14b</td>
</tr>
</tbody>
</table>

a Renal weight was measured in 10 killed mice per group.
b P < 0.001 versus db/m control.
c P < 0.05 versus db/db placebo.
through H), a heparan sulphate proteoglycan. It is postulated that such proteoglycans play an important role in holding up the anionic charge of the filtration barrier and thereby prevent loss of albumin. Moreover, we observed an increase in glomerular vascular endothelial growth factor (VEGF) expression in the db/db versus db/m mice. This was reduced in a dosage-dependent manner by treatment with CERA (Figure 3, I through L). Finally, we found a significant reduction of p-Akt expression in renal tissue of placebo-treated db/db mice in comparison with db/m controls (data not shown). This reduction of p-Akt in renal tissue was significantly ameliorated in CERA-treated mice. Again, the effect of CERA was dosage dependent.

**Effect of Phlebotomy in High-Dosage CERA–Treated Mice**

Phlebotomy in high-dose CERA–treated mice resulted in hematocrit levels that were comparable to baseline values before the start of CERA treatment (Table 3). SBP at the end of the treatment period in high-dosage CERA–treated mice and in phlebotomized mice was comparable (142 ± 6 versus 146 ± 8 mmHg; NS). In phlebotomized mice, kidney weight as well as the kidney-to-body weight ratio were not significantly different from those in high-dosage CERA–treated db/db mice (data not shown). In contrast, in phlebotomized, high-dosage CERA–treated mice, urinary albumin excretion after 6 and 13 wk of treatment was significantly lower as compared with mice that were treated with high-dose CERA only (Table 3). In general, albumin excretion was lower in both groups of db/db mice as compared with those in the first experimental series because in this experiment, we studied younger animals (4 wk of age). The immunohistochemical analysis of renal tissue revealed similar patterns of TGF-β, nephrin, and perlecan expression in both groups of db/db mice as compared with the high-dosage CERA–treated mice in the first experiment (Figure 4). Above that, TGF-β expression was comparable in mice that were treated with high-dosage CERA and in high-dosage CERA–treated, phlebotomized mice (Figure 4, A and B). This was also true for nephrin (Figure 4, C and D) and perlecan (Figure 4, E and F) tissue expression. In summary, TGF-β, nephrin, and perlecan expression was not significantly different in mice that were treated with high-dosage CERA (Figure 4, A, C, and E) and high-dosage CERA–treated, phlebotomized db/db mice (Figure 4, B, D, and F). Finally, we found a comparable expression of p-Akt expression in renal tissue of both groups of mice (data not shown).

**DISCUSSION**

In this study, we documented that long-term treatment with CERA has striking effects on different molecular pathways in

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**Table 3. Serum glucose and hematocrit in db/db mice treated with high-dosage CERA and high-dosage CERA accompanied by phlebotomy**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Serum Glucose (mmol/L)</th>
<th>Hematocrit (%)</th>
<th>Albuminuria (μg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 14</td>
<td>Baseline</td>
</tr>
<tr>
<td>db/db high-dosage CERA</td>
<td>8.1 ± 0.4</td>
<td>18.9 ± 1.4</td>
<td>56.9 ± 1.4</td>
</tr>
<tr>
<td>db/db high-dosage CERA + phlebotomy</td>
<td>8.5 ± 0.6</td>
<td>18.2 ± 2.6</td>
<td>57.3 ± 1.2</td>
</tr>
</tbody>
</table>

*a*P < 0.01 versus high-dosage CERA.

*b*P < 0.05 versus high-dosage CERA.

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**Figure 3.** Nephrin, perlecan, and vascular endothelial growth factor (VEGF) expression in renal tissue of db/db mice. Diabetic db/db mice were treated with saline (placebo) or CERA for 14 wk. Db/m nondiabetic mice were used as control. Immunohistochemistry was performed on cryosections. Representative pictures are shown for nephrin (A through D), perlecan (E through H), and VEGF (I through L). (A, E, and I) Db/m control mice. (B, F, and J) Db/db placebo-treated mice. (C, G, and K) Db/db mice that were treated with 0.4 µg CERA/kg per wk (low-dosage CERA). (D, H, and L) Db/db mice that were treated with 1.2 µg CERA/kg per wk (high-dosage CERA). Nephrin, perlecan, and VEGF expression in glomeruli was graded on a scale from 0 to 3. **P ≤ 0.01 versus db/m control; *P ≤ 0.05 versus(db/db placebo treatment; "P ≤ 0.01 versus db/db placebo treatment; "™P ≤ 0.01 versus low-dosage CERA.**
an experimental model of diabetic kidney damage: The db/db mouse. Long-term treatment with CERA not only reduced mediators of accumulation of extracellular matrix in diabetic nephropathy, such as TGF-β1, but also prevented the loss of nephrin in glomeruli of treated mice. To our knowledge, this is the first description of a positive effect of an erythropoiesis-stimulating agent in a model of nonischemic renal tissue injury. So far, positive effects of rHuEPO administration have been documented only in experimental models of acute ischemia/reperfusion kidney injury or chronic vascular injury to the kidney, similar to what has been shown in numerous studies in the heart. In these experimental settings, the main action of rHuEPO was inhibition of apoptosis in renal and cardiac tissue. However, in most of these studies, very high dosages of rHuEPO have been administered only once or for a short period to obviate potential adverse effects of rHuEPO therapy. Particularly, effects on the number and activation state of thrombocytes and the stimulation of platelet adherence to endothelium could mitigate the beneficial effects of a long-term high-dosage therapy on the kidney. In addition, changes in blood viscosity because of marked erythrocytosis and tissue generation of endothelin may work against the positive effects of rHuEPO. In line with these experimental data are our findings with high-dosage CERA treatment in db/db mice. We could clearly show beneficial dosage-dependent effects of CERA on molecular pathways of diabetic kidney damage, yet mesangial expansion was not reduced after 14 wk of high-dosage CERA treatment. As a consequence, urinary albumin excretion rate, which decreased by week 6 of treatment, increased again with ongoing high-dosage therapy. This result is in contrast with low-dosage CERA, for which the increase in albuminuria during the observation period was significantly reduced compared with placebo treatment, although this renoprotective effect of low-dosage CERA was not complete.

To explore further the confounding effect of increased hematocrit levels in this experimental setting, we performed an additional series of experiments in which high-dosage CERA–treated mice were phlebotomized to achieve hematocrit levels that were comparable to those at baseline (i.e., before the start of high-dosage CERA treatment). We could indeed show that this procedure prevented the adverse effects of increased hematocrit levels (i.e., reduced the increase in microalbuminuria in high-dosage CERA–treated animals, thereby exposing the tissue-protective potential of CERA). Further studies are warranted to elucidate the molecular mechanism(s) by which a rise in hematocrit with hematologically effective CERA dosages (and presumably also with rHuEPO) lessens the beneficial effects on the molecular pathways of diabetic nephropathy. Moreover, establishing a minimal effective dosage may also provide a new strategy to prevent progression in diabetic kidney disease as well as other conditions of chronic kidney injury.

We provide evidence for different mechanisms by which CERA may exert its protective effect on diabetic kidney damage. CERA treatment significantly reduced TGF-β1 expression in renal tissue of db/db mice. Overexpression of TGF-β1 plays an important integral role in the development of diabetic nephropathy, because this fibrogenic cytokine stimulates podocyte expression of collagen IV and VEGF, and the latter action in turn may increase the activity of the VEGF autocrine loop. Inhibition of TGF-β1 with a specific antibody almost completely prevented the mesangial expansion in db/db mice. Increased TGF-β1 levels can also cause podocyte detachment and/or apoptosis. Thus, inhibition of TGF-β1—
induced production of collagen IV by CERA may reduce mes- 
angial matrix expansion. In addition, decreased podocyte loss 
may be another beneficial effect of CERA-induced downregu-
lation of TGF-β1. CERA treatment could also directly diminish 
the activity of the VEGF autocrine loop. It has been docu-
mented that neutralization of VEGF with a systemically ad-
ministered anti-VEGF antibody markedly reduced the urinary 
albumin excretion in db/db mice and the streptozotocin type 1 
diabetes model.17,18 Expression of VEGF in the glomerulus is 
most pronounced in podocytes, where VEGF mRNA and pro-
tein expression is stimulated by high glucose concentrations 
and TGF-β1.12 Increased activity of the VEGF autocrine loop 
plays an important role in podocyte biology because VEGF by 
itself stimulates podocytes to produce collagen IV.13,19 The-
oretically, treatment with CERA could have mitigated intrarenal 
VEGF activity indirectly by ameliorating diabetic tissue dam-
age and/or through a direct effect on the VEGF/VEGF receptor 
pathway. Recently published experimental data provide evi-
dence for such a cross-link between the EPO/EPO receptor 
and the VEGF/VEGF receptor system, at least in the vascular sys-

tem.20

Finally, CERA may also directly act on podocytes, possibly 
by activating Akt, similar to what has been shown for rHuEPO 
in endothelial glomerular and tubular kidney cells.21 In addition,
the CERA-induced increase in nephrin and perlecan cell 
content may result in reduced glomerular basement mem-
brane permeability and albuminuria. Perlecan is a negatively 
charged heparan-sulfate proteoglycan that is responsible for 
permeselectivity of the negatively charged glomerular basement 
membrane, whereas nephrin, a transmembrane protein with a 
large extracellular portion, forms the molecular substrate of 
the slit diaphragm.22 It is interesting that an important target of 
nephrin-induced signaling is Bad, the proapoptotic protein of 
the Bcl-2 family that is also involved in erythrocyte prolifera-
tion.23 Thus, CERA-induced signaling and maintenance of ade-
quate nephrin content could prevent podocyte apoptosis, a 
truly beneficial effect given that terminally differentiated 
podocytes do not proliferate.23 In diabetes, nephrin protein 
production is downregulated, and the decrease in nephrin cor-
relates with the broadening of the foot process widths.24,25 In 
addition, the podocyte number and their density have been 
reported to be markedly reduced in glomeruli of patients with 
type 1 and type 2 diabetes.26,27 In this regard, protection of 
podocytes seems to be of paramount importance in preventing 
the development and progression of human diabetic nephrop-
athy.22,28,29

CONCLUSION

Long-term treatment with a long-acting erythropoiesis-stim-
ulating agent has striking dosage-dependent effects on molec-
ular pathways of diabetic kidney damage. However, in contrast 
to high-dosage CERA treatment, which resulted in a significant 
increase in hematocrit that abrogated these beneficial effects,

low-dosage therapy (as well as phlebotomy- in high-dosage–
treated animals) fully exposed the tissue-protective potential 
of CERA. Therefore, treatment with low-dosage CERA may be 
a feasible method of long-term tissue protection, and further 

studies are needed to establish minimal effective therapeutic 
dosages that are not associated with potentially harmful con-
sequences of an increase in hematocrit. This proposal is cor-
orborated by the results of recently published large trials in 
patients with chronic kidney disease, in which (almost) com-
plete correction of anemia with an erythropoiesis-stimulating 
agent did not retard progression.30,31

CONCISE METHODS

Animal Model and Study Protocol

The Animal Care Committee of Lower Saxony approved the study. 
We studied 75 male diabetic db/db (BKS.Cg-m+/+Leprdb) and 25 

male nondiabetic db/m control mice (Charles River Laboratories, Sulz-
feld, Germany). All mice were 6 wk of age at the beginning of the 
study. They were housed with 12:12-h light-dark cycles and had free 
access to food and water. Using a parallel-group study design, we 
randomly allocated the db/db mice to receive a weekly injection of (1 
0.9% NaCl (placebo; n = 25), (2) 0.4 µg CERA/kg body wt (low 
dosage; n = 25), and (3) 1.2 µg CERA/kg body wt (high dosage; n = 
25). CERA was provided by Roche Diagnostics (Penzberg, Germany). 
It is chemically synthesized through integration of amide bonds be-
tween amino groups of rHuEPO and methoxy polyethylene glycol-
succinimidyl butanoic acid. The resulting molecular weight of ap-
proximately 60,000 Da causes a considerably prolonged elimination 
half-life, thereby prolonging the action of CERA on its target tissues. 
Recently published reports have specified that the half-life of action 
of CERA in humans is approximately 130 h.32 We determined hemat-
ologically noneffective (low) and effective (high) dosages of CERA in 
an ancillary dosage-finding experiment.

The main study duration was 14 wk, and we assessed body weight 
of mice at baseline and every 2 wk after the start of treatment. In 
addition, SBP was measured in conscious mice with an occlusive tail-
cuff plethysmograph attached to a pneumatic pulse transducer (TSE 
BP system, Bad Homburg, Germany). For these measurements, we 
kept mice at 37°C. Furthermore, we assessed blood glucose with an 
Ascensia glucometer (Bayer Diagnostics, Leverkusen, Germany) and 
hematocrit with a vet scil abc (SCIL, Vierheim, Germany) at baseline 
and at regular intervals throughout the study. Finally, for the assess-
ment of 24-h albuminuria, we placed mice in separate mouse diuresis 
cages (Tecniplast, Hohenpeissenberg, Germany) with access to water 
(but not food) for 24 h. We measured urinary albumin concentration 
at baseline and after 6 and 13 wk with an ELISA specific for mouse 
albumin (Albuwell M; Exocell, Philadelphia, PA). SBP was measured 
by a noninvasive tail-cuff method with the BP-2000 BP analysis sys-
tem (Visitech Systems, Apex, NC). Ten mice from each group under-
went repeated measurements on two different days 2 wk before the 
study end.
Renal Histology and Immunohistochemistry
After 14wk of follow-up, we obtained renal tissue for morphologic and immunohistochemical analyses. For this purpose, 10 mice from each group were narcotized and the kidneys were perfused with Ringer lactate via the left ventricle. After the mice were killed, sagittal sections of the kidney were fixed in 4% neutral buffered formalin, embedded in paraffin, sectioned at 2 μm, and stained with the periodic acid-Schiff reagent. Coded tissue sections were analyzed by an investigator who was blinded with respect to the allocation of mice to the treatment groups. Forty glomeruli from each mouse were examined, and the amount of mesangial expansion was graded as follows: 0, no changes; 1, minor, mesangial expansion up to the diameter of two nuclei; 2, moderate, mesangial expansion with a diameter of 3 to 4 nuclei; 3, severe, mesangial expansion with a diameter of more than 4 nuclei. Similarly, arteriolar hyalinosis in the tissue sections was graded as follows: 0, no changes; 1, minor, few arterioles involved; 2, moderate, <50% of arterioles involved; 3, severe, >50% of arterioles involved.

Immunohistochemistry was performed on cryostat sections of the frozen kidneys or on paraffin sections as described previously. The following primary antibodies were used: Anti–TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. sc-146), anti-VEGF (Santa Cruz Biotechnology; cat. no. sc-152), anti–type IV collagen (Southern Biotechnology, Birmingham, AL; cat. no. 1340-01), polyclonal rabbit anti-perlecan (Research Diagnostics). For indirect immunofluorescence, nonspecific binding sites were blocked with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) for 30 min. Sections were then incubated with the primary antibody for 1 h. For fluorescence visualization of bound primary antibodies, sections were further incubated with Cy3-conjugated secondary antibodies (Jackson Immunoresearch) for 1 h. Specimens were analyzed using a Zeiss Axiosplan-2 imaging microscope with the computer program AxioVision 3.0 (Zeiss, Jena, Germany). Semiquantitative analysis of nephrin, perlecan, TGF-β1, and VEGF expression in 40 glomeruli per animal; of collagen IV and p-AKT expression in the kidney; and TGF-β1, collagen I, and p-AKT expression in the heart was done by using the following scoring system: 0, no; 1, weak; 2, moderate; and 3, strong expression. The investigator performing these immunohistochemical analyses had no knowledge of the treatment group assignment.

Effect of Phlebotomy in High-Dosage CERA–Treated Mice
In an additional experiment, we investigated the effect of phlebotomy in high-dosage CERA–treated db/db mice. Using a parallel-group study design, we randomly allocated 4-wk-old db/db mice to receive a weekly injection of (1) 1.2 μg CERA/kg body wt (high dosage; n = 20) and (2) 1.2 μg CERA/kg body wt (n = 20) plus phlebotomy. Phlebotomy was performed in narcotized mice at regular intervals throughout to keep hematocrit within normal (i.e., baseline) levels. The assessment of clinical and laboratory data and the immunohistochemical evaluation of renal tissue were identical as described previously. In these additional experiments, we analyzed only the expression of key molecules of diabetic injury in renal tissue (TGF-β1, nephrin, and perlecan).

Statistical Analyses
We used the InStat statistical program (InStat, San Jose, CA). The statistical significance was set at P < 0.05, and data are presented as means ± SEM. We compared baseline and end point characteristics with ANOVA and appropriately corrected t test for random data.

ACKNOWLEDGMENTS
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DISCLOSURES
None.

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