Life Sciences 135 (2015) 77–86

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Extracellular superoxide dismutase ameliorates streptozotocin-induced rat diabetic nephropathy via inhibiting the ROS/ERK1/2 signaling☆,☆☆

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ARTICLE INFO

Article history:
Received 6 December 2014
Received in revised form 26 March 2015
Accepted 25 April 2015
Available online 23 May 2015

Keywords:
Extracellular superoxide dismutase
Diabetic nephropathy
Mitogen activated protein kinase
Angiotensin II receptor 1
Transforming growth factor-beta

ABSTRACT

Aim: Diabetic nephropathy is the leading cause of end stage renal disease in developed countries throughout the world. The imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system is the main problem that is responsible for the progression of diabetic kidney disease. In this study, we investigated whether human extracellular superoxide dismutase (hEC-SOD) can prevent diabetic nephropathy in the rat model.

Main methods: Diabetic nephropathy symptoms were induced by intraperitoneal injection with 60 mg/kg streptozotocin (STZ) in male Sprague–Dawley (SD) rats. After daily supplement of rhEC-SOD (3200 U/kg/day) for 4 weeks, the serum or urine biochemical markers (glucose, creatinine, blood urea nitrogen, triglyceride, hemoglobin A1c, and microalbuminuria), histological changes, gene expressions (*phox47*, *opn*, and *gapdh*), and protein levels (TGF-β, AT1-R, phospho-p42/p44 MAPK, and p42/p44 MAPK) were determined.

Key findings: Results showed that rhEC-SOD administration could reverse SOD activity measured in kidney and diabetic-associated changes, including the fibrosis change, expression of collagen I, transforming growth factor-beta (TGF-β) and angiotensin II type I receptor (AT1-R), as well as the activation of the intracellular mitogen-activated protein kinase (MAPK) signaling pathway, associating with its inhibition of $p42^{MAPK}$/$p44^{MAPK}$ (ERK1/2) phosphorylation. Additionally, diabetic nephropathy up-regulated the expression of the *phox47* and *opn* genes, and these changes could also be suppressed. Though the proteinuria did not significantly reduce. Treatment with rhEC-SOD ameliorates STZ-induced diabetic nephropathy, leading to reduced death rates, kidney weight/body weight ratio, fibrosis change, and TGF-β1 expression through the down-regulation of ROS/ERK1/2 signaling pathway.

Significance: We conclude that rhEC-SOD can act as a therapeutic agent to protect the progression of diabetic nephropathy.

Abbreviations: AGEs, advanced glycation end products; AT1-R, angiotensin II type I receptor; EC-SOD, extracellular superoxide dismutase; ERK-1, $p44^{MAPK}$; ERK-2, $p42^{MAPK}$; GPX, glutathione peroxidase; HBD, heparin binding domain; IHC, immunohistochemistry staining; MAPK, mitogen-activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate; RAS, renal-angiotensin system; ROS, reactive oxygen species; STZ, streptozotocin; TGF-β, transforming growth factor-beta; VEGF, vascular endothelial growth factor.

☆ Funding: This research was supported by grant NSC-101-2313-B-005-014-MY3 from the National Science Council and was partly supported by the Ministry of Education, Taiwan, under the aiming top university plan (ATU-103-s0508). The funders had no role in this design, data collection and analysis, decision to publish, or preparation of the manuscript.

☆☆ Author contributions: C.W.K., H.L.C. and C.M.C. conception and design of research, C.J.S., Y.T.T., Y.H.C. and W.H.C. performed experiments; C.W.K., H.L.C., K.C.C. and C.M.C. analyzed data and prepared figures; Y.T.T., S.H.Y. and C.M.C. interpreted results of experiments; C.W.K. drafted manuscript; H.L.C., Y.T.T. and C.M.C. edited and revised manuscript; C.W.K., C.J.S., Y.T.T., H.L.C., W.H.C., K.C.C., S.H.Y. and C.M.C. approved the final version of manuscript.

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http://dx.doi.org/10.1016/j.lfs.2015.04.018
0024-3205/

1. Introduction

Diabetic nephropathy is the main cause of chronic kidney disease in patients who progress to end stage renal disease [7]. The characteristic pathological changes include an increased glomerular basement membrane width, diffuse mesangial sclerosis, hyalinosis, microaneurysms, and hyaline arteriosclerosis in the glomerulus [36]. Tubular and interstitial changes are also present [8,29]. Enhanced oxidative stress, the activation of the protein kinase C (PKC) and transforming growth factor-β (TGF-β)-SMAD signaling pathways, and the increased formation of advanced glycation end products (AGEs) are the main molecular pathways activated during diabetic nephropathy [49]. The balance between the production of reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), and the antioxidant defense system, which includes superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX), determines the degree of oxidative stress. Additionally, PKC, NADPH oxidase, and mitochondrial metabolism contribute to high glucose-induced ROS production [32]. ROS participate in positive feedback mechanisms that activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, PKC, and the renin–angiotensin system, which are located upstream of the ROS production pathway [19]. ROS further can up-regulate the expression of TGF-β and extracellular matrix deposition through the activation of a signal transduction cascade involving PKC, MAPK, and Janus kinase/signal transducers and activators of transcription, as well as downstream transcription factors, such as nuclear factor-κB, activated protein-1, and specificity protein [32]. Angiotensin II and TGF-β can also induce ROS formation through NADPH oxidase, resulting in a vicious circle that leads to the amplification of high glucose activated signaling.

Hyperglycemia-induced excess superoxide, which results from the formation of secondary reactive oxygen species, contributes to the characteristic diabetic kidney injury that has been observed in several studies [21]. Inhibiting NADPH oxidase, which generates superoxide, prevents mesangial matrix expansion in the kidney and the associated proteinuria in streptozotocin (STZ)-treated rats [5]. A series of in vitro and ex vivo studies, have also shown that SOD attenuates the formation of endothelial albumin permeability, vascular dysfunction, and peroxidation, as well as decreases the accumulation of glyco-oxidation products that is induced by diabetes or high concentrations of glucose [16–18,37,44,53].

The SOD mimetic tempol has been shown to decrease the expression of TGF-β and mesangial matrix expansion in the kidneys but does not affect proteinuria in STZ-treated diabetic rats [4]. Our previous report also showed that aerosolized recombinant human EC-SOD (rhEC-SOD) can prevent hyperoxia-induced lung injury [55].To date, the effect of recombinant EC-SOD on diabetic nephropathy has not been studied. The kinetic behavior of bovine erythrocyte Cu-Zn SOD had been investigated in Sprague–Dawley male rats after subcutaneous and oral administrations of doses 0.5, 5, and 20 mg/kg [39]. All the red blood cell SOD activity increased in 1 h ($P < 0.05$) and the maximum concentration was reached in 5 h for the two routes. The bioavailability was represented by the calculation of areas under curves after the administrations. To compare with the bioavailability after subcutaneous administration, the maximum bioavailability after oral administration was 14% for free SOD, 22% for SOD encapsulated into liposomes, and 57% when ceramides were added to liposomes. We administrated rhEC-SOD subcutaneously in our experiment in order to preserve the bioavailability. In this study, the anti-hyperglycemic effects of rhEC-SOD, which was produced from a synthetic cassette in the methylotrophic yeast *Pichia pastoris*, were investigated in the kidneys of STZ-treated diabetic rats for the first time.

2. Materials and methods

2.1. Production of human EC-SOD in P. pastoris

The construction, screening and production of rhEC-SOD were described in our previous study [11]. Briefly, the *hSOD3* cDNA fragment was amplified by PCR and cloned into the pPICZαA yeast expression vector. After the electroporation-stimulated transformation of *P. pastoris*, the pPICZαA-hSOD3-transformed colonies were selected using high levels of zeocin. The rhEC-SOD protein was produced and secreted into the culture medium after induction with methanol. Three liters of culture medium was concentrated by stirred-cell ultrafiltration (YM-10, Amicon, Danvers, MA). The precipitate was resuspended in 5 mM Tris buffer (pH 7.4) containing 50 mM NaCl and was dialyzed against the same buffer. The desalted fractions were separated and purified using a fast protein liquid chromatography (FPLC) system (AKTA purifier 10, Amersham Pharmacia Biotech., Arlington Heights, IL) [55]. We used different columns for purification. (A) HiTrap Q-Sepharose Fast flow. (B) Gel filtration Superdex 75. The activity of rhEC-SOD was evaluated using a water-soluble tetrazolium salt (WST-1) kit (Dojindo Molecular Technologies, Inc., Rockville, MD). The dosage of rhEC-SOD 3200 U/kg/day used in our study has the equal activity of tempol 200 mg/kg/day used in the previous study [4].

2.2. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Utilization Committee of National Chung Hsing University, Taiwan (IACUC Permit Number: 99-31). We monitor the rats in the morning (AM 9:00) and afternoon (PM 3:00) two times a day (7 days/week) for any signs of distress, lethargy, labored breathing, anorexia, or refusal to eat or drink. If rats developed any of these symptoms or anorexia with weight loss of more than 20–30 g over 2–3 days, rats were euthanized. All sacrifices with humane end points were performed by CO_2 inhalation, and all efforts were made to minimize

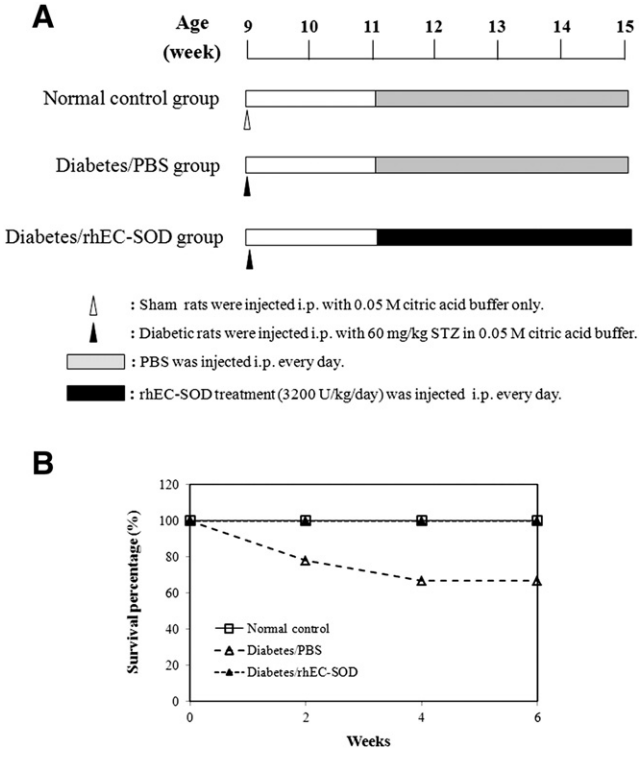

Fig. 1. (A) The schematics for the STZ-induced diabetic nephropathy and rhEC-SOD therapy. STZ (60 mg/kg body weight) was i.p. injected into the rats. In the diabetes/PBS group, PBS alone was administered daily for 4 weeks after STZ treatment. In the diabetes/rhEC-SOD group, rhEC-SOD was administered daily for 4 weeks after STZ treatment. (B) The survival rates of the rats following STZ-induced diabetic nephropathy and rhEC-SOD therapy (Normal control $n = 6$; Diabetes/PBS $n = 9$; Diabetes/rhEC-SOD $n = 6$). The administration of rhEC-SOD increased the survival rate of the diabetic rats.