European Journal of Pharmacology 744 (2014) 10–17

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Cardiovascular pharmacology

Combination therapy with losartan and L-carnitine protects against endothelial dysfunction of streptozotocin-induced diabetic rats

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ARTICLE INFO

Article history:
Received 19 February 2014
Received in revised form
17 September 2014
Accepted 18 September 2014
Available online 2 October 2014

Keywords:
Endothelial dysfunction
Losartan
L-carnitine
Oxidative stress
Diabetes

ABSTRACT

Endothelial dysfunction is a critical factor during the initiation of diabetic cardiovascular complications and angiotensin II appears to play a pivotal role in this setting. The present study aimed to investigate whether the combination therapy with losartan and the nutritional supplement, L-carnitine can provide an additional protection against diabetes-associated endothelial dysfunction and elucidate the possible mechanism(s) underlying this effect.

Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) (60 mg/kg) in rat. Effects of losartan (20 mg/kg, orally, 3 months) and L-carnitine (200 mg/kg, orally, 3 months) on tumor necrosis factor (TNF)-α, oxidative stress parameters, endothelial nitric oxide synthase expression (eNOS), and vascular function were evaluated.

Our results showed a marked increase in aortic superoxide anion (O_2^-) production and serum malondialdehyde (MDA) level alongside attenuating antioxidant enzyme capacities in diabetic rats. This was associated with a significant increase in anigiotensin II type 1 receptor gene expression and TNF-α serum level of diabetic rats alongside reducing aortic eNOS gene expression and nitric oxide (NO) bioavailability. The single or combined administration of losartan and L-carnitine significantly inhibited these changes. Additionally, the vascular endothelium-dependent relaxation with acetylcholine (ACh) in aortic diabetic rat was significantly ameliorated by the single and combined administration of losartan or L-carnitine. Noteworthy, the combination therapy exhibited a more profound response over the monotherapy.

Collectively, our results demonstrate that the combined therapy of losartan and L-carnitine affords additive beneficial effects against diabetes-associated endothelial dysfunction, possibly via normalizing the dysregulated eNOS and reducing the inflammation and oxidative stress in diabetic rats.

1. Introduction

Accumulating evidence indicates that the increased prevalence of cardiovascular diseases in diabetes has been attributed to the development of microvascular and macrovascular complications (Natali et al., 2000). Endothelial dysfunction plays a central role in diabetic vascular diseases (Liu et al., 2014). Conditions contributing to diabetic vascular remodeling and dysfunction include effects of oxidative stress and decreased nitric oxide (NO) bioavailability (De Vriese et al., 2000; Ceriello, 2006). NO production by the endothelial nitric oxide synthase (eNOS) is critically involved in maintaining the integrity and stability of the vascular endothelium, inhibiting platelet aggregation and leukocyte adhesion alongside maintaining blood flow (Caldwell et al., 2010). High glucose greatly increases endothelial reactive oxygen species production leading to the reduction of NO production in human endothelial cells (Taye et al., 2010).

On the other hand, activation of local renin–angiotensin system seems to be involved in the development of long-term complications of diabetes and large clinical trials confirming the beneficial effect of angiotensin converting enzyme inhibitors and angiotensin II receptor blockers in this setting (Hansson et al., 1999). Hyperglycemia stimulates production of angiotensin II, via up regulation of most of the cellular components of the renin–angiotensin system (Fiordaliso et al., 2000).

A growing body of evidence suggests that the main effector peptide of the renin–angiotensin system, angiotensin II, induces inflammatory molecules and contributes to the pathophysiology of cardiovascular disease (Schiffrin and Touyz, 2003; Granger et al., 2004). More importantly, proinflammatory cytokines, such as tumor necrosis factor (TNF)-α play an important role in hypertension and heart diseases (Jolda-Mydlowska and Salomon, 2003). TNF-α, can

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http://dx.doi.org/10.1016/j.ejphar.2014.09.032
0014-2999/

generate ROS and reactive nitrogen species, which may facilitate destruction of β-cells (Mathews et al., 2005). Likewise, TNF-α stimulates intracellular signaling cascades that promote apoptosis and matrix metalloproteinase expression (Sun et al., 2004). In addition, TNF-α is reported to downregulate eNOS expression in fat and muscle of obese rodents (Valerio et al., 2006). Recently, our group has shown that losartan could improve type 2 diabetes-associated metabolic abnormalities and inflammatory status via reducing TNF-α level (Mourad et al., 2012).

L-carnitine (β-hydroxy-γ-4-ntrimethyl aminobuytric acid), a quaternary ammonium compound serves as a cofactor required for the transport of long chain fatty acids into the mitochondria for energy production in peripheral tissues. It has been reported that L-carnitine inhibits free radicals generation preventing the impairment of fatty acid beta-oxidation in mitochondria and protects tissues from damage by repairing oxidized membrane lipids (Calo et al., 2006; Gulcin, 2006).

Although, the cardiovascular effects of L-carnitine and its analogs have already been reported by various experts in this field, its combinatory effect with losartan on diabetes-associated endothelial dysfunction has not been addressed.

The present study was conducted to analyze whether the combination therapy with losartan and L-carnitine can confer additive protection against diabetes-associated endothelial dysfunction and the possible underlying mechanism(s) involved.

2. Materials and methods

2.1. Chemicals

Losartan and L-carnitine were a kind gift from MEBACO Company, Egypt. Streptozotocin (STZ), acetylcholine (ACh) and phenylephrine were purchased from Sigma Chemical Company Inc., St Louis, MO, USA.

2.2. Animals

Adult male Wistar rats (200–240 g, 10–12 weeks old) were housed at room temperature with 12:12 h light/dark cycles and were given food and water ad libitum.

Experiments were conducted in accordance with the international ethical guidelines for animal care of the United States Naval Medical Research Centre, Unit No. 3, Abbaseya, Cairo, Egypt, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care international (AAALAC international). The adopted guidelines are in accordance with "Principles of Laboratory Animals Care" (NIH publication no. 85-23, revised 1985). The study protocol was approved by members of "the research ethics committee" and by the pharmacology and toxicology department, faculty of pharmacy, Minia University, Egypt.

STZ was freshly dissolved in 10 mmol/l citrate buffer, pH 4.5, and injected intraperitoneally at a single dose of 60 mg/kg for induction of diabetes in rat model (Coskun et al., 2005). The hyperglycemia was confirmed (2 days later) by measuring blood glucose levels using a SURESTEP Test Strip. Rats with blood glucose levels $\geq$ 300 mg/dl were considered diabetics. Rats were randomly divided into five groups of eight rats each, as following:

1. Control group: rats were normal non-diabetic rats, received the same volume of the solvent.
2. Diabetic group (STZ): rats were treated with STZ as described above.
3. Diabetic rats treated with losartan (STZ+LOS): diabetic rats were orally treated with losartan in a dose of 20 mg/kg, for 3 months (Jin et al., 2013).
4. Diabetic rats treated with L-carnitine (STZ+LC): diabetic rats were orally treated with L-carnitine in a dose of 200 mg/kg, for 3 months (Yurekli et al., 2011).
5. Diabetic rats treated with a combination of losartan (20 mg/kg, orally, 3 months) and L-carnitine (200 mg/kg, orally, 3 months) (STZ+LOS+LC).

The rats were killed and then blood samples were collected and centrifuged at 8000 × *g* for 10 min to obtain clear sera. The sera were used to determine blood glucose, TNF-α, MDA and antioxidant enzyme activities.

The thoracic aorta was dissected, and carefully cleaned from adhering tissue with special care. The first 2–3-mm segment of the aorta was used for vascular reactivity studies. A portion of the thoracic aorta was fixed in 10% neutral-buffered formalin and was used in histopatholgical analysis. The remaining portion of aorta was homogenized in cold potassium phosphate buffer (0.05 mmol/l, pH 7.4) and was centrifuged at 5000 × *g* for 10 min at 4 °C. The supernatant was kept at −80 °C for subsequent measurements. Total protein concentration was also determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemicals).

2.3. Measurement of antioxidant enzyme activities, oxidative stress biomarkers, and nitric oxide bioavailability

The reduced glutathione (GSH) serum content was determined spectrophotometrically as previously described (Spooner et al., 1981). Serum superoxide dismutase (SOD) activity was evaluated as previously described (Ouedraogo et al., 2007).

Serum MDA level was obtained spectrophotometrically as previously described (Satoh et al., 2005). Aortic tissues were homogenized in a cold Krebs-HEPES buffer (10 mmol/l glucose, 0.02 mmol/l Ca-Tritriplex, 25 mmol/l $NaHCO_3$, 1.2 mmol/l KH_2PO_4, 120 mmol/l NaCl, 1.6 mmol/l $CaCl_2 \cdot 2H_2O$, 1.2 mmol/l $MgSO_4 \cdot 7$ HO, and 5 mmol/l KCl, pH 7.4). Superoxide anion (O_2^-) was produced using lucigenin-derived chemiluminescence as described previously (Taye et al., 2010). O_2^- level was measured in the presence of lucigenin, (5 μmol/l) to minimize artifactual O_2^- production due to the redox cycling, incubated for 20 min. The reaction was started by the addition of NADPH (100 μmol/l), and the relative light units (RLU) of chemiluminescences were measured over a period of 30 min in a lumencense spectrometer. Results are expressed as count per min and normalized to the protein content in each sample.

Finally, the aortic nitrite/nitrate ratio (as an indicator of nitric oxide bioavailability) was assessed spectrophotometrically as previously described (Tsikas, 2007).

2.4. TNF-α estimation

Serum TNF-α level was assessed in this study using enzyme linked immunosorbent assay quantitative detection ELISA using a microplate reader as previously described (Intiso et al., 2004).

2.5. Determination of aortic eNOS and angiotensin II type 1 receptor gene expression by using reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated aortic homogenates using RNeasy Purification Reagent (Qiagen, Valencia, CA) according to manufacturer's instruction. The mRNAs were reverse transcribed into cDNA by using an Oligo(dT)12–18 primer and Superscript™ II RNase Reverse Transcriptase. This mixture was incubated at 42 °C for 1 h; the kit was supplied by SuperScript Choice System (Life Technologies, Breda, the Netherlands). eNOS gene was amplified and analyzed by the following forward primer: 5′-CATACAGAACCCAGGATGGGCT-3′, reverse primer: 5′-TCCTCAGGAGGTCTTGCACATA-3′.