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Chronic Activation of Endogenous Angiotensin-Converting Enzyme 2 Protects Diabetic Rats from Cardiovascular Autonomic Dysfunction

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Abstract

In this study we evaluated whether the activation of endogenous angiotensin-converting enzyme (ACE) 2 would improve the cardiovascular autonomic dysfunction of diabetic rats. Ten days after type 1 diabetes induction (Streptozotocin, STZ, 50mg/kg i.v.), the rats were orally treated with 1-[(2-dimethylamino)ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT), a newly discovered ACE2 activator (1mg/kg/day), or saline (equivalent volume) during 30 days. Autonomic cardiovascular parameters were evaluated in unanesthetized animals and an isolated heart preparation was used to analyze the cardiac function. Diabetes induced a significant decrease in the baroreflex bradycardia sensibility, as well as in the chemoreflex chronotropic response and parasympathetic tone. The XNT treatment improved these parameters by ~76% (0.82±0.09 vs. 1.44±0.17ΔPI/ΔmmHg), ~85% (−57±9 vs. −105±10 Δbpm) and ~205% (22±2 vs. 66±12 Δbpm), respectively. Also, XNT administration enhanced the bradycardia induced by the chemoreflex activation by ~74% in non-diabetic animals (−98±16 vs. −170±9 Δbpm). No significant changes were observed in the mean arterial pressure, baroreflex tachycardia sensibility, chemoreflex pressor response and sympathetic tone among any of the groups. Furthermore, chronic XNT treatment ameliorated the cardiac function of diabetic animals. However, the coronary vasoconstriction observed in diabetic rats was unchanged by ACE2 activation. These findings indicate that XNT protects against the autonomic and cardiac dysfunction induced by diabetes. Thus, our results evidenced the viability and effectiveness of oral administration of an ACE2 activator for the treatment of the cardiovascular autonomic dysfunction caused by diabetes.

Keywords

Baroreflex; Chemoreflex; ACE2 activation

Introduction

The current Diabetes Atlas predicts a global prevalence of diabetes by 7.8% (438 million people) in 2030 (IDF, 2011). One of the most serious, prevalent and poorly understood complications of the diabetes is the diabetic autonomic neuropathy (DAN), which is widely

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spread in the body and causes important impact in the quality of live and survival of diabetic patients (Kennedy *et al.*, 1995; Edwards *et al.*, 2008; Tesfaye *et al.*, 2010). Cardiovascular autonomic neuropathy (CAN) is the most studied and clinically important form of DAN. The CAN is classically related to parasympathetic and subsequent sympathetic dysfunction (Bennett *et al.*, 1975; Pop-Busui, 2010), which may result in silent myocardial ischemia, prolonged QT interval, arrhythmias and sudden death (Hume *et al.*, 1986; Nathan, 1993; Giunti *et al.*, 2007; Ieda *et al.*, 2008; Schonauer *et al.*, 2008). More importantly, epidemiological studies show that CAN increases the risk of cardiovascular mortality and cardiovascular events in individuals with diabetes (Maser *et al.*, 2003; Freeman, 2005; Giunti *et al.*, 2007).

The cardiovascular reflexes, acting through the autonomic modulation of the heart, lungs and vessels, are responsible for the maintenance of blood pressure (BP) and blood-gas composition. While the baroreflex modulates the beat-to-beat control of heart rate (HR) to correct fluctuations of the BP (Kirchheim, 1976), the chemoreflex regulates the respiration, the HR and BP to maintain the blood-gas composition (Wright, 1936; Gonzalez *et al.*, 1994). It has been shown that diabetic population presents an impairment of the cardiovascular reflexes and the earliest clinical indicator of CAN in these individuals is a decrease in the HR variability (Bernardi *et al.*, 1997; Frattola *et al.*, 1997; Tantucci *et al.*, 1997; Lefrandt *et al.*, 1999; Mancini *et al.*, 1999; Chessa *et al.*, 2002).

The renin-angiotensin system (RAS) is an important player in the neural cardiovascular control, as evidenced by its ability to modulate the baroreflex and chemoreflex activities (Allen, 1998; Paton & Kasparov, 1999; Phillips & de Oliveira, 2008; Allen *et al.*, 2009). While Angiotensin (Ang) II reduces the sensibility of the baroreflex, Ang-(1-7) via Mas receptor increases the baroreflex sensitivity (Campagnole-Santos *et al.*, 1992; Oliveira *et al.*, 1996; Britto *et al.*, 1997; Phillips & Sumners, 1998; Averill & Diz, 2000; Chaves *et al.*, 2000; Diz *et al.*, 2008a; de Moura *et al.*, 2010). In accordance with these data, angiotensin-converting enzyme (ACE) 2, the main Ang-(1-7)-forming enzyme, also improves the baroreflex responses (Diz *et al.*, 2008b; Yamazato *et al.*, 2011). Regarding the chemoreflex, it has been demonstrated that Ang II has an important role in the carotid body function by increasing the sensibility of the chemoreceptor cells and the sympathetic activity (Allen, 1998; Li *et al.*, 2006; Ding *et al.*, 2011).

Autonomic stimulation alters the myocardial mechanical performance and it has been suggested that CAN may be responsible for the cardiac dysfunction in diabetes (Scognamiglio *et al.*, 1998; Poirier *et al.*, 2003; Sacre *et al.*, 2010). Furthermore, imbalance of the autonomic system along with the impairment of the neural control of the cardiovascular system is related to poor prognosis and mortality in diabetes (Maser *et al.*, 2003; Freeman, 2005). Thus, considering the beneficial effects induced by the activation of the ACE2/Ang-(1-7)/Mas axis on the cardiovascular function and the lack in the literature of studies evaluating the role of ACE2 in CAN caused by diabetes, in the present study we hypothesized that the chronic activation of ACE2 would improve the cardiac and cardiovascular autonomic dysfunction observed in diabetic rats. To test this hypothesis, we conducted, for the first time, an oral treatment with the ACE2 activator 1-[(2-dimethylamino)ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT) in diabetic rats. XNT is a newly discovered ACE2 activator (Hernandez Prada *et al.*, 2008) and previous studies have demonstrated that activation of this enzyme is a promising strategy to treat cardiovascular and related diseases (Hernandez Prada *et al.*, 2008; Ferreira *et al.*, 2009; Fraga-Silva *et al.*, 2010; Ferreira *et al.*, 2011b).

Methods

Ethical approval

This study was conducted in 39 male Wistar rats (180–200 g) from CEBIO-Federal University of Minas Gerais (Belo Horizonte, MG, Brazil). Whenever possible the same animal was used in all protocols. The animals were housed in a temperature-controlled room (22–23°C) with a 12–12h light-dark cycle. Water and food were available *ad libitum*. All experimental protocols were performed in accordance with the Federal University of Minas Gerais (Brazil) Institutional Animal Care and Use Committee, which is in compliance with the NIH guidelines.

Diabetes induction and XNT treatment

Briefly, the animals were fasted for approximately 16h. After anesthesia with a ketamine/xylazine mixture (60:6mg/kg, i.p.), the animals were injected with streptozotocin (STZ; 50mg/kg i.v., Sigma, MO, USA) in sodium citrate buffer (10mmol/L, pH 4.5). Control non-diabetic (CTL) rats were injected with ~0.2mL of sodium citrate buffer (10mmol/L, pH 4.5, i.v.). Ten days after diabetes induction, the rats were assessed for blood glucose levels (CTL: n=10; XNT: n=9; STZ: n=10; STZ+XNT: n=10) using a glucometer (Accu-Chek® Compact Plus; Roche, IN, USA). The animals with fasting blood glucose concentration over 126mg/dL were considered diabetic. The treatment with XNT (1mg/kg/day, 0.1mL/100g of rat, gavage) or vehicle (saline pH 2–2.5; equivalent volume, gavage) was initiated ten days after diabetes induction and conducted for 30 days. Pilot experiments were performed in order to determine the lowest dose of XNT able to improve the baroreflex and chemoreflex activities in diabetic animals. Thus, after testing the doses of 0.6mg/kg/day and 1mg/kg/day, we chose the dose of 1mg/kg/day based on the effects observed in the baroreflex and chemoreflex activities. We have demonstrated in previous studies that XNT is able to activate ACE2 both *in vitro* (Hernandez Prada *et al.,* 2008) and *in vivo* (Ferreira *et al.*, 2009; Fraga-Silva *et al.*, 2010; Ferreira *et al.*, 2011b).

Cardiovascular parameters analysis

Under anesthesia with a ketamine/xylazine mixture (60:6 mg/kg, i.p.) a catheter (PE-10 connected to a PE-50) was inserted into the femoral artery (until abdominal aorta) and vein for blood pressure measurement and drug injections, respectively. The catheters were tunneled subcutaneously into the back of the neck to allow access when the animal was awake. After 24h of recovery, basal (CTL: n=10; XNT: n=9; STZ: n=10; STZ+XNT: n=10) and autonomic cardiovascular parameters were evaluated in unanesthetized animals. The arterial catheter was connected to a strain-gauge transducer coupled to a computer-based data acquisition system (MP100A, Biopac Systems Inc., CA, USA) in order to record pulsate arterial pressure (PAP). Mean arterial pressure (MAP) and HR were simultaneously calculated by the software Acqknowledge (Biopac Systems Inc., CA, USA) and continuously displayed.

Baroreflex control of the HR was determined by recording the reflex changes in the HR in response to transient increases in MAP produced by bolus injections of phenylephrine (1.0 μg/0.1mL/rat, i.v.) (CTL: n=7; XNT: n=5; STZ: n=7; STZ+XNT: n=7) or decreases in MAP induced by sodium nitroprusside (1.0 μg/0.1mL/rat, i.v.) (CTL: n=10; XNT: n=7; STZ: n=6; STZ+XNT: n=6), as previously described (Campagnole-Santos *et al.*, 1988). Control injections were performed with 0.1mL of isotonic sodium chloride (saline). The peak reflex changes of the HR corresponding to the maximum change in the MAP, which occurred immediately after the injections, were converted to changes in pulse interval (ΔPI=60.000/HR). For each animal, the ratio between changes in HR (as pulse interval, ΔPI, ms) and changes in MAP (ΔMAP, mmHg) was calculated (baroreceptor sensitivity index - BSI).

The chemoreflex was activated by intravenous injection of potassium cyanide (KCN: 40 μg/0.1mL) (CTL: *n*=7; XNT: *n*=7; STZ: *n*=7; STZ+XNT: *n*=8), in accordance with previous studies (Franchini & Krieger, 1993). The changes in MAP and HR (ΔMAP, mmHg and ΔHR, bpm) were analyzed at the peak of the responses.

The autonomic function was evaluated using sequential and alternated pharmacological blockage of muscarinic and β-adrenergic receptors with methyl-atropine (3mg/kg/0.2mL) and atenolol (8mg/kg/0.2mL), respectively, with an interval of 15 minutes between the injections. The intrinsic heart rate (IHR) was evaluated after 15 minutes of the double blockage with methyl-atropine and atenolol and it was used for calculation of the parasympathetic (IHR minus minimum HR) (CTL: *n*=8; XNT: *n*=7; STZ: *n*=6; STZ+XNT: *n*=7) and sympathetic (maximum HR minus IHR) (CTL: *n*=8; XNT: *n*=7; STZ: *n*=6; STZ +XNT: *n*=7) tones (Negrao *et al.*, 1992).

Isolated heart preparation

At the end of the invasive cardiovascular analysis, the rats (CTL: *n*=8; XNT: *n*=8; STZ: *n*=7; STZ+XNT: *n*=6) were heparinized (400IU, i.p.) and decapitated. The thorax was opened and the heart was carefully dissected, removed and placed in a cold Krebs-Ringer Solution (KRS: 118.4mM NaCl, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM, $MgSO_4.7H_2O$, 2.5mM $CaCl_2.2H_2O$, 11.7mM glucose and 26.5mM $NaHCO_3$) to preserve the heart before the perfusion. As described previously (Goes *et al.*, 1993), the hearts were perfused through an aortic stump with KRS at 37±1°C in a Langendorff system with constant pressure (65 mmHg) and oxygenation (5% CO_2 and 95%O_2). A force transducer (TSD 104 A, Biopac Systems Inc., CA, USA) was attached through a heart clip to the apex of the ventricles to record the contractile force using a data-acquisition system (Acqknowledge, Biopac Systems Inc., CA, USA). A diastolic tension of 1.0±0.2g was applied to the hearts. Coronary flow was measured by collecting the perfusate over a period of 1 minute at regular intervals. HR and ±dT/dt were calculated from the contractile tension recordings. After 30 minutes of stabilization, the functional parameters were recorded for an additional period of 30 minutes.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using Student's unpaired *t* Test (glycemia) or one-way ANOVA followed by the Newman-Keuls post-test. $P<0.05$ was considered statistically significant.

Results

Effects of XNT on hyperglycemia

As expected, ten days after induction of diabetes with streptozotocin (day 10 - D10), the animals presented hyperglycemia [80±3 mg/dL in control rats (CTL and XNT groups), *n*=19 vs. 289±23 mg/dL in diabetic rats (STZ and STZ+XNT groups), *n*=20, $P<0.05$]. At the end of the treatment (day 40 - D40), no further increase in glycemia was observed in diabetic animals treated with XNT when compared with diabetic rats treated with saline (STZ: 434±51 mg/dL, *n*=10 vs. STZ+XNT: 274±42 mg/dL, *n*=10, $P<0.05$).

Effects of diabetes and XNT on baseline and autonomic cardiovascular function

The baseline MAP and HR and autonomic cardiovascular parameters were assessed 24h after vascular catheterization in unanesthetized and freely moving animals. At baseline conditions, i.e. after adaptation of the animals to the experimental condition and before the evaluation of the reflexes and autonomic function, no significant effects induced by diabetes or by the XNT treatment were observed in MAP. However, diabetes caused a reduction in HR and the treatment of diabetic animals with XNT did not alter this parameter (Table 1).

Next, we evaluated the effects of chronic ACE2 activation on the impairment of the baroreflex, chemoreflex and autonomic tone induced by diabetes. Diabetic rats presented a reduced baroreflex bradycardia sensibility and the treatment of these animals with XNT improved this effect (STZ: 0.82±0.09 vs. STZ+XNT: 1.44±0.17 ΔPI/ΔmmHg, Fig. 1A). In control animals, XNT administration had no effect on the baroreflex bradycardia sensibility. Furthermore, no significant changes were observed in the baroreflex tachycardia sensibility among any of the groups (Fig. 1B). Regarding the chemoreflex, diabetic animals showed a decreased chemoreflex chronotropic response, which was completely reversed by the XNT treatment (STZ: −57±9 vs. STZ+XNT: −105±10 Δbpm, Fig. 2A). Interestingly, the treatment of control rats with XNT significantly enhanced the chemoreflex bradycardia (Fig. 2A). No significant changes were observed in the chemoreflex pressor response among any of the groups (Fig. 2B). In agreement with the impairment of the cardiovascular reflexes, diabetes induced an expressive attenuation of the parasympathetic tone and the chronic ACE2 activation efficiently normalized this effect (STZ: 22±2 vs. STZ+XNT: 66±12 bpm, Fig. 3A). Also, no significant effects were seen in the sympathetic tone among any of the groups (Fig. 3B). The IHR was reduced in diabetic rats and ACE2 activation did not alter this effect (Fig. 3C).

Effects of diabetes and XNT on cardiac function

After the *in vivo* studies, the animals were sacrificed and the cardiac function was evaluated using an isolated heart preparation. Chronic activation of ACE2 improved the reduction in the IHR induced by diabetes (Fig. 4A). Furthermore, the decrease in the ±dT/dt observed in hearts from diabetic animals was significantly attenuated by the XNT treatment (+dT/dt, STZ: 158±20 vs. STZ+XNT: 230±16 g/s, Fig. 4B and −dT/dt, STZ: 125±13 vs. STZ+XNT: 161±7 g/s, Fig. 4C). Concerning the coronary flow, we observed a decrease in diabetic animals treated or not with XNT, indicating that XNT did not affect the coronary vasoconstriction induced by diabetes (Fig. 4D). No significant changes were observed in systolic (Fig. 4E) and diastolic (Fig. 4F) tension among any of the groups.

Discussion

The present study was designed to evaluate the effects of chronic activation of intrinsic ACE2 on autonomic cardiovascular function of unanesthetized diabetic animals, as well as on cardiac function. The main findings of this study were: i) XNT treatment improved the baroreflex bradycardia sensibility, the chemoreflex chronotropic response and the parasympathetic tone of diabetic animals; ii) chronic ACE2 activation increased the bradycardia induced by the chemoreflex activation in non-diabetic animals; and iii) the cardiac function of diabetic rats was ameliorated by XNT treatment.

The CAN diagnosis in humans has been essentially functional and studies have been designed to understand its neural mechanisms. In this study, the XNT treatment normalized the parasympathetic and cardiovascular reflex dysfunction triggered by diabetes, suggesting a protective role of ACE2 in the parasympathetic neurotransmission in the heart and in the baroreflex and chemoreflex pathways. Several studies have shown that the neuronal alterations caused by diabetes in the autonomic system comprise both structural and functional changes. Li and co-workers (2010) have reported structural atrophy of vagal aortic afferent and cardiac efferent axons and terminals in chronic diabetes. Furthermore, it has been suggested that diabetes induces depression of the baroreceptor sensorial input, impairment of the ganglionic transmission and loss of preganglionic vagal efferent nerve fibers of the heart (Dall'Ago *et al.*, 1997; Mabe & Hoover, 2011). Regarding the central control of the autonomic nervous system, it seems that diabetic animals may have dysfunctional neurons in the nucleus ambiguous and central deficit in the baroreflex arc (Gu *et al.*, 2008; Yan *et al.*, 2009; Ai *et al.*, 2010). In addition, activation of Ang II/AT_1 inhibits

the parasympathetic neurotransmission in the heart (Potter, 1982; Du *et al.*, 1998; Kawada *et al.*, 2007). Indeed, the brain RAS and the local balance between the ACE/Ang II/AT_1 and ACE2/Ang-(1-7)/Mas axes have been established as an important regulator of the cardiovascular function (Crackower *et al.*, 2002; Xu *et al.*, 2011). Some studies targeting both axes demonstrated beneficial effects on the baroreflex activity and neurogenic hypertension by shifting the balance between the central ACE/Ang II/AT_1 and ACE2/Ang-(1-7)/Mas axes toward this latter branch (Yamazato *et al.*, 2007; Diz *et al.*, 2008a; Diz *et al.*, 2008b; Xia *et al.*, 2009; Feng *et al.*, 2010; Yamazato *et al.*, 2011). Thus, based on these evidences and that XNT is an ACE2 activator, this compound may decrease the Ang II levels and/or increase the Ang-(1-7) concentration, leading to modulation of peripheral pathways and of the central integration of the cardiovascular reflexes and autonomic function.

In addition to the effects of the ACE2 activation in diabetic animals, the chemoreflex bradycardia was exacerbated in non-diabetic rats treated with XNT. Considering that the effects of XNT in diabetic animals were observed in both baroreflex and chemoreflex cardiovagal component and that the baroreflex bradycardia was unchanged in non-diabetic animals, these data suggest that this compound increases the hypoxic sensibility of the chemoreceptors and/or enhances the central regulation of the chemoreflex independent of the presence of diabetes. The carotid body is the main oxygen peripheral arterial chemoreceptor in mammals. It has been described a local RAS in the carotid body (Lam & Leung, 2002). In pathological conditions associated to hypoxia, such as heart failure, it has been evidenced that the Ang II/AT_1 axis is hyperactive in the carotid body, thereby suggesting an enhancement of the chemoreceptor cells sensibility with consequent increase of the sympathetic activity (Allen, 1998; Li *et al.*, 2006; Ding *et al.*, 2011). Altogether, it is plausible to speculate that ACE2 might play a role in the central regulation of the baseline chemoreflex, preventing the impairment of this reflex in diabetic animals.

Looking at the results of the isolated heart preparation, XNT partially improved the cardiac dysfunction induced by diabetes. It has been reported that CAN may trigger cardiac dysfunction in diabetes (Scognamiglio *et al.*, 1998; Poirier *et al.*, 2003; Sacre *et al.*, 2010). Our findings indicated that the main target of XNT was the autonomic system and not the heart. This is supported by the observation that diabetic animals treated with XNT presented autonomic function comparable to control animals while the cardiac function was only partially improved. However, it is important to point out that the cardiac beneficial effects of XNT have been previously demonstrated. We have found that ACE2 activation induced by XNT improves the cardiac function and reduces the myocardial fibrosis in spontaneously hypertensive rats (SHR) by a mechanism involving ERK1/2 phosphorylation (Hernandez Prada *et al.*, 2008; Ferreira *et al.*, 2011b). In this way, further investigations using different approaches are needed to better understand the effects of ACE2 activation on diabetes-induced cardiac dysfunction. Also, at the present time, we can not exclude the possibility that the actions of XNT in the autonomic function were, at least in part, due to its possible beneficial effects on the cardiac structure.

Although the treatment with XNT improved the cardiac dysfunction in diabetic rats, no significant effects were observed in the reduction of the coronary flow observed in these animals. Classically, the effect of Ang II in vascular beds is vasoconstriction while the heptapeptide Ang-(1-7) causes vasodilation. However, it has been demonstrated that these effects depend on the dose and the vascular bed evaluated. For instance, Ang II at concentrations of 1–30 μM induces vasodilation (Toda & Miyazaki, 1981; Fukada *et al.,* 2005) whereas Ang-(1-7) at high concentrations (27–210nM) reduces the coronary flow in isolated rat hearts (Neves *et al.*, 1997). Thus, considering these evidences, it is pertinent to

suppose that the lack of effects in the coronary bed of XNT-treated animals may be related to the available amount of Ang II and/or Ang-(1-7) after ACE2 activation.

Resting bradycardia is an usual finding in streptozotocin-induced diabetic rats (Howarth *et al.*, 2007). In the present study, we observed resting bradycardia along with reduced IHR in unanesthetized diabetic rats, as well as in isolated diabetic rat hearts. Altogether, these data suggest that the bradycardia observed in diabetic hearts was mediated by alterations in the sinoatrial node (SAN) function. Importantly, isolated hearts from diabetic animals treated with XNT presented a slight improvement in the HR, suggesting that ACE2 has a role in the SAN. In fact, all components of the ACE2/Ang-(1-7)/Mas branch are expressed in the SAN of rats (Ferreira *et al.*, 2011a) and Ang II may induce apoptosis in cells of the SAN (Vongvatcharanon *et al.*, 2004).

Of note, chronic ACE2 activation prevented further increase in glycemia in diabetic rats, indicating a possible effect of XNT on pancreatic function. The role of ACE2 in the glucose homeostasis was recently reported by Bindom and co-workers (2010). ACE2 overexpression in the pancreas elicited an improvement in the fasting glycemia, glucose tolerance and islet insulin content along with an enhancement of the beta cells viability in type 2 diabetic mice (Bindom *et al.*, 2010). In addition, several studies have demonstrated that the major substrate for ACE2, Ang II, causes oxidative stress, inflammation and apoptosis in pancreatic beta cells (Tsang *et al.*, 2004; Lupi *et al.*, 2006; Kamper *et al.*, 2010; Saitoh *et al.*, 2010; Yuan *et al.*, 2010).

Along with the potential effects of XNT on the peripheral pathways and on the central integration of the cardiovascular reflexes and autonomic function, a further suitable mechanism of XNT action in diabetic animals could be the modulation of the inflammatory process elicited by the ACE/Ang II/AT_1 axis. Many studies have shown that the autonomic nervous system is able to modulate the immune response and the progression of inflammatory diseases. Stimulation of the vagus nerve may suppress innate immune responses and downregulate the expression of pro-inflammatory cytokines (Borovikova *et al.*, 2000; Tracey, 2002, 2007). On the other hand, it has been established that the ACE2/Ang-(1-7)/Mas axis possesses a anti-inflammatory role (Thomas *et al.*, 2010; Thatcher *et al.*, 2011; Zhong *et al.*, 2011) and that the chronic treatment with XNT can modulate the ACE activity and inhibit the expression of inflammatory cytokines (Ferreira *et al.*, 2009). Therefore, it is tempting to speculate that the prevention of further increase in glycemia along with the improvement in the parasympathetic tone, leading to a potential reduction in the inflammatory process, may represent a cardiovascular protective mechanism in diabetic animals treated with XNT.

It should be noted that XNT is a non-FDA-approved drug. This compound was initially described as an ACE2 activator in 2008 based on a virtual screening of its crystal structure (Hernandez Prada *et al.*, 2008). Since then it has been used to prove the concept that activation of endogenous ACE2 is a feasible strategy to treat cardiovascular and related diseases. Thus, XNT is a lead compound which the main objective is to serve as a “proof-of-the-concept”. Certainly, other compounds can be synthesized based on the XNT’s structure or may be discovered as an alternative to XNT for human testing. In fact, other ACE2 activators have been described (Kulemina & Ostrov, 2011).

In summary, in this study we demonstrated that chronic activation of endogenous ACE2 through oral administration of XNT protects against diabetes-induced cardiovascular autonomic and cardiac dysfunction. Therefore, ACE2 activation might be a feasible therapeutic strategy to prevent CAN and an adjuvant player in the treatment of the diabetic cardiomyopathy.

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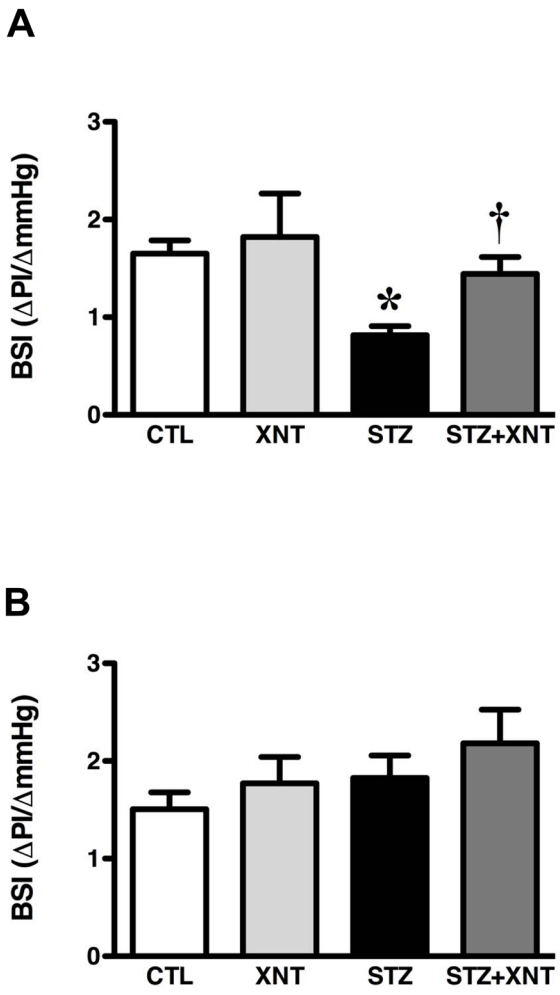

FIGURE 1.

Effects of diabetes and XNT on the baroreflex sensitivity. Sensibility index (ΔPI/ΔmmHg) of (**A**) baroreflex bradycardia and (**B**) baroreflex tachycardia of control and diabetic animals treated with saline (**A** - CTL: *n*=7 and STZ: *n*=7; **B** - CTL: *n*=10 and STZ: *n*=6) or with XNT (**A** - XNT: n=5 and STZ+XNT: n=7; **B** - XNT: *n*=7 and STZ+XNT: *n*=6). (*****)$P<0.05$ compared to control group treated with saline (CTL) and (†)$P<0.05$ compared to diabetes treated with saline (STZ). (One-way ANOVA followed by the Newman-Keuls post-test). BSI: baroreceptor sensibility index.

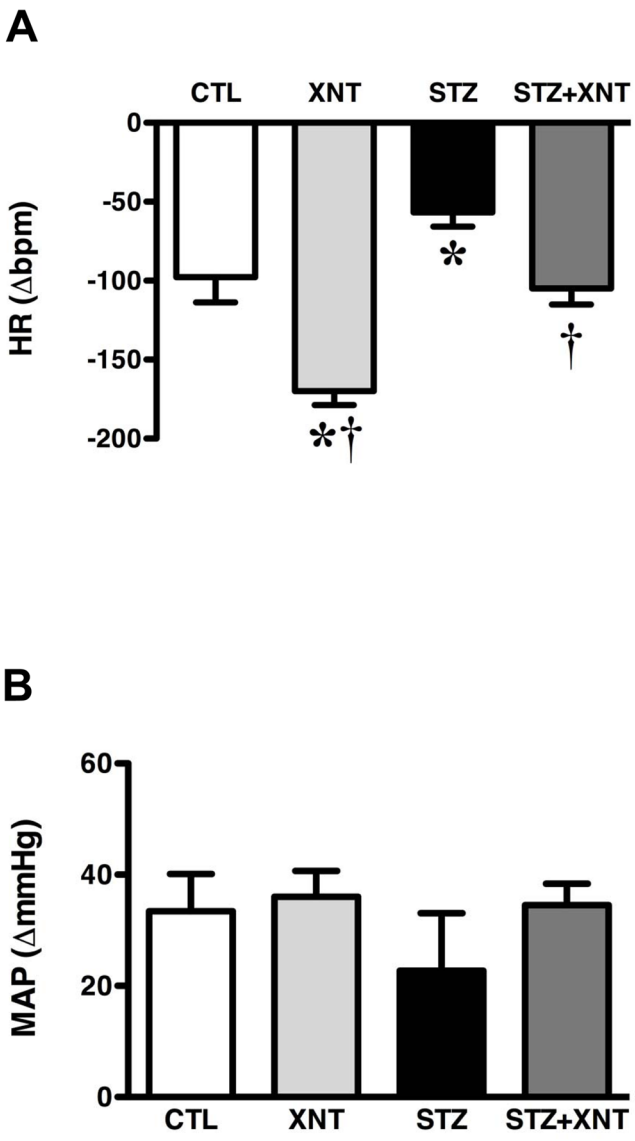

FIGURE 2.

Effects of diabetes and XNT on the chemoreflex activity. (**A**) Chronotropic (ΔHR) and (**B**) pressor responses (ΔmmHg) of the chemoreflex of control and diabetic animals treated with saline (CTL: *n*=7 and STZ: *n*=7) or with XNT (XNT: *n*=7 and STZ+XNT: *n*=8). **(*)***P*<0.05 compared to control group treated with saline (CTL) and (†)*P*<0.05 compared to diabetes treated with saline (STZ). (One-way ANOVA followed by the Newman-Keuls post-test).

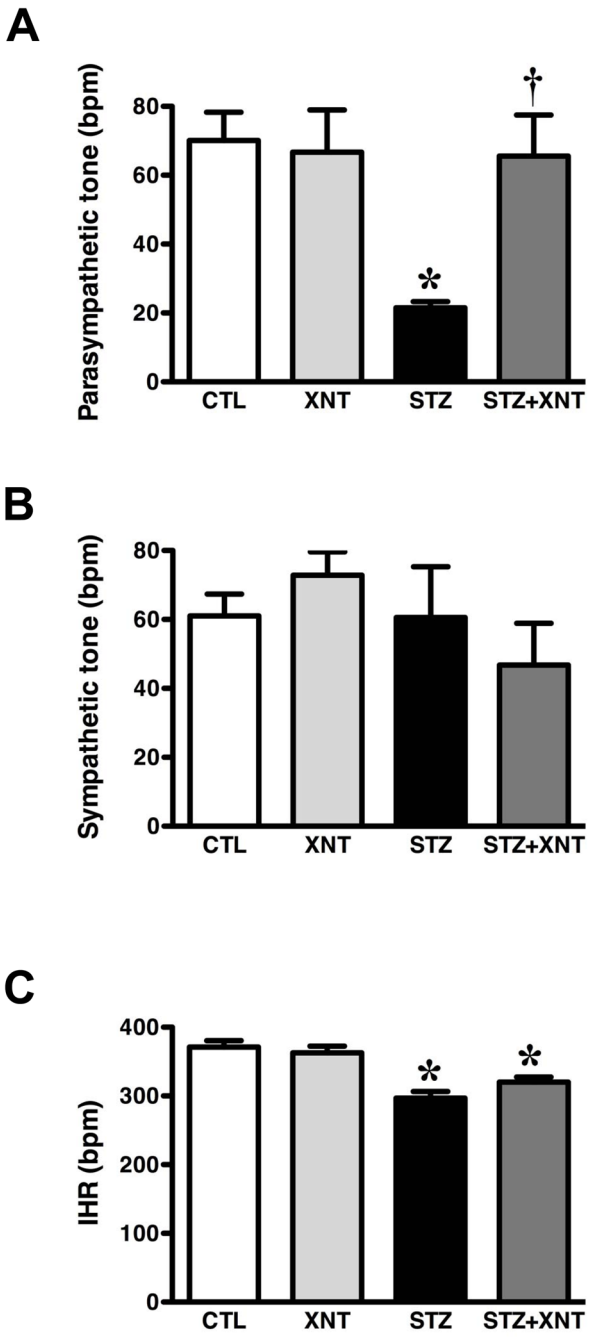

FIGURE 3.

Effects of diabetes and XNT on autonomic tone and intrinsic heart rate (IHR). (**A**) Parasympathetic tone (bpm); (**B**) sympathetic tone (bpm); and (**C**) IHR (bpm) of control and diabetic animals treated with saline (**A** - CTL: n=8 and STZ: n=6; **B** - CTL: n=8 and STZ: n=7; **C** - CTL: n=8 and STZ: n=8) or with XNT (**A** - XNT: n=7 and STZ+XNT: n=7; **B** - XNT: n=7 and XNT+STZ: n=5; **C** - XNT: n=8 and STZ+XNT: n=7). **(*)** P<0.05 compared to control group treated with saline (CTL) and (†) P<0.05 compared to diabetes treated with saline (STZ). (One-way ANOVA followed by the Newman-Keuls post-test).

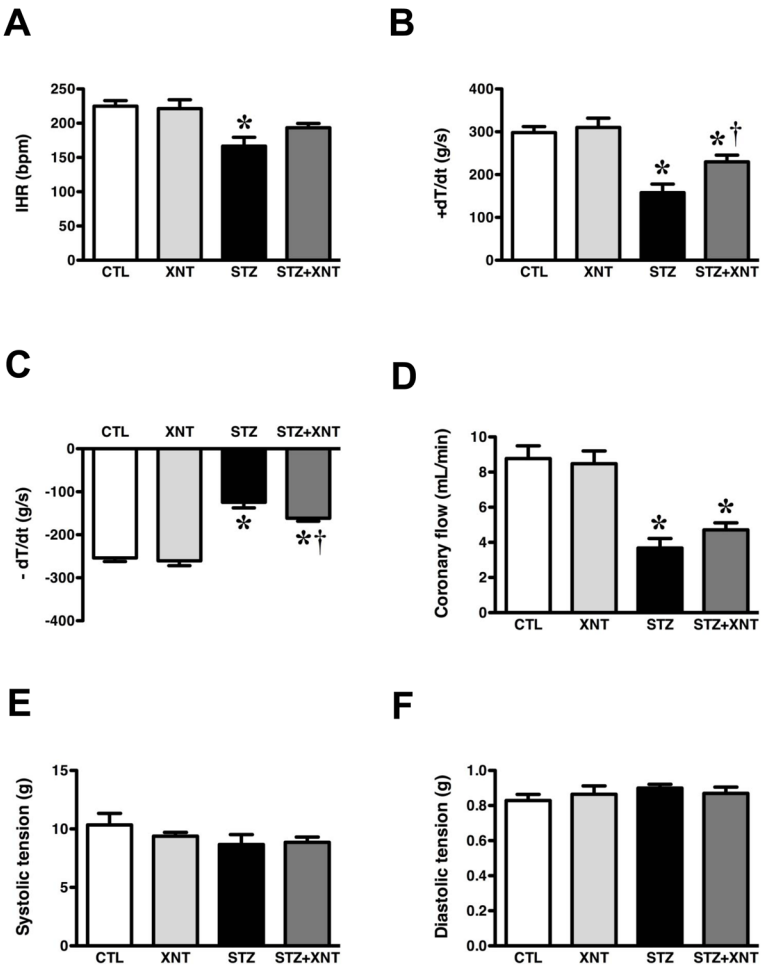

FIGURE 4.

Effects of diabetes and XNT on the cardiac function of isolated hearts. (**A**) Intrinsic heart rate (IHR, bpm); (**B**) +dT/dt (g/s); (**C**) −dT/dt (g/s); (**D**) coronary flow (mL/min); (**E**) systolic pressure (g); and (**F**) diastolic pressure (g) of control and diabetic animals treated with saline (CTL: n=7 and STZ: n=7) or with XNT (XNT: n=8 and STZ+XNT: n=6). **(*)** P<0.05 compared to control group treated with saline (CTL) and (†) P<0.05 compared to diabetes treated with saline (STZ). (One-way ANOVA followed by the Newman-Keuls post-test).

Table 1

Effects of diabetes and XNT on mean arterial pressure and heart rate of rats.

	CTL	XNT	STZ	STZ+XNT
MAP (mmHg)	112 ± 2	111 ± 1	109 ± 3	105 ± 2
HR (bpm)	330 ± 5	329 ± 7	299 ± 9*	298 ± 9*

*P<0.05 compared to control group (CTL). One-way ANOVA followed by the Newman-Keuls post-test.

MAP: mean arterial pressure and HR: heart rate.