Regulatory Peptides 177 (2012) 107–115

Contents lists available at SciVerse ScienceDirect

Regulatory Peptides

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

Oral administration of an angiotensin-converting enzyme 2 activator ameliorates diabetes-induced cardiac dysfunction

Tatiane M. Murça [a,e], Patrícia L. Moraes [a], Carolina A.B. Capuruço [b], Sérgio H.S. Santos [c], Marcos B. Melo [d], Robson A.S. Santos [d], Vinayak Shenoy [e], Michael J. Katovich [f], Mohan K. Raizada [e], Anderson J. Ferreira [a,*]

[a] Department of Morphology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[b] Department of Pediatrics, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[c] Department of Pharmacology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[d] Department of Physiology and Biophysics, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[e] Department of Physiology and Functional Genomics, College of Medicine, University of Florida, Gainesville, FL, USA
[f] Department of Pharmacodynamics, College of Pharmacy, University of Florida, Gainesville, FL, USA

ARTICLE INFO

Article history:
Received 24 November 2011
Received in revised form 9 April 2012
Accepted 5 May 2012
Available online 14 May 2012

Keywords:
Cardiomyopathy
ACE
Angiotensin-(1–7)
Angiotensin II
AMP-activated protein kinases
MAP kinases

ABSTRACT

We evaluated the hypothesis that activation of endogenous angiotensin-converting enzyme (ACE) 2 would improve cardiac dysfunction induced by diabetes. Ten days after diabetes induction (streptozotocin, 50 mg/kg, i.v.), male Wistar rats were treated with the ACE2 activator 1-[[2-(dimethylamino)ethyl]amino]-4-(hydroxymethyl)-7-[[(4-methylphenyl)sulfonyl]oxy]-9H-xanthen-9-one (XNT, 1 mg/kg/day, gavage) or saline (control) for 30 days. Echocardiography was performed to analyze the cardiac function and kinetic fluorogenic assays were used to determine cardiac ACE and ACE2 activities. Cardiac ACE2, ACE, Mas receptor, AT_1 receptor, AT_2 receptor and collagen types I and III mRNA and ACE2, ACE, Mas, AT_1 receptor, AT_2 receptor, ERK1/2, Akt, AMPK-α and AMPK-β_1 protein were measured by qRT-PCR and western blotting techniques, respectively. Histological sections of hearts were analyzed to evaluate the presence of hypertrophy and fibrosis. Diabetic animals presented hyperglycemia and diastolic dysfunction along with cardiac hypertrophy and fibrosis. XNT treatment prevented further increase in glycemia and improved the cardiac function, as well as the hypertrophy and fibrosis. These effects were associated with increases in cardiac ACE2/ACE ratios (activity: ~26%; mRNA: ~113%; and protein: ~188%) and with a decrease in AT_1 receptor expression. Additionally, XNT inhibited ERK1/2 phosphorylation and prevented changes in AMPK-α and AMPK-β_1 expressions. XNT treatment did not induce any significant change in AT_2 receptor and Akt expression. These results indicate that activation of intrinsic cardiac ACE2 by oral XNT treatment protects the heart against diabetes-induced dysfunction through mechanisms involving ACE, ACE2, ERK1/2, AMPK-α and AMPK-β_1 modulations.

1. Introduction

Diabetic patients might present a peculiar cardiovascular entity, the diabetic cardiomyopathy, characterized by diastolic and systolic dysfunction without coronariopathy and hypertension [1,2]. Cardiovascular diseases (CVD) are the most important cause of death in the diabetic population and diabetes increases two- to four-folds the risk of CVD [3]. Indeed, hyperglycemia is one of the metabolic and homeostatic abnormalities that increase the cardiovascular mortality in patients with diabetes [4,5].

It has been well-known that hyperactivity of the angiotensin-converting enzyme (ACE)/angiotensin (Ang) II/AT_1 receptor axis of the renin–angiotensin system (RAS) is associated with the establishment and progression of CVD and diabetes [6,7]. On the other hand, recent studies have reported that ACE2 holds beneficial cardiovascular actions, such as anti-hypertensive [8], anti-fibrotic [9,10], anti-oxidant [11], anti-inflammatory and anti-atherosclerotic [12,13] effects. This enzyme is an important member of the RAS since it catalyzes the hydrolysis of the C-terminal residue of Ang II to produce the cardioprotective peptide Ang-(1–7) [14–16]. Thus, the axis composed by ACE2, Ang-(1–7) and Mas, the receptor for this peptide, plays a critical protective role in balancing the deleterious effects of the ACE/Ang II/AT_1 receptor axis.

It is consensus that blockage of the ACE/Ang II/AT_1 receptor axis improves morbidity, mortality and cardiovascular events in patients with CVD or high-risk diabetes [17–21]. However, the need for new therapies emerges due to ACE inhibitor intolerance and no consistent proof of specific cardiovascular protection by blockage of AT_1 receptor that exceeds or efficiently synergizes the effect of ACE inhibition [22,23]. Recently, activation of the ACE2/Ang-(1–7)/Mas axis has been considered

* Corresponding author at: Department of Morphology, Av. Antônio Carlos, 6627, ICB, UFMG, 31.270-901, Belo Horizonte, MG, Brazil. Tel.: +55 31 3409 2811; fax: +55 31 3409 2810.
E-mail address: anderson@icb.ufmg.br (A.J. Ferreira).

0167-0115/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2012.05.093

a suitable approach for modulating the pathological effects of ACE/Ang II/AT_1 receptor axis hyperactivity in CVD. In fact, ACE2 is a key regulator of the heart function [24] and its deficiency mimics or enhances the cardiac dysfunction induced by ACE/Ang II/AT_1 receptor axis hyperactivity [9,13]. Genetic and pharmacological manipulation of ACE2 has been demonstrated to be an important strategy to treat diabetes. However, these studies have focused on organs other than the heart such as the kidney and pancreas [25–27]. For instance, Bindom et al. [25] reported an improvement in fasting glycemia, glucose tolerance and islet insulin content along with an enhancement of beta cell viability in type 2 diabetic mice overexpressing ACE2 [25].

To explore the therapeutic potential of ACE2, we have identified small-molecule ACE2 activators based on the virtual screening of its structure [28]. One of these compounds is the 1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl)sulfonyl oxy]-9Hxanthene-9-one (XNT). Acute administration of XNT induced a dose-dependent hypotensive response in spontaneously hypertensive rats (SHR) while chronic treatment with XNT improved the cardiac function and reversed the cardiac and renal fibroses in SHR [28]. Furthermore, XNT prevented the increase in right ventricular systolic pressure and hypertrophy in a monocrotaline-induced pulmonary hypertension model [29] and attenuated the thrombus formation in SHR [12]. Thus, since ACE2 causes beneficial end-organ outcomes in diabetes and reverses hypertension-induced cardiac fibrosis, we hypothesized that activation of endogenous ACE2 using XNT would produce therapeutic outcomes in diabetes-induced cardiac dysfunction. To test this hypothesis, physiological, histological and molecular analyses were conducted to evaluate the effects of chronic oral XNT treatment in diabetic cardiomyopathy.

2. Material and methods

2.1. Materials

The ACE2 activator, XNT, was synthesized by Alchem Laboratories Corp. (Alachua, FL, USA). Fluorogenic substrates for ACE2 (catalog ID: ES007) and ACE (catalog ID: ES005) were obtained from R&D Systems (Minneapolis, MN, USA). The following antibodies were used in the western blotting assays: ACE2 (Santa Cruz sc20998, Santa Cruz, CA, USA), ACE (Millipore CD143, Billerica, MA, USA), Mas (Alomone AAR-013, Jerusalem, Israel), AT_1 receptor (Santa Cruz sc1173, Santa Cruz, CA, USA), AT_2 receptor (Alomone AAR-012, Jerusalem, Israel), ERK1/2 (phosphorylated and total: Cell Signaling #9106, Danvers, MA, USA and Santa Cruz sc93/sc154, Santa Cruz, CA, USA, respectively), Akt (phosphorylated and total: Cell Signaling #9271 and #9272, Danvers, MA, USA, respectively), AMPK-α (phosphorylated and total: Cell Signaling #2535 and #2603, Danvers, MA, USA, respectively), AMPK-β_1 (phosphorylated and total: Cell Signaling #4181 and #4150, Danvers, MA, USA, respectively) and GAPDH (Sigma G8795, St. Louis, MO, USA). The following TaqMan® probes (Roche, Indianapolis, IN, USA) were utilized in the RT-PCR assays: ACE2, Rn01416923-m1; ACE, Rn00561094-m1; Mas, Rn00562673-s1; AT_1 receptor, Rn02132799-s1; AT_2 receptor, Rn00560677-s1; collagen type I, Rn00801649-g1; and collagen type III, Rn01437683-m1.

2.2. Methods

2.2.1. Animals, diabetes induction and XNT treatment

All experimental protocols were performed in accordance with the Federal University of Minas Gerais (Brazil) and the University of Florida Institutional Animal Care and Use Committees, which are in compliance with the NIH guidelines. The experiments were performed in male Wistar rats (180–200 g) obtained from CEBIO — UFMG (Belo Horizonte, MG, Brazil) and from Charles River Laboratories (Wilmington, MA, USA). They were housed in a light/dark cycle (12 h/12 h) room with standard rat chow and water ad libitum. Briefly, the rats were fasted (~16 h), anesthetized (ketamine:xylazine, 60:6 mg/kg, i.p.) and injected with streptozotocin (STZ; 50 mg/kg i.v., Sigma; St. Louis, MO, USA) to induce diabetes. Control non-diabetic (CTL) rats were injected with ~0.2 mL of sodium citrate buffer (10 mmol/L, pH 4.5). Ten days after diabetes induction, the rats were assessed for blood glucose levels (Accu-Chek®; Roche, Indianapolis, IN, USA). The animals with a fasting blood glucose concentration of over 126 mmol/L were considered diabetic. The treatment with XNT (1 mg/kg/day, gavage) or vehicle (saline pH 2–2.5; equivalent volume, gavage) was initiated 10 days after diabetes induction and conducted for 30 days. The dose of 1 mg/kg/day of XNT for oral administration was based on pilot experiments performed to determine the lowest amount of XNT that is able to induce cardiovascular effects. After testing the doses of 0.6 mg/kg/day and 1 mg/kg/day, we chose the dose of 1 mg/kg/day based on the effects observed [30]. We have demonstrated in previous studies that XNT is able to activate ACE2 both in vitro and in vivo [10,28].

2.2.2. Echocardiographic analysis

Transthoracic echocardiographic examination was performed using an Acuson Cypress™ machine equipped with an 8-MHz linear-array transducer (Siemens; Munich, Germany). The rats were anesthetized with a ketamine/xylazine mixture (60:6 mg/kg, i.p.) (CTL: n = 8; STZ: n = 7; STZ + XNT: n = 6). Left ventricular systolic and diastolic functions were assessed by ejection fraction (EF) and mitral inflow pulsed-wave Doppler, respectively. Three measurements were performed: 1) initial — at diabetes induction (day 0 — D0); 2) intermediate — ~28 days after diabetes induction (day 28 — D28); and 3) final — at the end of the experiments (day 40 — D40). Two-dimensional guided M-mode imaging at the papillary muscle level was used to measure the left ventricular end-systolic (LVESD) and end-diastolic (LVEDD) diameters and posterior wall thickness (LVPWT) during diastole. The EF was calculated from the M-mode echocardiogram using the equation: $EF(\%) = [(LVEDD^3 - LVESD^3)/LVEDD^3] \times 100$. Mitral inflow pulsed-wave Doppler velocity was recorded from the apical four-chamber view. All analyses were performed in a blinded way by the same echocardiographist and included morphological and functional parameters. Furthermore, to evaluate if the anesthesia used in our protocol could interfere in the parameters, we adjusted the data to heart rate and no significant differences were observed (data not shown).

2.2.3. Histological analysis

Heart beat was stopped in diastole using 10% KCl (i.v.). The hearts were fixed in 4% Bouin and stained with hematoxylin and eosin for cell morphometry (CTL: n = 4; STZ: n = 5; STZ + XNT: n = 4) or with picrosirius red for fibrosis (CTL: n = 3; STZ: n = 3; STZ + XNT: n = 4). Three sections (5 μm) from each animal were visualized in a light microscope (BX41®; Olympus, Center Valley, PA, USA), photographed (Q-Color3™; Olympus, Center Valley, PA, USA) under ×400 magnification and analyzed using the ImageJ software (http://rsbweb.nih.gov/ij/). The cardiomyocyte diameter of the left ventricular wall (~100 cardiomyocytes for each animal) was measured across the region corresponding to the nucleus. Only the cardiomyocytes cut longitudinally with nuclei and cellular limits visible were considered for analysis. Cardiac interstitial fibrosis of the left ventricle was measured by area percentage analysis. All analyses were performed in a blinded way by the same researcher.

2.2.4. Insulin sensitivity test

An insulin sensitivity test was performed in overnight fed rats 2 days before the end of the treatment (day 38 — D38). After intraperitoneal injection of insulin (0.75 U/kg body weight; Sigma, St. Louis, MO, USA), tail-blood samples were taken at 0, 15, 30, 60 and 90 min for measurement of blood glucose levels (Accu-Chek®; Roche, Indianapolis, IN, USA).

2.2.5. ACE and ACE2 activities

Enzymatic activity was measured in a microplate reader (BioTekSynergy™ 2; BioTek, Winooski, VT, USA), as previously described [28]. Briefly, left ventricle samples (ACE: 30 μg and ACE2: