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 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 the receptor in several in vivo and in vitro models of DN was significantly lower in podocyte-derived conditioned media treated with CERA compared with CERA alone (28). The receptor of AGEs (RAGE) is upregulated in cells such as 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 RAGE 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 may be initially partly compensated to a certain extent by podocyte hypertrophy and by covering the resulting decorated atrophy and thereby “nude” visceral space of the glomerular basement membrane by migrating processes of surviving podocytes (16). Yet, AGE-mediated reduced NR1P 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, NR1P expression is also decreased in kidney biopsies from patients with DN as well as in diabetic db/db mice (6). Independent from its hematopoetic 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 NR1P reduction and associated impaired cell migration. We hypothesized that in diabetic metabolism, the changes of p27Kip1 and NR1P 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 leprophoetin protective properties of EPO in overt DN in vivo in db/db mice, a well-characterized model of type 2 diabetes, focusing on p27Kip1 and NR1P 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-Dock7mLeprdb/+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 epoietin-β (NeoRecombin, Hoffmann-La Roche, Grenzach-Wyhlen, Germany) three times per week (n = 10) or 1.2 μg/kg ip continuous EPO receptor activator (CERA; MIRCERA, Hoffmann-La Roche) once per week (n = 10). Concentrations of epoietin-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, Norderstedt, Germany). At the end of the experiment, mice were placed in metabolic cages (Tecniplast, Buguggiate, Italy) to collect 24-h urine. To quantify albuminuria, the urinary albumin-to-creatinine ratio (ACR) was determined. Urinary albumin excretion was measured using an ELISA specific for mouse albumin (Cell Trend, Luckenwalde, Germany), and urinary creatinine was measured with a standard enzymatic assay (Cayman Chemicals, Ann Arbor, MI). Mice were killed 15 days after the onset of the study, and the kidneys were removed. One kidney per mouse was fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological and immunohistochemical experiments. Mouse serum glucose levels were measured with Fuji Dri-chem Slides Glu-PIII (Fujifilm Europe, Düsseldorf, Germany). At the end of the experiment, mice were killed 15 days after the onset of the study, and the kidneys were removed. One kidney per mouse was fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological and immunohistochemical experiments. Mouse serum glucose levels were measured with Fuji Dri-chem Slides Glu-PIII (Fujifilm Europe, Düsseldorf, Germany). At the end of the experiment, mice were killed 15 days after the onset of the study, and the kidneys were removed. One kidney per mouse was fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological and immunohistochemical experiments. Mouse serum glucose levels were measured with Fuji Dri-chem Slides Glu-PIII (Fujifilm Europe, Düsseldorf, Germany).

Nε-carboxymethyllysine serum concentrations. Nε-carboxymethyl-lysine (CML) is a common, chemically defined AGE species in vivo that accumulates in the diabetic milieu. CML serum levels were determined using an AGE-CML ELISA according to the manufacturer’s instructions (Microcat Biotechnologie, Bernried, Germany).

Immunohistochemistry and immunofluorescence. Deparaffinized kidney sections (4 μm thick) were subjected to heat-mediated antigen retrieval in citrate buffer (pH 6.0) and then incubated with 3% H2O2 for 10 min at room temperature to block endogenous peroxidase. As primary antibodies, polyclonal rabbit anti-CML (Roche Diagnostics, Penzberg, Germany), polyclonal rabbit anti-NRP1 (Santa Cruz Biotechnology, Santa Cruz, CA), and polyclonal rabbit anti-p27Kip1 (Cell Signaling Technology, Danvers, MA) antibodies were used. Staining was performed with Vectastain Elite ABC Kits (Vector Laboratories, Burlingame, CA) and aminomethylcarbazole as a chromogen. For negative controls, the primary antibody was replaced by rabbit IgG at the same concentration as the primary antibody. For the quantification of CML, NRP1, and p27Kip1, staining intensity was examined by an investigator who was unaware of the origin of the groups. For the quantitative assessment of CML, NRP1, 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: ×400). For each parameter, all images were taken under constant conditions as appropriate. After the glomerular area was highlighted, the mean densestrophic 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-synaptophysin 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, epoietin-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, epoietin-β, or CERA |
|---|---|---|---|---|---|---|---|
| | db/m Mice | | | db/db Mice | | | |
| | Placebo | Epoietin-β | CERA | Placebo | Epoietin-β | CERA |
| Body weight, g | 23.9 ± 1.2 | 22.5 ± 1.1 | 22.5 ± 0.8 | 44.4 ± 1.0±±± | 37.4 ± 2.7±±± | 40.7 ± 0.7±±± |
| Kidney weight, mg | 128.4 ± 7.6 | 139.8 ± 11.3 | 153.7 ± 7.8 | 170.0 ± 5.5±± | 158.9 ± 10.4 | 154.4 ± 7.8 |
| Serum glucose, mmol/l | 4.1 ± 0.8 | 3.3 ± 0.3 | 3.5 ± 0.2 | 7.8 ± 1.4± | 8.7 ± 1.8±± | 8.6 ± 1.4±± |
| Albumin-to-creatinine ratio, mg/g | 15.5 ± 2.8 | 27.2 ± 5.2 | 29.6 ± 3.5 | 119.3 ± 13.3±± | 59.3 ± 17.3±± | 73.7 ± 59.3±± |
| Hematocrit, % | 46.6 ± 0.3 | 45.0 ± 0.2 | 54.0 ± 0.2* | 4.9 ± 0.2 | 4.9 ± 0.2 | 4.9 ± 0.2 |
| Hemoglobin, mmol/l | 9.1 ± 0.2 | 8.3 ± 0.3 | 9.8 ± 0.3* | 9.5 ± 0.2 | 8.7 ± 0.3* | 9.1 ± 0.3 |
| Mean corpuscular volume, μm³ | 45.6 ± 1.5 | 49.1 ± 0.3* | 49.1 ± 0.4* | 49.5 ± 1.2 | 51.8 ± 0.5±± | 50.4 ± 1.2 |
| Mean corpuscular hemoglobin, fmol | 0.91 ± 0.01 | 0.91 ± 0.01 | 0.89 ± 0.01 | 0.94 ± 0.01† | 0.97 ± 0.01††† | 0.94 ± 0.01††† |
| Mean corpuscular hemoglobin concentration, mmol/l | 20.0 ± 0.7 | 18.4 ± 0.1* | 18.0 ± 0.1* | 19.5 ± 0.6 | 18.6 ± 0.3 | 18.8 ± 0.4 |

Values are means ± SE; n = 10 mice/group. CERA, continuous erythropoietin receptor activator. *P < 0.05 and **P < 0.01, epoietin-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.

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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 epoetin-β or CERA at the dosage used (20 IE/kg ip epoetin-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 epoetin-β or CERA for only 2 wk (20 IE/kg ip epoetin-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 epoetin-β as well as CERA (20 IE/kg ip epoetin-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 NRP1 similar to nondiabetic mice. We have previously shown that BSA-AGE induces p27Kip1 upregulation and NRP1 downregulation in podocytes. Both effects were abolished by epoetin-β or CERA in culture (29). To test these parameters in vivo, we studied the expression of p27Kip1 (Fig. 2) and NRP1 protein (Fig. 3) in the glomeruli of control animals and in db/db animals treated with placebo and EPO (20 IE/kg ip epoetin-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, A and B) and significantly reduced staining of NRP1 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 NRP1 (Fig. 3C) protein with the podocyte-specific marker synaptopodin. These findings indicate that the diabetes-induced effects on podocytes p27Kip1 and NRP1 protein, similar to previous cell culture experiments, were abrogated by epoetin-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
red 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-
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 decreased by EPO application.

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 p27 Kip1 is upregulated, whereas glomerular NRP1 expression is downregulated, compared with nondiabetic db/m mice of the same treatment group; ***P < 0.001 vs. nondiabetic db/m mice of the same treatment group. 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 diabetes-induced podocyte loss. Representative nuclear staining of the podocyte marker Wilms’ tumor-1 (WT-1; Cy-3; A) and the podocyte number (B) showed in placebo-treated db/db mice a significantly lower podocyte number compared with nondiabetic db/m mice as well as EPO-treated db/db mice. Magnification: ×400. One hundred glomerular cross sections were quantified per group (n = 10 mice/group). ##P < 0.01 and ###P < 0.001 vs. nondiabetic db/m mice of the same treatment group; **P < 0.01 and ***P < 0.001 vs. the placebo-treated diabetic group.

Fig. 4. A and B. Podocyte number. EPO treatment (20 IE/kg ip epoetin-β or 1.2 μg/kg ip CERA) protected glomeruli from diabetes-induced podocyte loss. Representative nuclear staining of the podocyte marker Wilms’ tumor-1 (WT-1; Cy-3; A) and the podocyte number (B) showed in placebo-treated db/db mice a significantly lower podocyte number compared with nondiabetic db/m mice as well as EPO-treated db/db mice. Magnification: ×400. One hundred glomerular cross sections were quantified per group (n = 10 mice/group). ##P < 0.01 and ###P < 0.001 vs. nondiabetic db/m mice of the same treatment group; **P < 0.01 and ***P < 0.001 vs. the placebo-treated diabetic group.
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 darbepoetin 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 NR1P1 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


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