Angiotensin-Converting Enzyme 2 Activation Improves Endothelial Function

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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-(dimetilamino)etil]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.[1] 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.[1,2]

The renin–angiotensin system is a pivotal modulator of the vascular function and its hyperactivity is involved in the endothelial dysfunction.[3,4] The renin–angiotensin system is regulated by 2 opposite axes.[5,6] 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 AT_1 receptor, promoting vasoconstriction, proliferation, and oxidative stress.[4,7] Thus, this axis is composed by ACE, Ang II, and AT_1 receptor. The second axis, formed by ACE2, angiotensin-(1–7) [Ang-(1–7)], and Mas,[5] is activated by Ang-(1–7) binding to its own receptor Mas.[8] Generally, this axis promotes opposite effects to those elicited by the ACE/Ang II/AT_1 receptor branch.[5,6] Ang-(1–7) may be generated by different enzymatic pathways[9]; however, it has been proposed that ACE2 is the main enzyme involved in the Ang-(1–7) formation.[10,11] ACE2 is a monocarboxypeptidase that

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primarily catalyzes the conversion of Ang II into Ang-(1–7),[10] 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.[6]

The role of the ACE/Ang II/AT_1 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.[3,4,7] Treatment with free radical scavengers, such as superoxide dismutase, catalase, and tempol, reduces blood pressure and vascular damage in response to Ang II.[12,13] Moreover, it was reported that activation of the ACE2/Ang-(1–7)/Mas axis ameliorates the endothelial function in many animal models.[14] Indeed, short-term infusion of Ang-(1–7) improved endothelial response to acetylcholine,[15] and Mas deficiency caused endothelial dysfunction and increases in blood pressure associated with elevation of the ROS production.[16]

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,[17] on endothelial dysfunction. XNT was discovered on the basis of crystal structure of ACE2 using a virtual screening strategy.[17] 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 reductions in extracellular signal–regulated kinases expression.[17,18] Moreover, XNT reduced pulmonary hypertension induced by monocrotaline,[19] attenuated thrombus formation and platelet attachment to vessels of hypertensive rats,[20] and ameliorated cardiac and autonomic function of diabetic rats.[21,22]

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,[17] 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 (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

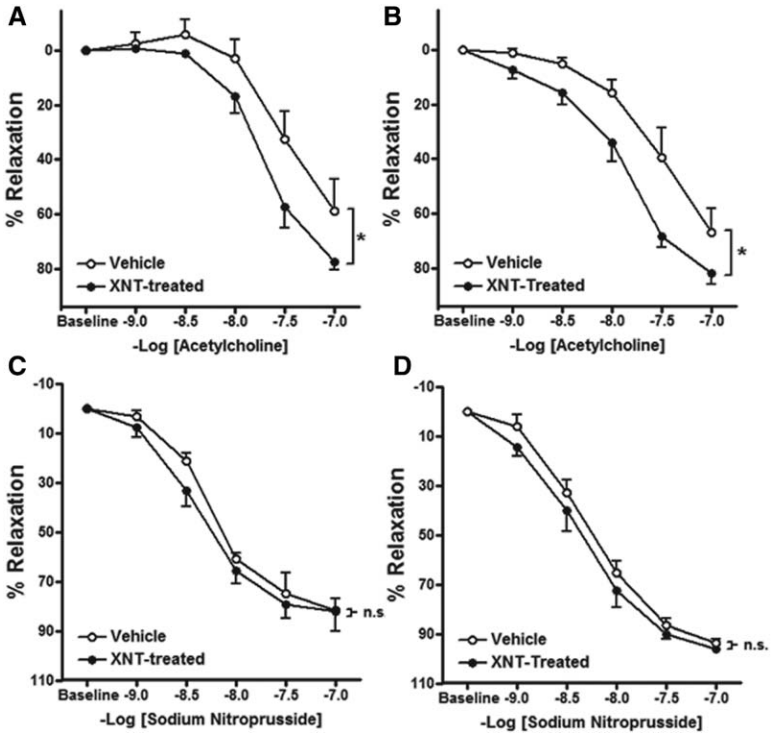

Figure 1. 1-[[2-(Dimetilamino)etil]amino]-4-(hidroximetil)-7-[[(4-metilfenil)sulfonil]oxi]-9H-xantona-9 (XNT) improves endothelial function of hypertensive and diabetic rats. Vasodilation produced by increasing cumulative concentrations of acetylcholine (**A**) and sodium nitroprusside (**C**) in aortic rings of XNT-treated or untreated hypertensive rats. Vasodilatory effects induced by increasing cumulative concentrations of acetylcholine (**B**) or sodium nitroprusside (**D**) in aortic rings of XNT-treated or untreated diabetic rats. *P<0.05 (2-way ANOVA followed by the Bonferroni multiple comparison test). Each point represents the mean±SEM (n=7–10). n.s. indicates nonsignificant.

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.[6,17–20,23–27] 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.[28] Nowadays, there is only 1 Ang-(1–7) receptor identified (ie, Mas receptor).[8] Usually, this receptor is blocked by A-779 and D-pro7-Ang-(1–7).[29] 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.[28] 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

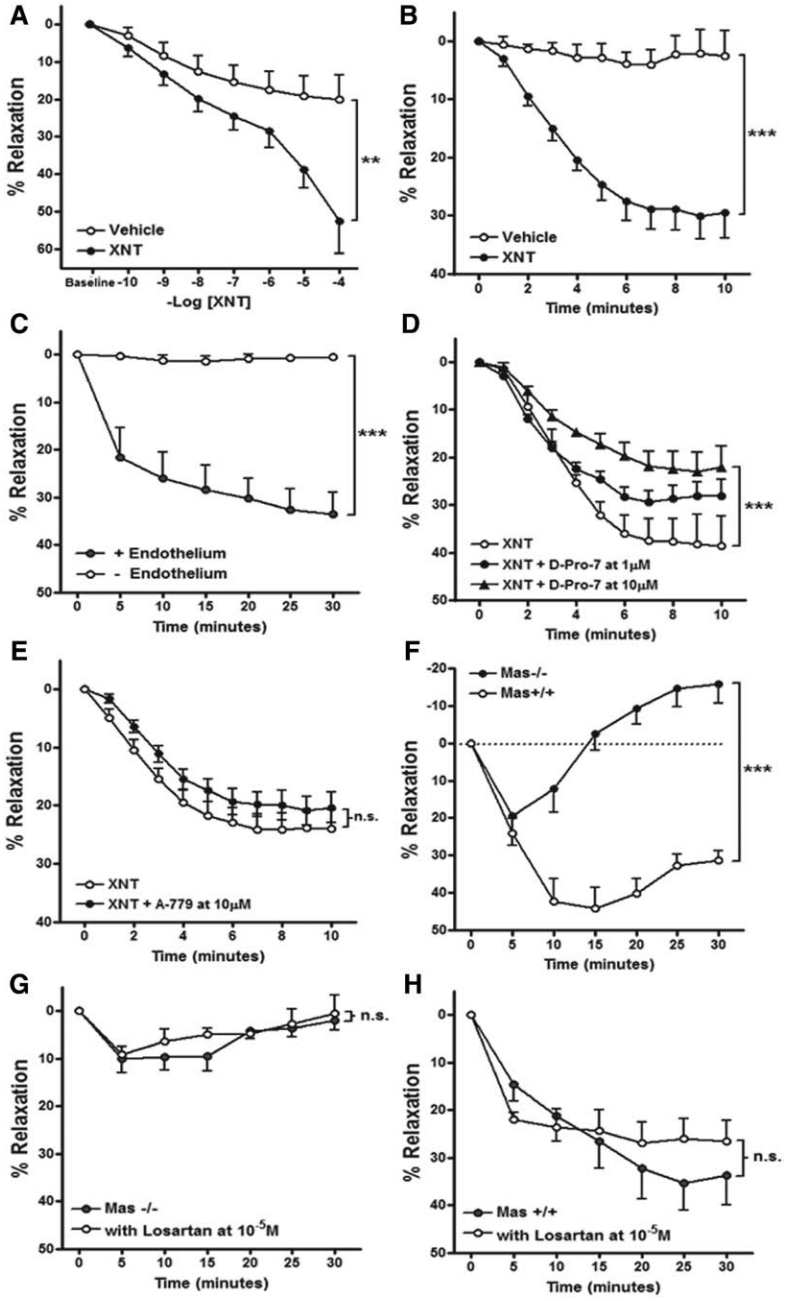

Figure 2. 1-[[2-(Dimetilamino)etil]amino]-4-(hidroximetil)-7-[[(4-metilfenil)sulfonil]oxi]-9H-xantona-9 (XNT) produces Mas-mediated vasodilation. Vasodilation produced by increasing cumulative concentrations of XNT (**A**) and time curve of the submaximal concentration (10 μmol/L) of XNT (**B**). The vasodilatory effects of XNT were dependent on the intact endothelium (**C**). The effects of XNT (10 μmol/L) were partially blocked by D-pro7-Ang-(1–7) (**D**) but not by A-779 (**E**). Also, the vasorelaxant effect of XNT (10 μmol/L) was absent in aortic rings of $Mas^{-/-}$ mice after 5 minutes of incubation (**F**). The AT_1 receptor antagonist losartan did not affect the response of XNT in $Mas^{-/-}$ (**G**) and $Mas^{+/+}$ (**H**) mice. $^{**}P<0.01$ and $^{***}P<0.001$ (2-way ANOVA followed by the Bonferroni multiple comparison test). Each point represents the mean±SEM (n=8–10). n.s. indicates nonsignificant.

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.[18] 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.[30] Under pathological conditions, elevation of the ROS content, the so-called oxidative stress, results in vascular dysfunction.[31] In fact, consistent evidences indicate that ROS play an important role in the development of organ damage in diabetes mellitus and hypertension.[30,32] 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,

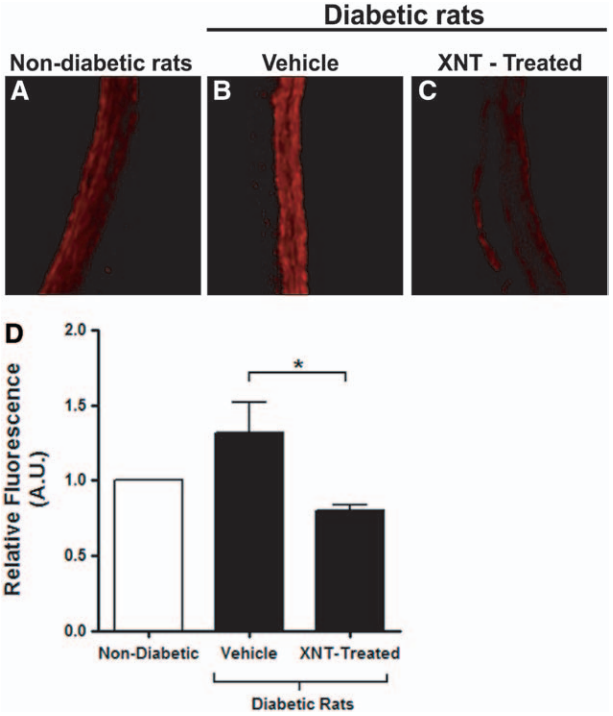

Figure 3. 1-[[2-(Dimetilamino)etil]amino]-4-(hidroximetil)-7-[[(4-metilfenil)sulfonil]oxi]-9H-xantona-9 (XNT) treatment reduces reactive oxygen species (ROS) content in aortic rings of diabetic rats. Representative photomicrographs of descending thoracic aorta sections showing the ROS production using 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).

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.

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Novelty and Significance

What Is New?

- Our study indicates that activation of intrinsic angiotensin-converting enzyme 2, a recently described member of the renin–angiotensin system, using a small molecule angiotensin-converting enzyme 2 activator named 1-[[2-(dimetilamino)etil]amino]-4-(hidroximetil)-7-[[(4-metilfenil) sulfonil]oxi]-9H-xantona-9 (XNT) improves endothelial function by decreasing the generation of reactive oxygen species.

What Is Relevant?

- The balance between release and function of endothelium-derived nitric oxide and reactive oxygen species production is critical in endothelial dysfunction. Therapies targeting the reestablishment of this balance might have significant implications in hypertension and cardiovascular disease management. Thus, our study proposes angiotensin-converting enzyme 2 activation as a potential target for treating hypertension and related cardiovascular diseases.

Summary

Our results show that XNT administration ameliorates the endothelial function of hypertensive and diabetic rat vessels by attenuation of the oxidative stress. Moreover, XNT elicits an endothelial-dependent vasorelaxation response, which is mediated by Mas.