Erythropoietin ameliorates podocyte injury in advanced diabetic nephropathy in the \textit{db/db} mouse

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Running Title: Erythropoietin and podocytes injury in DN

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ABSTRACT

Podocyte damage and accumulation of advanced glycation end-products (AGEs) are characteristic 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 cell cycle inhibitor p27^Kip1 and to the inhibition of neuropilin 1 (NRP1). We previously demonstrated that treatment with erythropoietin is associated with protective effects for podocytes in vitro. db/db mice with overt DN aged 15-16 weeks were treated with either placebo, or epoetin-β, or CERA (continuous erythropoietin receptor activator) for 2 weeks. db/db mice compared with non-diabetic db/m controls revealed the expected increases in body weight, blood glucose, albumin-to-creatinine ratio (ACR) and AGE-accumulation. Whereas no differences in body weight, hyperglycemia and AGEs were observed among the diabetic groups receiving epoetin-β resp. CERA and placebo indicating that epoetin-β/CERA treatment does not interfere with the development of diabetes in this model. However, the ACR were significantly lower in db/db mice treated with epoetin-β or CERA. Furthermore, kidney weights in db/db mice were increased compared with the db/m controls indicating renal hypertrophy, whereas the increase in renal weight in epoetin-β- or CERA-treated db/db was significantly lower than in the placebo-treated controls. Induction of p27^Kip1 and suppression of NRP1 were significantly reduced in the epoetin-β resp. 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 the NRP1 and p27^Kip1 expressions as well as the number of podocytes returned to normal level. Our data show for the first time that
medication of overt DN with erythropoietin for a short time can ameliorate albuminuria and podocyte loss.

**Key Words:** Erythropoietin (EPO), CERA, diabetic nephropathy (DN), podocytes, *db/db* mouse

**INTRODUCTION**
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 multi-ligand member of the immunoglobulin 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 diabetic nephropathy 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 Neuropilin-1 (NRP1) was reduced by AGEs (6,7). Due to their quiescent phenotope, podocytes cannot re-entry 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 de-coated and
thereby “nude” visceral space of the glomerular basement membrane by migrating processes of surviving podocytes (16). Yet, AGE-mediated reduced NRP1 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, NRP1 expression is also decreased in kidney biopsies from patients with DN as well as in diabetic db/db mice (6).

Independently from its hematopoetic effects, erythropoietin may be protective for several tissues including heart, brain and kidney mainly, presumably by prevention of ischemic damage and by anti-apoptotic pro-survival effects (14,24). Podocytes express erythropoietin receptors and therefore can respond to haematopoietic growth factor stimulation (11). Others, elegantly showed in vivo and in vitro that treatment with erythropoietin or its analogues ameliorated podocyte injury by protective effects on nephrin expression and the cytoskeleton, by reduction of apoptosis or by activation of pro-survival intracellular pathways (13,17,30). More recently, in a model of kidney allograft injury, the non-hemodynamic nephro-protective potential of erythropoietin substitution was outlined by comparison with blood transfusions (10).

Recently, we demonstrated in cultured podocytes that in AGE-mediated injury the addition of erythropoietin prevented p27Kip1 mediated cell-cycle arrest and podocyte hypertrophy as well as NRP1 reduction and associated impaired cell migration (29). We hypothesize, that in diabetic metabolism the changes of p27Kip1 and NRP1 expression and their functional consequences on podocytes can be ameliorated in a mouse model of overt DN by erythropoietin. Therefore, the present study was performed to evaluate the potential nephroprotective properties of erythropoietin in overt DN in vivo in db/db, a well-characterized model of type 2 diabetes mice
focusing on p27Kip1 and NRP1 expressions and on the related functional changes in podocytes.

**METHODS**

*Animal Model and Study Protocol*

All animal experiments were approved by the local ethics committee and were done in accordance with the German Animal Protection Law. We studied diabetic *db/db* (B6.Cg-Dock7m *Lepr*db/++/J) and non-diabetic *db/m* mice (Jackson Laboratory, Bar Harbor, ME) as controls. The *db/m* and *db/db* animals were treated with either 20 I.E./kg i.p. epoetin-β (NeoRecormon, Hoffmann-La Roche, Grenzach-Wyhlen, Germany) three times per week (n = 10) or 1.2 µg/kg i.p. CERA (continuous erythropoietin receptor activator, MIRCERA, Hoffmann-La Roche, Grenzach-Wyhlen, Germany) once per week (n = 10). The concentrations of epoetin-β and CERA were chosen according to generally used doses in clinical practice applying to patients with renal disease, and previous published experiments (30). Non-diabetic *db/m* (n = 10) and diabetic *db/db* (n = 10) were injected with 0.9% i.p. NaCl (placebo) as the control. All animals were maintained in a pathogen-free facility and had free access to water and were on the standard rodent chow. All mice were 16 weeks old at the beginning of the study and only male mice were used to control for potential hormonal effects. We assessed hematocrit, hemoglobin, MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), and MCHC (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 (Techniplast, Buguggiate, Italy) to collect the 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, USA). 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 studies. Mouse serum glucose levels were measured with Fuji Dri-chem Slides Glu-PiII (Fujifilm Europe, Düsseldorf, Germany).

*Nε-Carboxymethyllysine (CML) serum concentrations*

CML is a common, chemically defined AGE species *in vivo* accumulating in the diabetic milieu. The CML serum levels were determined by using the AGE-CML ELISA according to the manufacturer's instructions (Microcoat Biotechnologie GmbH, 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 a polyclonal rabbit anti-CML (Roche Diagnostics, Penzberg, Germany), a polyclonal rabbit anti-NRP1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and a polyclonal rabbit anti-p27Kip1 (Cell Signaling Technology, Inc., Danvers, MA, USA) antibody were used. Staining was performed with the Vectastain® Elite ABC Kits (Vector Laboratories, Burlingame, CA, USA) and aminoethylcarbazole as a chromogen. For negative controls, the primary antibody was replaced by rabbit immunoglobulin in the same concentration as the primary antibody. For quantification of CML, NRP1 and p27Kip1, staining intensity was examined by an investigator who was unaware of the origin of the groups. For 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 AG, Germany). Ten non-overlapping 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 highlighting the glomerular area, the mean densitometric grey levels were measured. Finally, the average of grey 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% H_2O_2 incubation. Mouse anti-synaptopodin antibody was purchased from Acris (Herford, Germany) and rabbit anti-WT1 C-terminal antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Staining was performed using an anti-mouse IgG-Alexa 488- respectively anti-rabbit IgG-Cy3-linked secondary antibody (Life Technologies, Carlsbad, CA, USA).

**Statistical Analysis**

The values given in this article are presented as mean ± S.E.M. Results were analyzed using SPSS statistics (IBM company, Armonk, NY). The Kruskal-Wallis test was used for multigroup comparison followed by the Mann-Whitney rank sum test to compare two groups of mice. A P value of ≤ 0.05 was 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 for modeling DN in type 2 diabetes (2). The underlying genetic background is susceptible to diabetic complications such as nephropathy. db/db mice in the age until 6 weeks show body weights and blood glucose levels, which are similar to those of the db/m littermates; db/db mice become obese and develop hyperglycemia with the age of 6 to 8 weeks (20). The DN in these mice is characterized by albuminuria, podocyte loss, and mesangial matrix expansion (2). In contrast to previous publications (e.g. 20,30), we used mice in the age of 16 weeks 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 (Figure 1). As expected diabetic db/db mice exhibited significantly increased in body weight, hyperglycemia and Nε-Carboxymethyllysine (CML) serum concentrations as well as glomerular CML-accumulation compared to the non-diabetic db/m mice. Epoetin-β- or CERA-treatment (20 I.E./kg i.p. epoietin-β, 1.2 µg/kg i.p. CERA; n = 10) exhibited no significant effects on these parameters, suggesting that any possible effect 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 compared with the db/m mice, presumably due to volume depletion associated with the development of type 2 diabetes. No increase of hemoglobin or hematocrit were detected in treated diabetic mice in comparison to the
placebo-treated diabetic animals, indicating that epoetin-β or CERA in the used dosage (20 I.E./kg i.p. epoetin-β, 1.2 µg/kg i.p. CERA) did not influence hematopoiesis in db/db mice. However, the mean corpuscular hemoglobin concentration (MCHC) was slightly reduced in EPO-treated mice, which is a known effect of erythropoietin therapy (5) (Table 1). Menne et al. and Schiffer et al. have been observed a less marked increase in kidney weight in long-term (14 weeks) CERA-treated db/db mice as compared with placebo-treated diabetic mice (20,30). Interestingly, we found that the diabetes-induced increased kidney weight, as a crude parameter of DN-associated renal hypertrophy, was reduced in db/db mice when treated with epoetin-β or CERA for only two weeks (20 I.E./kg i.p. epoetin-β, 1.2 µg/kg i.p. CERA; n = 10) and no more significant to non-diabetic animals (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 I.E./kg i.p. epoetin-β, 1.2 µg/kg i.p. 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 p27^Kip1 and NRP1 similarly to non-diabetic mice

We showed previously that bovine serum albumin-AGE induces p27^Kip1 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 p27^Kip1 (Figure 2) and NRP1 protein (Figure 3) in the glomeruli of control animals and in the db/db animals treated with placebo and EPO (20 I.E./kg i.p. epoetin-β, 1.2
µg/kg i.p. CERA; n = 10) using immunohistochemical staining. Podocytes of untreated diabetic \(db/db\) mice showed a markedly increased \(p27^{Kip1}\) protein staining (Figure 2, A and B) and a significantly reduced staining of NRP1 protein (Figure 3, A and B) compared with non-diabetic \(db/m\) animals. In contrast, in podocytes of EPO-treated diabetic \(db/db\) mice respective values were similar to those of non-diabetic animals (Figure 2, A and B; Figure 3, A and B). To demonstrate the podocyte specific alteration of both proteins, we performed immunofluorescence double staining of \(p27^{Kip1}\) (Figure 2C) and NRP1 (Figure 3C) protein with the podocyte-specific marker synaptopodin. These findings indicate that the diabetes-induced effects on podocytes \(p27^{Kip1}\) and NRP1 protein, similar to previous cell culture experiments, were abrogated by epoetin-\(\beta\) 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 (WT-1) is restricted to podocytes (33). By using the WT-1 C-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-\(\beta\) or with CERA (Figure 4), indicating a role of erythropoietin in regeneration of the filtration barrier even after short-term administration.
DISCUSSION

Anemia is characteristic of DN (1,39) and may occur earlier compared to other nephropathies (8,18). There is controversial evidence 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 erythropoietin receptor activation in DN using a dose of erythropoetin that does not induce hematological change. We focused on podocyte damage in db/db mice. db/db mice developed a progressive nephropathy during the course of the disease with early glomerular hyperfiltration (41). Although hemodynamic factors (e.g. glomerular hypertension and hyperfiltration), thickening of the GBM, 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 a 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), the ACR was also decreased by erythropoietin 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 (RAGE) (9). In this experiment increased CML levels indicating typical AGE accumulation in db/db mice was shown. Several studies have documented that both body weight as well as kidney weight are significantly increased in diabetic db/db mice (20,30,42). 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 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 $p27^{Kip1}$ and a decrease in NRP1 expression (6,7,28) and that in diabetic $db/db$ mice the glomerular expression of $p27^{Kip1}$ is upregulated whereas the glomerular NRP1 expression is downregulated compared to non-diabetic controls (6,41). On the other hand, we have documented that $p27^{Kip1}$ 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 $p27^{Kip1}$ expression and increasing the suppressed NRP1 expression (29). In our current study, short-term erythropoietin treatment protected glomeruli from $db/db$ mice from induction of $p27^{Kip1}$ as well as NRP1 reduction, indicating that the erythropoietin receptor activators induce several protective cellular mechanisms in vivo, which prevent the $p27^{Kip1}$- and NRP1-dependent effects, such as cell cycle arrest, cellular hypertrophy, decreased cell viability and proliferation as well as reduced podocyte migration. $p27^{Kip1}$-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 signalling 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 (protein kinase B) and JAK/PI3K pathways in podocytes by epoetin-β and CERA (20,30). Expression of the erythropoietin receptor and erythropoietin mediated phosphorylation has been proven in cultured immortalized mouse podocytes recently (29). In the glomeruli from adult mice the erythropoietin receptor was shown to be
expressed in podocytes as well as in tubular and endocapillary cells (11). However not all could confirm 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 subsequently increased proteinuria in DN is caused by the decrease in the 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 WT-1 staining showed that erythropoietin in particular mediated podocyte protection by reduced podocyte loss in the db/db mice 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 counteracting the increase of TGF-β1 and VEGF expressions (20). As described recently, in vitro and in vivo treatment with erythropoietin analogue 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 erythropoietin or the analog CERA. In addition, not only chronic but also short-term treatment of EPO ameliorated increased albuminuria and renal hypertrophy, independently from hematopoetic effects and without influencing the development of diabetes as well as AGE accumulation. Furthermore, changes of
p27^Kip1 and NRP1 expression in podocytes of db/db mice, were reversed by erythropoietin.

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DISCLOSURE

All the authors declared no competing interests.
REFERENCES


34 Tanji N, Markowitz GS, Fu C, Kislinger T, Taguchi A, Pischetsrieder M, Stern D, Schmidt AM, D'Agati VD. Expression of advanced glycation end products


LEGENDS TO FIGURES

Fig. 1. A-C. Advanced glycation end-products. Compared with db/m control mice the diabetic db/db mice show significantly increased serum CML levels (A), and CML deposition in the kidney (B, C), whereas no significant changes were observed between the untreated and EPO-treated (20 I.E./kg i.p. epoietin-β, 1.2 µg/kg i.p. CERA) diabetic groups. (B) Representative CML staining (magnification: 400 x). The red-bordered glomeruli show the areas used for quantification (see method section). (C) Semiquantitative analysis of CML-IHC. Data represent mean values ± SEM. Statistical significance compared to db/m mice of the same treatment indicated by # p < 0.05, ### p < 0.001; n = 10 per group.

Fig. 2. A-C. p27Kip1 expression. The protein expression of p27Kip1 is significantly upregulated in diabetic db/db mice compared to non-diabetic db/m, and returned to non-diabetic levels after EPO treatment (20 I.E./kg i.p. epoietin-β, 1.2 µg/kg i.p. CERA). Representative p27Kip1 staining (A) and semiquantitative analysis of p27Kip1-immunohistochemistry (B). Magnification: 400 x; The red-bordered glomeruli show the areas used for quantification (see method section). # p < 0.05 versus non-diabetic db/m mice of the same treatment; * p < 0.05, ** p < 0.01 versus diabetic placebo group; n = 10 per 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 (DAPI). Magnification: 400 x. Scale bars: 20 µm.

Fig. 3. A-C. NRP1 expression. Treatment with epoetin-β as well as CERA (20 I.E./kg i.p. epoietin-β, 1.2 µg/kg i.p. CERA) ameliorates the suppression of neuropilin 1
(NRP1) expression. Representative NRP1 staining (A) and semiquantitative analysis of NRP1 stainings (B) show that the diabetes-induced suppression of NRP1 protein is reversed after EPO-treatment. Magnification: 400 x; The red-bordered glomeruli show the areas used for quantification (see method section). # p < 0.05 versus non-diabetic db/m mice of the same treatment; ** p < 0.01, *** p < 0.001 versus diabetic placebo group; n = 10 per group. (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 (DAPI). Magnification: 400 x. Scale bars: 20 µm.

Fig. 4. A and B. Podocyte number. Erythropoietin-treatment (20 I.E./kg i.p. epoietin-β, 1.2 µg/kg i.p. CERA) protects glomeruli from diabetes-induced podocyte loss. Representative nuclear staining of the podocyte marker WT-1 (Cy-3) (A) and the podocyte number (B) show in placebo-treated db/db mice a significantly lower podocyte number compared with non-diabetic db/m mice as well as EPO-treated db/db mice. Magnification: 400 x; ## p < 0.01, ### p < 0.001 versus non-diabetic db/m mice of the same treatment; ** p < 0.01, *** p < 0.001 versus diabetic placebo group; 100 glomerular cross sections were quantified per group (n = 10 per group).
Figure 1 A-C
Figure 2 A-C
Figure 3 A-C
Figure 4 A, B
Table 1. Clinical/laboratory data and parameters of kidney function in non-diabetic db/m mice and in db/db mice treated with placebo and epoetin-β or CERA.

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* treatment versus placebo of the same genotype.

# db/db versus db/m of the same treatment.
CERA, continuous erythropoietin receptor activator.

ACR, albumin-to-creatinine ratio.

MCV, mean corpuscular volume.

MCH, mean corpuscular hemoglobin.

MCHC, mean corpuscular hemoglobin concentration.

Values are means ± SEM (n = 10).