Erythropoietin ameliorates podocyte injury in advanced diabetic nephropathy in the *db/db* mouse

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Loeffler I, Rüster C, Franke S, Liebisch M, Wolf G. Erythropoietin ameliorates podocyte injury in advanced diabetic nephropathy in the *db/db* mouse. *Am J Physiol Renal Physiol* 305: F911–F918, 2013. First published July 3, 2013; doi:10.1152/ajprenal.00643.2012.—Podocyte damage and accumulation of advanced glycation end products (AGEs) are characteristics of diabetic nephropathy (DN). The pathophysiology of AGE-challenged podocytes, such as hypertrophy, apoptosis, and reduced cell migration, is closely related to the induction of the cell cycle inhibitor p27Kip1 and to the inhibition of neurpin 1 (NRP1). We have previously demonstrated that treatment with erythropoietin is associated with protective effects for podocytes in vitro. *db/db* mice with overt DN aged 15–16 wk were treated with either placebo, epoetin-β, or continuous erythropoietin receptor activator (CERA) for 2 wk. *db/db* mice compared with nondiabetic *db*m control mice revealed the expected increases in body weight, blood glucose, albumin-to-creatinine ratio, and AGE accumulation. Whereas there were no differences in body weight, hyperglycemia and AGEs were observed among diabetic mice that received epoetin-β compared with CERA and placebo treatment, indicating that epoetin-β/CERA treatment does not interfere with the development of diabetes in this model. However, the albumin-to-creatinine ratio was significantly lower in *db/db* mice treated with epoetin-β or CERA. Furthermore, kidney weights in *db/db* mice were increased compared with *db/m* control mice, indicating renal hypertrophy, whereas the increase in renal weight in epoetin-β- or CERA-treated *db/db* mice was significantly lower than in placebo-treated control mice. Induction of p27Kip1 and suppression of NRPI were significantly reduced in the epoetin-β treatment group versus the CERA treatment group. Furthermore, erythropoietin treatment diminished the diabetes-induced podocyte loss. Together, independently from hematopoietic effects, epoetin-β or CERA treatment was associated with protective changes in DN, especially that NRPI and p27Kip1 expressions as well as numbers of podocytes returned to normal levels. Our data show, for the first time, that medication of overt DN with erythropoietin for a short time can ameliorate albuminuria and podocyte loss.

erythropoietin; continuous erythropoietin receptor activator; diabetic nephropathy; podocytes; *db/db* mouse

PROGRESSIVE PODOCYTE INJURY, characterized by decreased density and number, hypertrophy, and foot process effacement, plays a central role in the development of diabetic nephropathy (DN) in both type 1 and type 2 diabetes (32). In DN, renal injury, including damage of podocytes, is partially mediated by the enhanced formation and accumulation of advanced glycation end products (AGEs) (9). The receptor of AGEs (RAGE) is a multiligand member of the Ig superfamily of cell surface molecules with key ligands such as AGEs and S100/calgranulins in diabetic tissues, which is principally expressed by podocytes (37). In DN, RAGE is upregulated in cells such as glomerular podocytes and endothelial cells in both humans and mice (34, 37). Blockade of the AGE/RAGE pathway or deletion of the receptor in several in vivo and in vitro models of DN is associated with improved podocyte survival and therefore with reduced progression of the disease (16, 19, 21, 26).

In previous podocyte cell culture studies, AGE-mediated hypertrophy and cell cycle arrest was associated with induction of the cell cycle inhibitor p27Kip1 (28), whereas expression of neurpin 1 (NRP1) was reduced by AGEs (6, 7). Due to their quiescent phenotype, podocytes cannot reenter into the mitotic cell cycle. Podocyte loss is closely linked to proteinuria and eventually to the development of glomerulosclerosis. However, podocyte loss can be initially partly compensated to a certain extent by podocyte hypertrophy and by covering the resulting decoated and thereby “nude” visceral space of the glomerular basement membrane by migrating processes of surviving podocytes (16). Yet, AGE-mediated reduced NRPI expression resulted in decreased podocyte migration and therefore could contribute to the development of glomerulosclerosis by adherence of the “nuded” glomerular basement membrane to Bowman’s capsule (6). In vivo, NRPI expression is also decreased in kidney biopsies from patients with DN as well as in diabetic *db/db* mice (6).

Independent from its hematopoietic effects, erythropoietin (EPO) may be protective for several tissues, including the heart, brain, and kidney mainly, presumably by prevention of ischemic damage and by antiapoptotic prosurvival effects (14, 24). Podocytes express EPO receptors and therefore can respond to hematopoietic growth factor stimulation (11). Others (13, 17, 30) have elegantly shown in vivo and in vitro that treatment with EPO or its analogs ameliorated podocyte injury by protective effects on nephrin expression and the cytoskeleton, by reduction of apoptosis, or by activation of prosurvival intracellular pathways. More recently, in a model of kidney allograft injury, the nonhemodynamic nephroprotective potential of EPO substitution was outlined compared with blood transfusions (10).

Recently, we (29) demonstrated in cultured podocytes that in AGE-mediated injury the addition of EPO prevented p27Kip1 mediated cell cycle arrest and podocyte hypertrophy as well as NRPI reduction and associated impaired cell migration. We hypothesized that in diabetic metabolism, the changes of p27Kip1 and NRPI expression and their functional consequences on podocytes can be ameliorated in a mouse model of overt DN by EPO. Therefore, the present study was performed to evaluate the potential nephroprotective properties of EPO in overt DN in vivo in *db/db* mice, a well-characterized model of type 2 diabetes, focusing on p27Kip1 and NRPI expressions and on the related functional changes in podocytes.

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METHODS

Animal model and study protocol. All animal experiments were approved by the local ethics committee and were done in accordance with German Animal Protection Law. We studied diabetic db/db (B6.Cg-Dock7m Leprdb/+; +/+j) mice and nondiabetic db/m mice (Jackson Laboratory, Bar Harbor, ME) as controls. db/m and db/db animals were treated with either 20 IE/kg ip epoetin-β (NeoReccorm, Hoffmann-La Roche, Grenzach-Wyhlen, Germany) three times per week (CERA; MIRCERA, Hoffmann-La Roche) once per week (B6.Cg-Dock7m Leprdb/+; +/+j). Concentrations of epoetin-β and CERA were chosen according to generally used doses in clinical practice applied to patients with renal disease and previous published experiments (30). Nondiabetic db/m mice (n = 10) and diabetic db/db mice (n = 10) were injected with 0.9% ip NaCl (placebo) as the control. All animals were maintained in a pathogen-free facility, had free access to water, and were fed standard rodent Chow. All mice were 16 wk old at the beginning of the study, and only male mice were used to control for potential hormonal effects. We assessed hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration with the pocH-100iV DIFF instrument (Sysmex, Burlington, CA) and aminomethylcarbazole as a chromogen. For negative controls, the primary antibody was replaced by rabbit Ig at the same concentration as the primary antibody. For the quantification of CML, NRPl, and p27Kip1, staining intensity was examined by an investigator who was unaware of the origin of the groups. For the quantitative assessment of CML, NRPl, and p27 staining intensities, sections were stained in one batch for each parameter, respectively. For imaging and documentation, a computer-assisted microscope with digital camera and AxioVision 4.8 software was used (Carl Zeiss). Ten nonoverlapping glomeruli of each individual kidney sample were scanned in the monochrome mode of the camera (magnification: x400). For each parameter, all images were taken under constant conditions as appropriate. After the glomerular area was highlighted, the mean densitometric gray levels were measured. Finally, the average of gray labels obtained for each individual kidney sample was used as an equivalent for the respective staining intensity.

For immunofluorescence, deparaffinized kidney sections were treated as described above without 3% H2O2 incubation. Mouse anti-synaptopodin antibody was purchased from Acris (Herford, Germany), and rabbit anti-Wilms’ tumor-1 COOH-terminal antibody was from Santa Cruz Biotechnology. Staining was performed using antimouse IgG-Alexa 488, and anti-rabbit IgG-Cy3-linked secondary antibodies, respectively (Life Technologies).

Statistical analysis. Values given in this article are presented as means ± SE. Results were analyzed using SPSS statistics (IBM, Armonk, NY). The Kruskal-Wallis test was used for multigroup comparisons followed by the Mann-Whitney rank-sum test to compare two groups of mice. P values of ≤0.05 were considered significant.

RESULTS

EPO administration did not influence the development of diabetes. We used the db/db mouse model because that is currently the most widely used mouse to model DN in type 2 diabetes (2). The underlying genetic background is susceptible to diabetic complications such as nephropathy. db/db mice until the age of 6 wk show body weights and blood glucose levels that are similar to those of their db/m littermates; db/db mice become obese and develop hyperglycemia within the ages of 6–8 wk (20). DN in these mice is characterized by albuminuria, podocyte loss, and mesangial matrix expansion (2). In contrast to previous publications (e.g., Refs. 20 and 30), we used mice at the age of 16 wk with already overt features of diabetes type 2 and DN, which were subsequently treated with placebo, epoetin-β, or CERA. The development of diabetes was monitored by measuring body weight, serum glucose (Table 1), and serum AGES (Fig. 1). As expected, diabetic

Table 1. Clinical/laboratory data and parameters of kidney function in nondiabetic db/m mice and db/db mice treated with placebo, epoetin-β, or CERA

<table>
<thead>
<tr>
<th>db/m Mice</th>
<th>db/db Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Placebo</strong></td>
<td><strong>Epoetin-β</strong></td>
</tr>
<tr>
<td><strong>Placebo</strong></td>
<td><strong>Epoetin-β</strong></td>
</tr>
<tr>
<td><strong>Body weight, g</strong></td>
<td>$23.9 ± 1.2$</td>
</tr>
<tr>
<td><strong>Kidney weight, mg</strong></td>
<td>$1284 ± 7.6$</td>
</tr>
<tr>
<td><strong>Serum glucose, mmol/l</strong></td>
<td>$4.1 ± 0.8$</td>
</tr>
<tr>
<td><strong>Albumin-to-creatinine ratio, mg/g</strong></td>
<td>$155.5 ± 2.8$</td>
</tr>
<tr>
<td><strong>Hematocrit, %</strong></td>
<td>$0.46 ± 0.03$</td>
</tr>
<tr>
<td><strong>Hemoglobin, mmol/l</strong></td>
<td>$9.1 ± 0.2$</td>
</tr>
<tr>
<td><strong>Mean corpuscular volume, μm³</strong></td>
<td>$45.6 ± 1.5$</td>
</tr>
<tr>
<td><strong>Mean corpuscular hemoglobin, fmol</strong></td>
<td>$0.91 ± 0.01$</td>
</tr>
<tr>
<td><strong>Mean corpuscular hemoglobin concentration, mmol/l</strong></td>
<td>$20.0 ± 0.7$</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 10$ mice/group. CERA, continuous erythropoietin receptor activator. *$P < 0.05$ and **$P < 0.01$, epoetin-β or CERA treatment vs. placebo treatment for the same genotype; †$P < 0.05$, ††$P < 0.01$, and †††$P < 0.001$, db/db vs. db/m mice of the same treatment group.
of EPO on renal function is due to EPO receptor activation and signaling and not due to correction of glucose and/or AGE levels.

**EPO influences renal hypertrophy and albuminuria of diabetic db/db mice.** Assessment of hematological parameters showed slightly elevated hematocrit and hemoglobin values in db/db mice compared with db/m mice, presumably due to volume depletion associated with the development of type 2 diabetes. No increases of hemoglobin or hematocrit were detected in treated diabetic mice compared with placebo-treated diabetic mice, indicating that epiotin-β or CERA at the dosage used (20 IE/kg ip epiotin-β or 1.2 µg/kg ip CERA) did not influence hematopoiesis in db/db mice. However, the mean corpuscular hemoglobin concentration was slightly reduced in EPO-treated mice, which is a known effect of EPO therapy (5) (Table 1). Menne et al. (20) and Schiffer et al. (30) found a less marked increase in kidney weight in long-term (14 wk) CERA-treated db/db mice compared with placebo-treated diabetic mice. Interestingly, we found that diabetes-induced increased kidney weight, as a crude parameter of DN-associated renal hypertrophy, was reduced in db/db mice when they were treated with epiotin-β or CERA for only 2 wk (20 IE/kg ip epiotin-β or 1.2 µg/kg ip CERA, n = 10) and no more significant compared with nondiabetic mice (Table 1). Furthermore, the significantly higher urinary ACR in db/db mice revealed that the animals developed DN, assuming that albuminuria is an essential functional feature of DN (43). As shown in Table 1, treatment of diabetic mice with epiotin-β as well as CERA (20 IE/kg ip epiotin-β or 1.2 µg/kg ip CERA, n = 10) resulted in a significantly reduced albuminuria. These data suggest that even short-term EPO treatment ameliorates the renal hypertrophy as well as proteinuria in diabetic mice.

**Diabetic db/db mice treated with EPO show an expression of p27Kip1 and NRPI similar to nondiabetic mice.** We have previously shown that BSA-AGE induces p27Kip1 upregulation and NRPI downregulation in podocytes. Both effects were abolished by epiotin-β or CERA in culture (29). To test these parameters in vivo, we studied the expression of p27Kip1 (Fig. 2) and NRPI protein (Fig. 3) in the glomeruli of control animals and in db/db animals treated with placebo and EPO (20 IE/kg ip epiotin-β or 1.2 µg/kg ip CERA, n = 10) using immunohistochemical staining. Podocytes of untreated diabetic db/db mice showed markedly increased p27Kip1 protein staining (Fig. 2) and significantly reduced staining of NRPI protein (Fig. 3, A and B) compared with nondiabetic db/m mice. In contrast, in podocytes of EPO-treated diabetic db/db mice, the respective values were similar to those of nondiabetic mice (Figs. 2, A and B, and 3, A and B). To demonstrate the podocyte-specific alteration of both proteins, we performed immunofluorescence double staining of p27Kip1 (Fig. 2C) and NRPI (Fig. 3C) protein with the podocyte-specific marker synaptotodin. These findings indicate that the diabetes-induced effects on podocytes p27Kip1 and NRPI protein, similar to previous cell culture experiments, were abrogated by epiotin-β as well as CERA.

**The induced podocyte loss in diabetic db/db mice is influenced by EPO treatment.** In the mature glomerulus, the expression of Wilms’ tumor-1 is restricted to podocytes (33). Using the Wilms’ tumor-1 COOH-terminal antibody 1, one can identify podocytes accurately and conveniently (33). However, podocyte loss in the kidneys of the diabetic animals was...
reduced after treatment with epoetin-β or CERA (Fig. 4), indicating a role of EPO in the regeneration of the filtration barrier even after short-term administration.

DISCUSSION

Anemia is characteristic of DN (1, 39) and may occur early compared with other nephropathies (8, 18). There is controversial evidence as to whether the correction of anemia may halt or slow the progression of DN (3, 25, 27). However, overzealous correction of anemia in diabetes has been associated with severe side effects and is certainly not recommended (31).

In the present study, we investigated the potential protective effects of EPO receptor activation in DN using a dose of EPO that does not induce hematological changes. We focused on podocyte damage in db/db mice. db/db mice develop progressive nephropathy during the course of the disease with early glomerular hyperfiltration (41). Although hemodynamic fac-

Fig. 2. A–C: p27Kip1 expression. Protein expression of p27Kip1 was significantly upregulated in diabetic db/db mice compared with nondiabetic db/m mice and returned to nondiabetic levels after EPO treatment (20 IE/kg ip epoetin-β or 1.2 μg/kg ip CERA). A and B: representative p27Kip1 staining (A) and semiquantitative analysis of p27Kip1 immunochemistry (B). Magnification: ×400. The red-bordered glomeruli show the areas used for quantification (see METHODS). n = 10 mice/group. #P < 0.05 vs. nondiabetic db/m mice of the same treatment group; *P < 0.05 and **P < 0.01 vs. the placebo-treated diabetic group. C: podocyte-specific expression of p27Kip1. Immunofluorescence double staining with p27Kip1 (Cy-3) and synaptopodin (Alexa 488) demonstrates the podocyte-specific upregulation of the expression of p27Kip1 in placebo-treated db/db mice. Nuclear staining was done with 4',6-diamidino-2-phenylindole (DAPI). Magnification: ×400. Scale bars = 20 μm.
tors (e.g., glomerular hypertension and hyperfiltration), thickening of the glomerular basement membrane, and loss of negatively charged proteoglycans are important factors for the development of proteinuria, recent research has focused on the primary role of podocyte pathology in this process (42). In the present study, overt DN was assumed because db/db mice exhibited significantly increased ACR. Whereas previous studies (20, 30) monitored the effect of long-term EPO treatment on the onset and progression of DN, we focused our interest on the effects of EPO on already fully developed DN. Interestingly, similarly to the work of Menne et al. (20), ACR was also decreased by EPO application in our short-treatment study. In DN, injury to podocytes is mediated by the enhanced formation and accumulation of AGEs and a marked upregulation of their specific receptors (RAGEs) (9). In this experiment, increased CML levels, indicating typical AGE accumulation, in db/db mice were shown. Several studies (20, 30, 42) have documented that both body weight as well as kidney weight are

Fig. 3. A–C: neuropilin 1 (NRP1) expression. Treatment with epoetin-β as well as CERA (20 IE/kg ip epoetin-β or 1.2 μg/kg ip CERA) ameliorated the suppression of NRP1 expression. Representative NRP1 staining (A) and semiquantitative analysis of NRP1 stainings (B) showed that the diabetes-induced suppression of NRP1 protein was reversed after EPO treatment. Magnification: ×400. The red-bordered glomeruli show the areas used for quantification (see METHODS). n = 10 mice/group. #P < 0.05 vs. nondiabetic db/m mice of the same treatment group; **P < 0.01 and ***P < 0.001 vs. placebo-treated diabetic mice. C: podocyte-specific expression of NRP1. The podocyte-specific downregulation of the expression of NRP1 in placebo-treated db/db mice is shown by immunofluorescence double staining with NRP1 (Cy-3) and synaptopodin (Alexa 488). Nuclear staining was done with DAPI. Magnification: ×400. Scale bars = 20 μm.
significantly increased in diabetic db/db mice. In agreement with previous studies (20, 30), we observed a reduction in kidney weight in both EPO-treated groups even after short-term therapy, suggesting that there is a positive effect of EPO on diabetic hypertrophy.

We have previously shown that changes in podocyte pathophysiology, which are implicated in the development and progression of DN, are closely linked with the induction of the cell cycle inhibitor p27Kip1 and a decrease in NRP1 expression (6, 7, 28) and that in diabetic db/db mice, glomerular expression of p27Kip1 is upregulated, whereas glomerular NRP1 expression is downregulated, compared with nondiabetic db/m mice (6, 41). On the other hand, we have documented that p27Kip1 knockout mice are, at least partially, protected from DN (40). Furthermore, we have recently reported that treatment with both EPO molecules (epoetin-β or CERA) protected cultured podocytes from AGE-mediated damage by reducing the enhanced p27Kip1 expression and increasing the suppressed NRP1 expression (29). In the present study, short-term EPO treatment protected glomeruli from db/db mice from the induction of p27Kip1 as well as NRP1 reduction, indicating that EPO receptor activators induce several protective cellular mechanisms in vivo, which prevent the p27Kip1- and NRP1-dependent effects, such as cell cycle arrest, cellular hypertrophy, decreased cell viability and proliferation, and reduced podocyte migration. p27Kip1-mediated podocyte hypertrophy and decreased podocyte migration caused by NRP1 suppression are likely linked to podocytopathy, eventually leading to glomerulosclerosis (6). Other studies on EPO-induced signaling in podocytes have also shown that EPO exerts cell-protective effects leading to improved podocyte survival (20, 30, 35) and demonstrated the phosphorylation of Akt (PKB) and JAK/phosphatiylinositol 3-kinase pathways in podocytes by epoetin-β and CERA (20, 30). Expression of the EPO receptor and EPO-mediated phosphorylation have been recently proven in cultured immortalized mouse podocytes (29). In the glomeruli from adult mice, the EPO receptor was shown to be expressed in podocytes as well as in tubular and endocapillary cells (11). However, not all studies confirmed these results (12).

In addition to structural abnormalities of podocytes in DN, such as foot process widening, the number and density of podocytes have been reported to be markedly reduced in patients and animal models with type 1 and 2 diabetes (4, 15, 22, 30, 33, 36, 38). Broadening of foot process widths and the subsequently increased proteinuria in DN is caused by the decrease in nephrin protein, which is exclusively expressed by podocytes and predominantly localized to the slit diaphragm (22, 36). In agreement with our own results, it has been shown that chronic CERA administration ameliorates diabetes-induced podocyte loss in developing DN (30). Determination of the podocyte number by Wilms’ tumor-1 staining showed that EPO, in particular, mediated podocyte protection by reduced...
podocyte loss in the db/db mouse treatment group. One possible explanation could be that CERA may affect different molecular pathways of diabetic kidney damage, for example, by preventing the loss of glomerular nephrin and perlecan content and by counteracting the increase of transforming growth factor-β1 and VEGF expressions (20). As recently described, in vitro and in vivo treatment with the EPO analog darboepoetin resulted in ameliorated podocyte injury and decreased proteinuria in experimental nephrotic syndrome and was accompanied by preservation of the cytoskeleton and nephrin expression as well as by reduced apoptosis (13, 17).

Taken together, to the best of our knowledge, it has been shown for the first time in this study that not only developing but also overt DN can be positively influenced by EPO or the analog CERA. In addition, not only chronic but also short-term treatment of EPO ameliorated increased albuminuria and renal hypertrophy, independently from hematopoietic effects and without influencing the development of diabetes as well as AGE accumulation. Furthermore, changes of p27Kip1 and NRPI expression in podocytes of db/db mice were reversed by EPO.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


