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Simvastatin ameliorates diabetic nephropathy by attenuating oxidative stress and apoptosis in a rat model of streptozotocin-induced type 1 diabetes

Nawal M. Al-Rasheed[a], Nouf M. Al-Rasheed[a], Yieldez A. Bassiouni[a,b], Iman H. Hasan[a,*], Maha A. Al-Amin[a], Hanaa N. Al-Ajmi[a], Ayman M. Mahmoud[c,*]

[a] Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Saudi Arabia
[b] Department of Pharmacology, Faculty of Medicine, Alexandria University, Egypt
[c] Physiology Division, Department of Zoology, Faculty of Science, Beni-Suef University, Egypt

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ABSTRACT

Statins are HMG-CoA reductase inhibitors with lipid-lowering effect and commonly used to reduce cardiovascular risk in diabetic patients. The present study investigates the ameliorative effect of simvastatin (SIM) on diabetic nephropathy in rats, pointing to its anti-apoptotic and anti-oxidative stress efficacies. Diabetes was induced by a single intraperitoneal injection of 55 mg/kg body weight streptozotocin (STZ) and both control and diabetic rats received 10 mg/kg SIM for 90 days. Treatment with SIM diminished the body weight loss, blood glucose and, serum creatinine, urea and uric acid in diabetic rats. SIM improved the creatinine clearance rate and urinary levels of creatinine, urea and albumin in STZ-induced rats. Lipid peroxidation and nitric oxide (NO) were significantly increased in the diabetic kidney whereas reduced glutathione, SOD and catalase were declined. Bax and caspase-3 showed a significant increase and Bcl-2 was decreased in the kidney of diabetic rats. SIM administration reduced lipid peroxidation and NO, and improved antioxidants and the expression of apoptotic markers. Diabetic rats showed increased collagen deposition in the kidney, atrophied irregular renal corpuscles with collapsed glomeruli and tubules with degenerated epithelial lining, an effect that was reversed following treatment with SIM. In conclusion, SIM can protect against diabetic nephropathy by attenuating oxidative stress and apoptosis.

1. Introduction

Diabetes mellitus is a serious life-long disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. The incidence of diabetes is dramatically increasing and the number of patients with diabetes is expected to increase to 693 million by 2045 [1]. Long-term hyperglycemia is associated with the micro- and macrovascular complications that affects multiple organs, including the eyes and kidneys. This has made diabetic nephropathy (DN) one of the most common complications in patients with diabetes [2]. It has been estimated that approximately a quarter of the diabetic patients develop nephropathy [3]. It is worth stressing that early treatment may keep the kidney disease from getting worse. If left untreated, the patient shows gradually declined glomerular filtration rate (GFR) and persistence of microalbuminuria which progresses to manifest proteinuria. Finally, the kidneys fail and end-stage renal disease (ESRD) usually follows. DN is one of the most important causes of ESRD which affects 15–25% of type 1 and 30–40% of type 2 diabetes patients [4].

The pathogenesis of DN is complex, multifactorial and involves many pathways. Hyperglycemia is the main driving force for the progressive destruction of the glomeruli in diabetes. Persistent elevated blood glucose produces mechanical tension and hemodynamic changes to the glomeruli, which lead to alteration in downstream transcription factors and gene expression [5]. These changes induce various pathways of oxidative stress and liberate numerous cytokines and growth factors [6]. Hyperglycemia-mediated increased generation of reactive oxygen species (ROS) activates various redox-sensitive signaling molecules, leading to cellular dysfunction and injury and ultimately micro- and macrovascular complications [7]. In addition, excessive ROS production can inactivate endogenous antioxidants, and provoke chromatin condensation, DNA fragmentation and accelerated apoptosis of renal epithelial cells [8]. Therefore, mitigating hyperglycemia-induced oxidative stress and apoptosis can prevent DN.

Statins are lipid-lowering medications often prescribed to help lower blood cholesterol levels [9]. This class of drugs are the first-line treatment widely prescribed for coronary artery disease and to reduce

* Corresponding authors at: Physiology Division, Department of Zoology, Faculty of Science, Beni-Suef University, Beni-Suef, 62514, Egypt.
E-mail addresses: ihasan@ksu.edu.sa (I.H. Hasan), ayman.mahmoud@science.bsu.edu.eg (A.M. Mahmoud).

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the risk of stroke and heart attack [9]. Statins work by inhibiting hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase and recently their beneficial effects have been attributed to their anti-inflammatory [10] and anti-oxidant properties [11]. Simvastatin (SIM) is a statin drug with pleotropic effects, including hypocholesterolemic, antineoplastic and anti-inflammatory [12–14]. Recently, we demonstrated the protective effect of SIM against diabetic cardiomyopathy in rats [15]. We showed that treatment of diabetic rats with SIM reduced hyperlipidemia and diminished oxidative stress, inflammation and apoptosis. Therefore, we aimed in the present study to evaluate potential of SIM to attenuate hyperglycemia-induced oxidative stress and apoptosis in a rat model of DN. Streptozotocin (STZ)-induced type 1 diabetes in rats has been suggested as a useful model to study early changes in DN [16]. In addition, STZ-induced type 1 diabetic rodents develop renal injury with similarities to human DN [17].

2. Materials and methods

2.1. Chemicals and reagents

STZ, SIM, malondialdehyde (MDA), pyrogallol, reduced glutathione (GSH), hydrogen peroxide, thiobarbituric acid and Griess reagent were purchased from Sigma-Aldrich (USA). Creatinine and urea assay kits were purchased from Quimica Clinica Aplicada (Amposta, Spain). Rat Albumin ELISA kit was supplied by Assaypro (St. Charles, Mo., USA). Anti-caspase-3 (ab13847) was supplied by Abcam (Cambridge, MA, USA), and antibodies against Bcl-2-associated X protein (Bax; Cat. No. sc-7480), B-cell lymphoma 2 (Bcl-2; Cat. No. sc-7382) and β-actin (Cat. No. sc-47778) were purchased from Santa Cruz Biotechnology (USA). All other chemicals and reagents were supplied by Sigma-Aldrich or other standard commercial suppliers.

2.2. Experimental animals and treatments

Adult male Wistar rats weighing 160–200 g, obtained from the Animal Care Center at the College of Pharmacy, King Saud University (Riyadh, Saudi Arabia), were included in the present study. The animals were maintained at normal atmospheric temperature (23 ± 2 °C) and relative humidity of 50–60% on a 12 h light/dark cycle. The rats were supplied a standard laboratory diet of known composition and water *ad libitum*. All animal procedures were approved by the Institutional Research Ethics Committee, College of Pharmacy, King Saud University (Riyadh, Saudi Arabia).

To induced type 1 diabetes, overnight fasted rats received a single intraperitoneal (i.p.) injection of STZ (55 mg/kg body weight), dissolved in 0.1 M cold citrate buffer (pH 4.5). The corresponding control rats received a single i.p. injection of citrate buffer. Seventy-two hr after STZ injection, blood glucose levels were determined using MEDISAFE MINI blood glucose reader (TERUMO Corporation, Tokyo, Japan) and rats with fasting glucose levels more than 200 mg/dl were selected for further treatments.

Sixteen control and 16 diabetic rats were divided into 4 groups, each comprising 8 rats, as following:

- (Control): Rats received 0.9% sodium chloride (NaCl) for 90 days by oral gavage.
- (Control + SIM): Rats received 10 mg/kg simvastatin [18] dissolved in 0.9% NaCl by oral gavage for 90 days.
- (Diabetic): STZ-induced diabetic rats received 0.9% NaCl by oral gavage for 90 days.
- (Diabetic + SIM): STZ-induced diabetic rats 10 mg/kg simvastatin dissolved in 0.9% NaCl by oral gavage for 90 days.

One day before the end of the study, the rats were housed in individual metabolic cages to collect the urine for 24 h. Urine samples were pooled to assay creatinine, urea and albumin levels. Overnight fasted rats were then sacrificed and blood samples were collected. Immediately after sacrifice, the kidneys were rapidly excised, rinsed in ice-cold saline and weighed. Each kidney was cut into two halves. One half was fixed in 10% neutral buffered formalin for histopathological and immunohistochemical studies. The other half was minced, and a 10% (w/v) homogenate was prepared using 0.1 M phosphate buffered saline (PBS; pH 7.4). The homogenate was centrifuged, and the supernatant was used to assay lipid peroxidation, GSH, nitric oxide (NO), catalase (CAT) and superoxide dismutase (SOD). for the estimation of renal antioxidant parameters. Other samples from the kidney were kept frozen in liquid nitrogen and stored at −80 °C for western blot analysis.

2.3. Biochemical assays

Creatinine and urea were assayed according to the methods of Larsen [19] and Coulombe and Favreau [20] respectively, using reagent kits purchased from Quimica Clinica Aplicada (Amposta, Spain). Serum and urine albumin levels were determined using ELISA kit supplied by Assaypro (St. Charles, Mo., USA), following the manufacturer's instructions.

Lipid peroxidation in the kidney homogenate of control and diabetic rats was determined by assaying MDA, according to the method of Ohkawa et al. [16]. Kidney NO levels were determined as nitrite using Griess reagent [21]. GSH levels, and activity of SOD and CAT were measured in the kidney homogenates following the methods of Beutler et al. [22], Marklund and Marklund [23], and Cohen et al. [24], respectively.

2.4. Histopathology and immunohistochemistry

Immediately after sacrifice, samples from the kidneys of control and diabetic rats were fixed in 10% neutral buffered formalin for 24 h. The fixed samples were processed to prepare 4-μm-thick paraffin sections. The prepared sections were stained with hematoxylin and eosin (H&E) to examine the structure of the kidney or with Masson's Trichrome stain for the detection of interstitial fibrosis.

Other sections from the kidney of control and diabetic rats were processed for the immunohistochemical detection of caspase-3. Briefly, the sections were blocked by immersion in 3% hydrogen peroxide (H_2O_2) solution for 5 min. After washing in Tris-buffered saline (TBS; pH 7.6) for 10 min, the slides were incubated with protein block (Novocastra, UK) for 5 min to block the non-specific binding of antibodies. The sections were probed with rabbit polyclonal anti-caspase-3 (1:100 dilution), washed in TBS three times and then incubated with biotinylated IgG (Novocastra, UK) for 30 min. After washing in TBS, diaminobenzedine (DAB) substrate was added and the sections were counterstained with Mayer's hematoxylin. The sections were mounted and examined. Negative control slides were processed through the same steps with omission of the primary antibody. Image analysis of caspase-3 immuno- staining was performed using ImageJ (NIH, USA), and values were presented as % relative to control.

2.5. Western blot

The effect of SIM on the apoptosis markers Bax and Bcl-2 in the kidney of control and diabetic rats was determined using western blotting as previously described [15]. Briefly, the frozen kidney samples were homogenized in RIPA buffer supplemented with proteinase inhibitors and the concentration of proteins was determined using Bradford reagent. Forty μg proteins were separated on SDS-PAGE and electrotransferred onto nitrocellulose membranes. After blocking in 5% skimmed milk in TBS/Tween20 (TBST), the membranes were probed with anti-Bax, anti-Bcl-2 and anti-β-actin (Santa Cruz Biotechnology, USA) overnight at 4 °C. After washing in TBST, the membranes were incubated with the secondary antibody and developed using enhanced chemiluminescence kit (BIO-RAD, USA). The developed blots were