Renin-Angiotensin System

Angiotensin-Converting Enzyme 2 Activation Improves Endothelial Function


Abstract—Diminished release and function of endothelium-derived nitric oxide coupled with increases in reactive oxygen species production is critical in endothelial dysfunction. Recent evidences have shown that activation of the protective axis of the renin–angiotensin system composed by angiotensin-converting enzyme 2, angiotensin-(1–7), and Mas receptor promotes many beneficial vascular effects. This has led us to postulate that activation of intrinsic angiotensin-converting enzyme 2 would improve endothelial function by decreasing the reactive oxygen species production. In the present study, we tested 1-[(2-(dimethylamino)ethyl)amino]-4-(hidroximetil)-7-[(4-metilfenil)sulfonil]oxi]-9H-xantona-9 (XNT), a small molecule angiotensin-converting enzyme 2 activator, on endothelial function to validate this hypothesis. In vivo treatment with XNT (1 mg/kg per day for 4 weeks) improved the endothelial function of spontaneously hypertensive rats and of streptozotocin-induced diabetic rats when evaluated through the vasorelaxant responses to acetylcholine/sodium nitroprusside. Acute in vitro incubation with XNT caused endothelial-dependent vasorelaxation in aortic rings of rats. This vasorelaxation effect was attenuated by the Mas antagonist D-pro7-Ang-(1–7), and it was reduced in Mas knockout mice. These effects were associated with reduction in reactive oxygen species production. In addition, Ang II–induced reactive oxygen species production in human aortic endothelial cells was attenuated by preincubation with XNT. These results showed that chronic XNT administration improves the endothelial function of hypertensive and diabetic rat vessels by attenuation of the oxidative stress. Moreover, XNT elicits an endothelial-dependent vasorelaxation response, which was mediated by Mas. Thus, this study indicated that angiotensin-converting enzyme 2 activation promotes beneficial effects on the endothelial function and it is a potential target for treating cardiovascular disease. (Hypertension. 2013;61:1233-1238.) • Online Data Supplement

Key Words: angiotensin-(1–7) ■ diabetes mellitus ■ endothelium dysfunction ■ oxidative stress ■ renin–angiotensin system

Endothelial cells play a central role in maintaining vascular homeostasis and their dysfunction is associated with many forms of cardiovascular and metabolic diseases. Diminished production and function of endothelium-derived nitric oxide and other vasoprotective factors and the exaggerated production of reactive oxygen species (ROS) and vasoconstrictors eventually lead to endothelial dysfunction, resulting in elevated vascular tone, which contributes to the development and progression of cardiovascular and metabolic diseases.

The renin–angiotensin system is a pivotal modulator of the vascular function and its hyperactivity is involved in the endothelial dysfunction. The renin–angiotensin system is regulated by 2 opposite axes. The effector of the first one is angiotensin II (Ang II), which is generated by coordinated enzymatic reactions involving, mainly, the angiotensin-converting enzyme (ACE). This peptide acts primarily through the AT1 receptor, promoting vasoconstriction, proliferation, and oxidative stress. Thus, this axis is composed by ACE, Ang II, and AT1 receptor. The second axis, formed by ACE2, angiotensin-(1–7) [Ang-(1–7)], and Mas, is activated by Ang-(1–7) binding to its own receptor Mas. Generally, this axis promotes opposite effects to those elicited by the ACE/Ang II/AT1 receptor branch. Ang-(1–7) may be generated by different enzymatic pathways; however, it has been proposed that ACE2 is the main enzyme involved in the Ang-(1–7) formation. ACE2 is a monocarboxypeptidase that...
primarily catalyzes the conversion of Ang II into Ang-(1–7), thereby contributing to the balance between the 2 peptides and, consequently, it is a key modulator of the 2 axes of the renin–angiotensin system.

The role of the ACE/Ang II/AT₁ axis in the development and progression of the endothelial dysfunction is well recognized, especially in terms of ROS production by endothelial and vascular smooth muscle cells. Treatment with free radical scavengers, such as superoxide dismutase, catalase, and tempol, reduces blood pressure and vascular damage in response to Ang II. Moreover, it was reported that activation of the ACE2/Ang-(1–7)/Mas axis ameliorates the endothelial function in many animal models. Indeed, short-term infusion of Ang-(1–7) improved endothelial response to acetylcholine, and Mas deficiency caused endothelial dysfunction and increases in blood pressure associated with elevation of the ROS production.

Altogether, these findings led us to postulate that activation of intrinsic ACE2 would improve endothelial dysfunction by decreasing the production of ROS. To test this hypothesis, we evaluated the effects of 1-[2-(dimetilamino)etil]amino]-4-(hidroximetil)-7-[[(4-metilfenil)sulfonil]oxi]-9H-xantona-9 (XNT), a small molecule that has been reported to activate intrinsic ACE2, on endothelial dysfunction. XNT was discovered on the basis of crystal structure of ACE2 using a virtual screening strategy. Administration of this compound in spontaneously hypertensive rats (SHRs) decreased blood pressure, improved cardiac function, and reversed myocardial and perivascular fibrosis through a mechanism involving reduction of extracellular signal–regulated kinases expression. Moreover, XNT reduced pulmonary hypertension induced by monocrotaline, and Mas deficiency caused endothelial dysfunction and increases in blood pressure associated with elevation of the ROS production.

To examine the effects of chronic XNT treatment on the endothelial function, the vasorelaxant responses to ACh (acetylcholine) and SNP (sodium nitroprusside) were evaluated in aortic rings from hypertensive and diabetic rats. The vasodilatory responses to ACh were markedly enhanced in both SHRs and diabetic rats (Figure 1A) and diabetic Wistar rats (Figure 1B) treated with XNT. In contrast, the endothelial-independent responses to SNP were not affected by XNT when compared with vessels from untreated SHRs (Figure 1C) and diabetic rats (Figure 1D). These results showed that the endothelium-dependent vascular responses were improved by ACE2 activation in SHRs and diabetic rats.

**Methods**

The procedures used for the measurement of ACE2 activity, isolated aortic ring preparation, radioimmunoassay, Western blotting, and immunohistochemistry are described in the online-only Data Supplement.

**Animals**

Male Sprague-Dawley rats and SHRs (12 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA). Male Wistar rats (12 weeks of age) were obtained from the CEBIO-Federal University of Minas Gerais (Belo Horizonte, MG, Brazil). Male wild-type (Mas⁺⁺) and Mas knockout (Mas −/−) mice (FVB/N background, 12 weeks of age) were bred at the transgenic animal facility of the Laboratory of Hypertension, Federal University of Minas Gerais (Belo Horizonte, MG, Brazil). All animals were kept in temperature-controlled rooms with 12/12-hour light/dark cycle and had free access to water and food. All experimental protocols were performed in accordance with the University of Florida (Gainesville, FL) and the Federal University of Minas Gerais (Brazil) Institutional Animal Care and Use Committees, which are in compliance with the National Institutes of Health guidelines.

**Statistical Analysis**

The results are presented as mean±SEM. Two-way ANOVA with Bonferroni multiple comparison post test was used to compare the curves obtained in the ACE2 activity and aortic ring preparation protocols. In addition, 1-way ANOVA followed by the Bonferroni post test was used to analyze the Western blotting, immunohistochemistry, and ROS production data, and Student t test was used to analyze the radioimmunoassay results. All statistical analyses were considered significant when P≤0.05.

**Results**

**XNT Improves Endothelial Function of SHRs and Diabetic Rats**

In accordance with previous studies by Hernández Prada et al., we observed that incubation of rhACE2 with XNT in vitro increased the activity of ACE2, thereby confirming the ability of XNT to activate this enzyme (Figure S1 in the online-only Data Supplement). Similar data were observed when aortic samples from normal and diabetic animals were incubated with the fluorogenic substrate (Figure S2). As a consequence, ACE2 activation significantly increased the concentration of Ang-(1–7) in plasma of diabetic animals, but not in aorta (Figure S3). Although ~30% of decrease in plasma Ang II levels was observed in diabetic-treated rats, it did not reach statistical significance (Figure S4). Importantly, the ACE2 activity in diabetic rats was significantly lower when compared with normal animals (Figure S2). Treatment with XNT was unable to change the ACE2 protein expression in diabetic rats (Figures S5 and S6).

To examine the effects of chronic XNT treatment on the endothelial function, the vasorelaxant responses to ACh (acetylcholine) and SNP (sodium nitroprusside) were evaluated in aortic rings from hypertensive and diabetic rats. The vasodilatory responses to ACh were markedly enhanced in both SHRs and diabetic rats (Figure 1A) and diabetic Wistar rats (Figure 1B) treated with XNT. In contrast, the endothelial-independent responses to SNP were not affected by XNT when compared with vessels from untreated SHRs (Figure 1C) and diabetic rats (Figure 1D). These results showed that the endothelium-dependent vascular responses were improved by ACE2 activation in SHRs and diabetic rats.

**XNT Produces Vasorelaxant Responses Associated With Mas Activation**

XNT caused concentration-dependent vasorelaxation in aortic rings of Sprague-Dawley rats preconstricted with phenylephrine (Figure 2A). Using the submaximal concentration of 10 μmol/L, XNT promoted a time-dependent vasorelaxation with maximal effect reached after 7 minutes (Figure 2B). To evaluate the participation of the endothelium in the vasorelaxant effects of XNT, aortic rings of rats with or without intact endothelium were incubated with this compound. It was found that the XNT effects were dependent on the endothelial cells (Figure 2C).

To address the mechanism by which XNT produces vasorelaxation, the vessels were preincubated with 2 different Mas antagonists. It was observed that the vasorelaxant effect of XNT was attenuated by D-pro7-Ang-(1–7) (10 μmol/L; Figure 2D). Interestingly, preincubation with A-779 (10 μmol/L), a classical Mas antagonist, did not change its vasorelaxant activity (Figure 2E). On the basis of possible participation of Mas in the vasorelaxant effects of XNT, we further evaluated the actions of this compound in vessels of Mas⁻/⁻ mice. We found that XNT at 10 μmol/L caused similar vasorelaxation in Mas⁻/⁻ and Mas⁺⁺ mice during the first 5 minutes of incubation. However, after this initial period the XNT effect
was absent in Mas−/− mice when compared with Mas+/+ mice (Figure 2F). The initial vasodilatory effect observed in Mas−/− mice was not associated with Ang II degradation by ACE2 because incubation with losartan did not affect the vasodilator response of XNT (Figures 2G and 2H).

**XNT Attenuates Oxidative Stress**

Consistent evidences indicate that ROS play an important role in the development of endothelial dysfunction. Thus, we investigated the participation of the oxidative stress in the effects of XNT on the endothelial function of diabetic Wistar rats. Specifically, we evaluated the ROS production in aortic vessels of diabetic animals. Diabetes mellitus caused an increase in the generation of ROS, which was significantly reduced by XNT treatment (Figure 3). No significant changes were observed in the expression of catalase, superoxide dismutase, and NOX2 in aorta of diabetic rats treated or not with XNT (Figure S7).

In addition, we tested whether XNT is able to reduce the Ang II–inducing ROS production in human aortic endothelial cells. It was observed that XNT treatment attenuated the ROS production stimulated by Ang II. XNT alone did not change the basal level of ROS (Figure S8).

**Discussion**

The beneficial role of ACE2 in the pathophysiology of cardiovascular and metabolic diseases is currently under intensive investigation. In fact, the involvement of this enzyme in cardiac function, hypertension, atherosclerosis, and other cardiovascular diseases has recently been demonstrated. The most significant finding of the present study is that pharmacological ACE2 activation using XNT exerts protective effects on endothelial function. Furthermore, we showed that this action involves the Mas receptor and reduction of ROS production.

As the main ACE2 enzymatic function is degrading Ang II with consequent production of Ang-(1–7), the likely mechanism underlying the XNT effects is balancing the bioavailability of these 2 peptides. Indeed, we observed that the Mas antagonist D-pro7-Ang-(1–7) attenuated the vasorelaxant response elicited by XNT, thereby indicating the involvement of Ang-(1–7)/Mas in this effect. Unexpectedly, A-779, a classical Mas antagonist, did not affect this action. In spite of these apparent contradictory results, these findings are in keeping with previous studies showing that the vasorelaxant effect of Ang-(1–7) in aortic rings of Sprague-Dawley rats was blocked by D-pro7-Ang-(1–7) but not by A-779. Nowadays, there is only 1 Ang-(1–7) receptor identified (ie, Mas receptor). Usually, this receptor is blocked by A-779 and D-pro7-Ang-(1–7). However, as mentioned above, in certain situations one of these antagonists is not efficient or is only partially effective in blocking the Ang-(1–7) effects. This strongly suggests the existence of other unidentified Ang-(1–7) receptors. To further evaluate the role of Ang-(1–7)/Mas in the effects of XNT, we measured the plasma and aortic Ang-(1–7) levels and tested the XNT effects in isolated aortic rings of Mas-deficient mice. It was observed that ACE2 activation significantly increased the concentration of this peptide in the plasma. The results obtained in Mas−/− mice showed that, during the first 5 minutes of incubation, XNT induced vasorelaxation in Mas−/− and Mas+/+ mice. However, after 10 minutes the effects of XNT were absent in Mas−/−, indicating that Mas is involved in the vascular response of XNT. One may suggest that the initial vasodilatory effect observed in Mas−/− mice is caused by degradation of Ang II by ACE2. Nevertheless, this hypothesis is not plausible because incubation of XNT associated with losartan did not block this vasodilatory response. Therefore, further experiments are required to explain this observation. Altogether, these findings...
indicated that the vasorelaxant effects of XNT are dependent on Ang-(1–7)/Mas axis.

The partial blockade (=50%) of the XNT actions by D-pro7-Ang-(1–7) was an expected finding because ACE2 is an enzyme with a dual role within the renin–angiotensin system (ie, it degrades Ang II with consequent production of Ang-(1–7)). Thus, the residual effect of XNT in the presence of D-pro7-Ang-(1–7) might be caused by the reduction of the Ang II content. However, we did not observe any significant decrease in plasma Ang II levels in diabetic rats treated with XNT. Furthermore, increases in ACE2 protein and mRNA expression are a frequent finding when using ACE2 activators. This suggests that these compounds not only induce their beneficial effects by forming Ang-(1–7) and degrading Ang II, but a nonidentified mechanism is also present.

Nevertheless, in our study, XNT was unable to increase the ACE2 protein expression maybe because of the duration of the experimental protocol. Thus, further investigations are necessary to clearly identify the mechanisms of action of XNT.

The ROS are intracellular and intercellular second messengers that modulate the endothelial function. Under pathological conditions, elevation of the ROS content, the so-called oxidative stress, results in vascular dysfunction. In fact, consistent evidences indicate that ROS play an important role in the development of organ damage in diabetes mellitus and hypertension. Ang II elicits many of its pathophysiological effects by stimulating ROS generation through the reduction of the nicotinamide adenine dinucleotide phosphate oxidase activity. Moreover, treatment with free radical scavengers, such as superoxide dismutase, catalase,
and tempol, reduces the vascular damage in response to Ang II.12,13 In contrast, it has been reported that Ang-(1–7) via Mas ameliorates endothelial dysfunction in animal models by decreasing the oxidative stress.14–16 In accordance with these findings, we showed that chronic treatment with XNT reduced the ROS content in aorta of diabetic rats to a similar level observed in nondiabetic animals. In addition, XNT treatment was also able to reduce the Ang II–induced ROS production in human aortic endothelial cells. No significant changes were observed in the aortic expression of catalase, superoxide dismutase, and NOX2 among any of the groups. Taking into account that these enzymes are critical ROS scavengers, our results suggest that the modulation of the expression of these enzymes is not a mechanism by which XNT reduces the ROS production in aorta. Moreover, it is pertinent to note that the effects of XNT on ROS generation must be evaluated under in vivo condition to further confirm the association between XNT treatment and oxidative stress reduction.

In attempting to explain the mechanisms by which XNT improves the endothelial function of diabetic rats, acute experiments evaluating the role of the endothelium and Mas receptor were run in rings extracted from normal animals. Thus, the translation of these data obtained in normal rats to diabetic and hypertensive animals must be done carefully. However, they are an evidence of the mechanisms involved in the beneficial endothelial actions of XNT in these pathological conditions. Also, the hypertensive model was less explored in our study and it was used with the intention of adding more generality to our data.

Perspectives

Our present study demonstrated that XNT improves the endothelial function of hypertensive and diabetic rats. These actions involved the Mas receptor and reduction of ROS production. Thus, these results indicate that pharmacological ACE2 activation by XNT promotes beneficial effects on the endothelial function and that this compound is a lead molecule to develop potential therapeutic strategies and drugs to treat cardiovascular and metabolic diseases by improving the endothelial function.

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Disclosures

None.

References


Figure 3. 1-[2-(Dimetilamino)etilamino]-4-(hidroximetil)-7-[[4-metilfenil(sulfonil)oxi]-9H-xantona-9 (XNT) treatment reduces reactive oxygen species (ROS) content in aortic rings of diabetic rats. Representative photomicrographs of descending thoracic aorta sections showing the ROS production using DHE (dihydroethidium; 2 μmol/L) in nondiabetic rats (A), vehicle-treated diabetic rats (B), and XNT-treated diabetic rats (C). Quantification of ROS content (D). *P<0.05 (1-way ANOVA followed by the Bonferroni multiple comparison test). Each column represents the mean±SEM (n=6–8) of relative fluorescence in arbitrary unit (AU).


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ANGIOTENSIN-CONVERTING ENZYME 2 ACTIVATION IMPROVES ENDOTHELIAL FUNCTION

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METHODS

Experimental Model of Diabetes

Diabetes was induced in Wistar rats using streptozotocin (STZ; Sigma-Aldrich, USA; Cat# S0130), as described elsewhere. Briefly, anesthetized rats (100mg/kg ketamine and 10mg/kg xylazine) were injected with STZ (50mg/kg), intravenously. The animals were deprived of food twelve hours before the STZ injection. After ten days, blood glucose levels were measured using a glucometer and the treatment with XNT (1mg/kg per day, during four weeks, gavage) was initiated. At the end of the XNT treatment, the measurement of the blood glucose levels was repeated and the animals were sacrificed.

Isolated Aortic Rings Preparation

Isolated aortic rings were used to evaluate the acute and chronic (SHR and diabetic animals) vascular effects of XNT. Aortic rings (4mm) from the descending thoracic aorta, free of adipose and connective tissues, were set up in gassed (95%O₂ and 5%CO₂) Krebs-Henseleit solution (110.8mmol/L NaCl, 5.9mmol/L KCl, 25.0mmol/L NaHCO₃, 1.1mmol/L MgSO₄, 2.5mmol/L CaCl₂, 2.3mmol/L NaH₂PO₄ and 11.5mmol/L glucose) at 37°C under a tension of 1g for 1 hour to equilibrate. The vessels from mice were stabilized with 0.5g of tension. Mechanical activity was recorded isometrically using a force transducer (World Precision Instruments, USA), amplified (Model TMB-4; World Precision Instruments, USA) and stored in a personal computer equipped with an analogue-to-digital converter board (AD16JR; World Precision Instruments, USA) utilizing the CVMS data acquisition/recording software (World Precision Instruments, USA). The effects of XNT were evaluated in aortic rings pre-constricted with phenylephrine (0.1µmol/L). In the acute protocols (Sprague-Dawley rats, n=8 to 10), XNT was added into the bath in increasing cumulative concentrations (0.1nmol/L to 100µmol/L) or as an unique submaximal concentration (10µmol/L) after the stabilization of the response to phenylephrine. To evaluate the role of Mas, D-pro²-Ang-(1-7) (1µmol/L and 10µmol/L) or A-779 (10µmol/L) was added into the bath associated to the XNT (10µmol/L). Aortic rings of Mas⁺/⁺ and Mas⁻/⁻ mice were incubated with the submaximal concentration (10µmol/L) of XNT in the absence or presence of losartan after the stabilization of the response to phenylephrine. In addition, the acetylcholine (ACh, at 1nmol/L to 1µmol/L) and sodium nitroprusside (SNP, at 1nmol/L to 1µmol/L) vasorelaxant responses were used to evaluate the endothelial function of chronically XNT-treated SHR (1mg/kg per day, for four weeks, osmotic minipumps, model 2ML4-Alzet®) and of chronically XNT-treated diabetic rats (Wistar rats, 1mg/kg per day, four weeks, gavage). Also, the participation of the endothelium in the vasorelaxant effects of XNT was examined by incubating this compound with aortic rings from normal Wistar rats with or without intact endothelium.

Measurement of ACE2 Activity

The enzymatic activity (n=5) of human recombinant ACE2 (rhACE2; R&D systems, USA; Cat# 933-ZN) and of aorta samples from normal and diabetic animals (n=6-8) was determined using a fluorogenic substrate (fluorogenic peptide VI; R&D systems, USA; Cat# ES007) in the presence or absence of XNT. Enzymatic activity was measured with a Spectra Max Gemini EM Fluorescence Reader (Molecular Devices, USA), as previously described. Samples were read every 30 seconds for, at least, 40 minutes immediately after the addition of the fluorogenic peptide substrate at 37°C. The data obtained using tissue samples were represented as the average of all readings.
Angiotensin-(1-7) Measurement

Aortas (n=5) were homogenized with 0.045 N HCl in ethanol (10 ml/g of tissue) containing 0.90 µmol/l p-hydroxymercuribenzoate, 131.50 µmol/l of 1,10-phenanthroline, 0.90 µmol/l phenylmethylsulfonyl fluoride (PMSF), 1.75 µmol/l pepstatin A, 0.032% EDTA, and 0.0043% protease-free bovine serum albumin (BSA) and evaporated. After evaporation, the samples were dissolved in 0.003% trifluoracetic acid (TFA). Blood samples (n=8) were collected and transferred to polypropylene tubes containing 1 mmol/l p-hydroxymercuribenzoate, 30 mmol/l of 1,10-phenanthroline, 1 mmol/l PMSF, 1 mmol/l pepstatin A, and 7.5% EDTA (50 µl/ml of blood). After centrifugation, plasma samples were frozen in dry ice and stored at -80°C. Ang II and Ang-(1-7) was extracted onto a BondElut phenylsilane cartridge (Varian). The columns were preactivated by sequential washes with 10 ml of 99.9% acetonitrile/0.1% heptafluorobutyric acid (HFBA) and 10 ml of 0.1% HFBA. Sequential washes with 10 ml of 99.9% acetonitrile/0.1% HFBA, 10 ml of 0.1% HFBA, 3 ml of 0.1% HFBA containing 0.1% BSA, 10 ml of 10% acetonitrile/0.1% HFBA, and 3 ml of 0.1% HFBA were used to activate the columns. After sample application, the columns were washed with 20 ml of 0.1% HFBA and 3 ml of 20% acetonitrile/0.1% HFBA. The adsorbed peptide was eluted with 3 ml of 99.9% acetonitrile/0.1% HFBA into polypropylene tubes rinsed with 0.1% fat-free BSA. After evaporation, Ang II and Ang-(1-7) levels were measured by radioimmunoassay (RIA), as previously described. Protein concentration in the crude homogenates was determined by the Bradford method.

Western Blotting

Descending thoracic aorta of diabetic rats (Wistar rats, n=4 to 10) were collected and homogenized in lysis buffer containing 9mol/l ureia and 2% CHAPS with freshly added protease inhibitor mix (GE Healthcare, UK; Cat# 80-6501-23). Thirty micrograms of protein were separated by electrophoresis on a 10% polyacrylamide gel and transferred to nitrocellulose membranes. Non-specific bindings were blocked with TBS-T (Tris-base at 3%, Tween 20, pH 7.6) containing 5% non-fat skim milk. Membranes were probed with one of following specific primary antibodies: anti-catalase (1:1000, Cell Signaling Technology, USA; Cat# 8841), anti-SOD (1:1000, Cell Signaling Technology, USA; Cat# 2770), anti-NOX2 (1:250, Santa Cruz Biotechnology, USA; Cat# SC-130549), anti-ACE2 (1:500, Gene Tex, CA, USA; Cat# GTX15348) or anti-GAPDH (1:5000, Santa Cruz Biotechnology, USA; Cat# sc-166545) followed by incubation with secondary antibodies. Immunoreactive bands were quantified by densitometry.

Immunohistochemistry

Paraffin-embedded ventricular sections (6µm, n=4 to 6 sections) were first incubated with 0.3% H₂O₂ in phosphate-buffered saline (PBS) for 15 minutes followed by incubation with 2% BSA in PBS containing 0.3% Triton X100 for 1 hour. Sections were incubated overnight at 4°C with the anti-ACE2 antibody (1:250, Gene Tex, CA, USA; Cat# GTX15348) diluted in PBS containing 0.3% Triton X100 and 0.3% BSA. After four or five rinses in PBS, biotinylated goat anti-rabbit IgG secondary antibody was added for 1 hour followed by incubation with avidin-biotin-peroxidase complex reagents (Dako LSAB+System-HRP, Dako, Inc., Carpinteria, CA, USA) for 1 hour. The sections were stained with diaminobenzidine solution for 4 minutes and counterstained with hematoxylin. Each step was followed by washing the sections with PBS.
containing 0.3% Triton X100. Sections incubated without primary antibodies were used as negative controls. The sections were analyzed using an Olympus BX 41 microscope (Olympus, Inc., Irving, TX, USA). Five fields of each section were sequentially photographed under 40x objective. The strongest labeling area of the positive labeled tissue was measured using the Image Pro-Plus software and the results were expressed in percentage of occupied area. The segmentation was based in the pixels number of the strongest labeling area.

Detection of Reactive Oxygen Species

To detect ROS production in aorta of diabetic rats (Wistar rats, n=6 to 8), 30µm-cryosections of the descending thoracic aorta were stained with dihydroethidium (DHE; Sigma-Aldrich, USA, Cat# 37291) at 2µmol/L in PBS for 15 minutes at 37°C. The slices were washed with PBS and examined on a fluorescence microscope equipped with a digital imaging system (Carl Zeiss MicroImaging, USA). Furthermore, the intracellular levels of ROS (n=9 to 12 experiments) in human aortic endothelial cells (HAEC; Cascade Biologics, USA; Cat# C-006-5C) were also measured using DHE, as described elsewhere. Briefly, cells were grown in glass slides in a humidified 5% CO2/95% O2 atmosphere at 37°C. ROS production was stimulated by Ang II at 0.1µmol/L in the presence or absence of XNT at 1µmol/L. After 20 minutes, the cells were washed twice with PBS and loaded with DHE at 2µmol/L for 5 minutes. HAEC were washed with PBS and examined on a fluorescence microscope (Carl Zeiss MicroImaging, USA). DHE fluorescence intensity of acquired digital images was quantified by the NIH software Image J.

REFERENCES
RESULTS

Supplemental Figure S1. XNT enhances the activity of recombinant human ACE2 (rhACE2). The fluorescence resulted from the breakdown of the fluorogenic substrate by rhACE2 in the presence or absence of XNT. *P<0.001 (Two-way ANOVA followed by the Bonferroni's multiple comparison test). Each point represents the mean ± SEM (n=5) of relative fluorescence in arbitrary unit (A.U.).

Supplemental Figure S2. XNT enhances the activity of aortic ACE2 of normal (CTRL) and diabetic (STZ) rats. The fluorescence resulted from the breakdown of the fluorogenic substrate by ACE2 in the presence or absence of XNT. *P<0.05 (Student t-test). The data represent the average of all readings (n=6-8 in each group). A.U.: arbitrary unit (A.U.).
Supplemental Figure S3. Ang-(1-7) levels in (A) plasma and (B) aorta of diabetic (STZ) rats treated or not with XNT. ACE2 activation significantly increased the concentration of Ang-(1-7) in the plasma of diabetic rats. *P<0.05 (Student t-test).
Supplemental Figure S4. Plasma Ang II levels in diabetic (STZ) rats treated or not with XNT. No significant changes were observed between the groups (Student $t$-test).

Supplemental Figure S5. Angiotensin-converting enzyme 2 (ACE2) protein expression in aorta of control (non-diabetic) and diabetic rats treated or not with XNT. Representative blot and quantification of the expression. A total of 30µg of protein was applied to the gel. Data were normalized using GAPDH. One-way ANOVA followed by the Bonferroni’s multiple comparison test. The data are presented as mean ± SEM (n=4 to 5).
Supplemental Figure S6. Expression of Angiotensin-converting enzyme 2 (ACE2) in aorta of control (non-diabetic) and diabetic rats treated or not with XNT. Representative photomicrographs of (A) control, (B) diabetic rat treated with saline and (C) diabetic rat treated with XNT. The negative control (inset) was obtained by omitting the primary antibody from the incubation procedure. (D) Quantification of ACE2 in aorta of control (non-diabetic) and diabetic rats treated or not with XNT. One-way ANOVA followed by the Bonferroni’s multiple comparison test. The data are presented as mean ± SEM. Scale bar represents 50 µm.
Supplemental Figure S7. Catalase, superoxide dismutase (SOD) and NOX2 protein expression in aorta of diabetic rats. Representative blots and quantification of the expression of (A) catalase, (B) SOD and (C) NOX2 in aorta of control (non-diabetic) and diabetic rats treated or not with XNT. For each blot a total of 30 µg of protein was applied to the gel. Data were normalized using GAPDH. One-way ANOVA followed by the Bonferroni’s multiple comparison test. The data are presented as mean ± SEM (n=6 to 10).
Supplemental Figure S8. XNT attenuates the Ang II-induced reactive oxygen species (ROS) production in human aortic endothelial cells (HAEC). The cells were incubated with Ang II (0.1µmol/L) in the presence or absence of XNT (1µmol/L). ROS production was detected using dihydroethidium (DHE; 2µmol/L). Representative photomicrographs of HAEC showing the ROS production in control cells (A), Ang II-treated cells (B), XNT-treated cells (C) and Ang II+XNT-treated cells (D). Quantification of ROS content (E). **p<0.01 and ***p<0.001 (One-way ANOVA followed by the Bonferroni’s multiple comparison test). Each column represents the mean ± SEM (n=9 to 12 experiments) of relative fluorescence in arbitrary unit (A.U.).