Original Article

MOLECULAR METABOLISM

Targeting erythropoietin protects against proteinuria in type 2 diabetic patients and in zebrafish

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ABSTRACT

Objective: Adult human kidneys produce erythropoietin (EPO), which regulates red blood cell formation; however, whether EPO also functions directly on kidney development and controls diabetic kidney disease remains unknown. Here we analyzed the role of EPO in kidney development and under hyperglycemic conditions in zebrafish and in humans.

Methods: Diabetic patients and respective controls were enrolled in two cohorts. Serum EPO level and urine protein change upon human EPO administration were then analyzed. Transient knockdown and permanent knockout of EPO and EPOR in renal TG(WT1B:EGFP) zebrafish were established using the morpholino technology and CRISPR/Cas9 technology. Zebrafish embryos were phenotypically analyzed using fluorescence microscopy, and functional assays were carried out with the help of TexasRed labeled 70 kDa Dextran. Apoptosis was determined using the TUNEL assay and Annexin V staining, and caspase inhibitor zVADfmk was used for rescue experiments.

Results: In type 2 diabetic patients, serum EPO level decreased with the duration of diabetes, which was linked to reduced kidney function. Human recombinant EPO supplementation ameliorated proteinuria in diabetic nephropathy patients. In zebrafish, loss-of-function studies for EPO and EPOR, showed morphological and functional alterations within the pronephros, adversely affecting pronephric structure, leading to slit diaphragm dysfunction by increasing apoptosis within the pronephros. Induction of hyperglycemia in zebrafish embryos induced pronephros alterations which were further worsened upon silencing of EPO expression.

Conclusions: EPO was identified as a direct renal protective factor, promoting renal embryonic development and protecting kidneys from hyperglycemia induced nephropathy.

Keywords Erythropoietin; Type 2 diabetes; Diabetic nephropathy; Zebrafish

1. INTRODUCTION

Diabetic nephropathy (DN) [1], a common diabetes microvascular complication, ranks first as the cause of end-stage renal disease (ESRD) globally [2]. Accumulating evidence suggests that renal hyperfiltration and renal injury, oxidative stress, and apoptotic signals are all involved in the pathogenesis of DN [3–6]. It was also shown that polymorphisms in some genes were associated with a heightened incidence of diabetic nephropathy [7,8]. However, genetic mechanisms for the onset of diabetic nephropathy are still poorly understood and thus limit the therapeutic options for patients.

Erythropoietin (EPO) is a glycoprotein hormone traditionally considered essential for erythropoiesis [9,10]. Recent studies revealed a relationship between EPO and the progression of diabetic complications [11–13]. In addition, single nucleotide polymorphisms (SNPs) in the human *EPO* gene were suggested to be a potential contributing factor for the development of DN in various global populations [14]. Functional EPO and EPOR expression have been demonstrated in both human and murine kidney tubular epithelial, glomerular endothelial, and mesangial cells, suggesting that EPO may play a physiological role in both renal development and renal disease states through interactions with EPOR [15]. In adult human, EPO is mainly produced in kidney interstitial fibroblast and then released into circulation to exert its subsequent effects [16,17]. As a result, global EPO silencing animal models are required to explore its underlying functions. However, studies using EPO knockout or knockdown animal models beyond the embryonic stage have rarely been reported, likely due to embryonic lethality [18–21]. In addition, the mechanisms by which EPO functions in renal system in vivo remains largely unknown.

With the development of novel genome-editing techniques and renal reporter lines and due to its translucency [22], the zebrafish is an attractive model for carrying out research in various biomedical fields, including renal and diabetes studies [23]. The two to three day-old zebrafish pronephros consists simply of two glomeruli, which fuse at the embryonic midline and are connected by pronephric tubules to the bilateral pronephric ducts [24]. The simplicity and rapid development

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Abbreviations and Acronyms

DM	diabetes mellitus
DN	diabetic nephropathy
EPO	erythropoietin
EPOR	erythropoietin receptor
hEPO	human recombinant erythropoietin
hpf	hour post fertilization
dpf	day post fertilization
hpi	hour post injection

of pronephros in zebrafish make it an useful model for investigating the morphology and function of kidney in the context of a developing organism [25]. In addition, it was reported that pronephros cell types are also highly conserved between zebrafish and mammals [24]. Thus, the present studies took advantage of the zebrafish model to dissect the developmental pathways and regulations in renal development and pathogenesis under hyperglycemic conditions.

In this study, we provide evidence that EPO is a clinically-protective factor in the progression of diabetic complications. In addition, by analyzing transient and permanent loss-of-function models for EPO and EPOR in zebrafish, we have identified EPO as an essential regulator of pronephros development and function by interacting with its receptor EPOR and thereby repressing apoptosis. In summary, the present study identifies EPO as an active renal antiapoptotic factor, protecting kidney from hyperglycemia-induced damage and proteinuria in an EPOR-dependent manner both in zebrafish and in type 2 diabetic patients.

2. MATERIAL AND METHODS

2.1. Serum EPO concentrations from DM and non-DM patients

Blood samples from DM and non-DM patients were collected on admission after overnight fasting and stored at −70 °C prior to analysis. Type 2 diabetes was diagnosed on initial admission, and the diabetes duration was recorded accordingly. The diagnosis of Type 2 diabetes was made according to AACE Diagnostic Criteria for Glucose Abnormalities [26]. Exclusion criteria included patients with uncorrected anemia, active EPO supplementary treatment, dialysis, cardiovascular disease, cancer, and GFR <30 ml/min. The demographic data are listed in Supplementary Table 1. Erythropoietin was measured using a sandwich enzyme-linked immunoassay (Quantikine® IVD EPO ELISA and Quantikine® Immunoassay R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. The study protocol was approved beforehand by the Medical Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University, and the procedures followed were in accordance with the institutional guidelines. This study complied with the Declaration of Helsinki, and formal consent was obtained from all patients.

2.2. Study design and participants of hEPO injection in end-stage diabetic nephropathy patients

Consecutive patients (Supplementary Table 2) admitted to the nephrology department of the First Affiliated Hospital of Xi'an Jiaotong University for diabetic nephropathy between May 2013 and March 2017 were selected. The inclusion criteria were: 1) confirmed admission diagnosis of Type 2 diabetic nephropathy, and 2) continuous follow-up measurements of urine protein concentration and 24 h urine protein collection. The exclusion criteria were: 1) diabetic ketosis or nonketotic hyperosmolar coma, and 2) severe nondiabetic disease with expected survival of less than 1 year and unwillingness to participate. A patient was included only once. Information about present medication and a detailed medical history were obtained via hospital medical records. Diabetic nephropathy and proteinuria were defined according to the universal definition criteria by the American Diabetes Association criteria [26]. Twenty-four h urine protein was collected from the first morning urine and urine protein concentration were measured using biuret techniques [27]. Urine protein concentration change and 24 h urine protein change was defined as the subtracted level of urine protein or 24 h urine protein by 2 follow-ups. Written informed consent was obtained from all study participants, with ethnic committee approval at the First Affiliated Hospital of Xi'an Jiaotong University.

2.3. Gene profile analysis and co-expression analysis

The gene expression data GSE30528 [28], including 9 diabetic kidney disease and 13 control glomeruli samples from micro dissected human kidney, and the microarray gene expression data GSE30529 [28], including 10 diabetic kidney disease and 12 control tubule samples from micro dissected human kidney, were obtained from the Gene Expression Omnibus [29] database. Pearson's correlation coefficient method [30] was utilized to identify the co-expression as well as differential co-expression of EPO and EPOR along with kidney pathogenesis related genes, including Notch1, WT1, Pax1, NPHS1, and NPHS2 in the kidney section from DN patients and control. For co-expression analysis of EPO and proteinuria related genes, GeneMANIA (http://genemania.org/; accessed April, 2017), a real-time multiple association network integration algorithm for predicting gene function, was used for analyzing gene−gene interactions in the study [31].

2.4. Zebrafish lines

Embryos of the *TG(WT1B:EGFP)* line were raised and staged as described. Embryos were kept in E3 medium (5 mM NaCl, 0.17 mM KCL, 0.33 mM $CaCl_2$, 5−10% methylene blue) at 28.5 °C with 0.003% 1-phenyl-2-thiourea (PTU) (Sigma) to suppress pigmentation and staged according to somite number or hours post-fertilization (hpf).

2.5. Inhibitors and reagents

Texas-Red® tagged 70 kDa dextran (Molecular Probes) was used for renal functional assays. Proteinase K (10 mg/ml stock) was utilized for embryo digestion and linearized plasmid treatment (Roche Recombinant PCR grade). Zebrafish embryos were subjected to 300 μM of the pan caspase inhibitor, zVAD-fmk (Sigma−Aldrich) incubation at 24 hpf for 24 h.

2.6. Injections of morpholinos and intracardiac injection of human EPO in zebrafish

EPO (ENSDARG00000055163.7, HYPERLINK: http://www.ensembl.org/Danio_rerio/Gene/Summary?db=core;g=ENSDARG00000055163;r=7:21624135-21652094) and EPOR (ENSDARG00000090834.4, HYPERLINK: http://www.ensembl.org/Danio_rerio/Gene/Summary?db=core;g=ENSDARG00000090834;r=3:14394007-14414315) morpholinos were selected as previously described [32,33] and produced by Gene Tools™ (Philomath, OR, USA). Morpholino sequences are listed in Supplementary Table 3. Dose escalation studies were performed to determine submaximal morpholino concentrations (Supplementary Figure 2C, D). EPO, EPOR and Pdx1 morpholino were diluted to 4 or

