Angiotensin-Converting Enzyme 2–Independent Action of Presumed Angiotensin-Converting Enzyme 2 Activators

Studies In Vivo, Ex Vivo, and In Vitro

Philipp K. Haber, Minghao Ye, Jan Wysocki, Christoph Maier, Syed K. Haque, Daniel Batlle

Abstract—Angiotensin (Ang)-converting enzyme 2 (ACE2) is a key enzyme in the metabolism of Ang II. XNT (1-[2(dimethylamino)ethylamino]-4-(hydroxymethyl)-7-[4-methylphenyl] sulfonyl oxy)-9H-xanthene-9-one) and diminazene have been reported to exert various organ-protective effects, which are attributed to the activation of ACE2. To test the effect of these compounds, we studied Ang II degradation in vivo and in vitro as well as their effect on ACE2 activity in vivo and in vitro. In a model of Ang II–induced acute hypertension, blood pressure (BP) recovery was markedly enhanced by XNT (slope with XNT, −3.26±0.2 versus −1.62±0.2 mmHg/min without XNT; P<0.01). After Ang II infusion, neither plasma nor kidney ACE2 activity was affected by XNT. Plasma Ang II and Ang (1–7) levels also were not significantly affected by XNT. The BP-lowering effect of XNT seen in wild-type animals was also observed in ACE2 knockout mice (slope with XNT, −3.09±0.30 versus −1.28±0.22 mmHg/min without XNT; P<0.001). These findings show that the BP-lowering effect of XNT in Ang II–induced hypertension cannot be because of the activation of ACE2. In vitro and ex vivo experiments in both mice and rat kidney confirmed a lack of enhancement of ACE2 enzymatic activity by XNT and diminazene. Moreover, Ang II degradation in vitro and ex vivo was unaffected by XNT and diminazene. We conclude that the biological effects of these compounds are ACE2-independent and should not be attributed to the activation of this enzyme. (Hypertension. 2014;63:774-782.) ● Online Data Supplement

Key Words: diminazene ■ hypertension ■ renin-angiotensin system ■ 1-[(2-dimethylamino)ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl)sulfonyloxy]-9H-xanthene-9-one

Since the discovery of angiotensin (Ang)-converting enzyme 2 (ACE2) in 2000,1,2 our understanding of the renin–angiotensin system has been greatly expanded, and an important role for the ACE2–Ang (1–7)–Mas receptor axis within the overall renin–angiotensin system is now generally accepted.3–5 This system operates as an antagonist to the system’s pressor branch (ACE–Ang II–Ang II type 2 receptor) and seems to exert primarily organs specific effects.3–21 Thus, this is a promising target for new therapeutic strategies to treat hypertension.5,14,17,19 Cardiovascular,10,15 and kidney disease.11,13,18,21 The monodoppeptidase ACE2 itself is a promising therapeutic target because it efficiently degrades Ang II, a pressor peptide, to form the sepatpeptide Ang (1–7).22,23 The latter peptide has vasodilatory, anti-inflammatory, and antifibrotic effects that have been reported to be because of the activation of the Mas receptor.3,12,16

In the mouse, the administration of human recombinant (r) ACE219 and murine rACE220 have been shown to lower plasma Ang II effectively with the attendant formation of Ang (1–7). The development of compounds that activate ACE2 could have potential therapeutic use, particularly in the chronic setting where the intravenous administration of rACE2 might not be practical. In 2008, the group led by Raizada22 introduced 2 ACE2 activators: XNT (1-[2(dimethylamino)ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl)sulfonyl oxy]-9H-xanthene-9-one) and resorcinolnaphthalein. These compounds were identified out of a library consisting of approximately 140,000 compounds from the National Cancer Institute Developmental Therapeutics Program via structure-based virtual screening using the DOCK v5.2 package.22 XNT was prioritized over resorcinolnaphthalein for research use because of significantly favorable solubility properties.22 Three years later, diminazene (DIZE),23 an agent already established in veterinary medicine, for example, as a treatment option against trypanosomiasis,24 was identified via virtual screening as an ACE2 activator. In the following years, XNT and DIZE have been used successfully to treat a vast array of conditions, such as hypertension,22,25 pulmonary hypertension,26–28 cardiac and renal fibrosis,22 and glaucoma,29 in experimental rat or mice models. The premise of the therapeutic benefit has been attributed to ACE2 activation and the conversion of Ang II to Ang (1–7) 23,24,26–36 However, in none of these studies it was demonstrated that

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ACE2 activation had taken place by demonstrating the enhanced conversion of Ang II to Ang (1–7). Moreover, the effect on ACE2 activity was generally not reported in vivo.\textsuperscript{22,23,25–35} We examined the effect of XNT on Ang II–induced hypertension in an ACE2 knockout (KO) line and found that this compound was effective in lowering BP, thereby indicating that its action is ACE2-independent. We, therefore, designed further studies to comprehensively evaluate the effect of XNT and DIZE on ACE2 activity and Ang II in vitro, in vivo, and ex vivo.

\textbf{Methods}

\textbf{Animal Models}

Male wild-type (WT) and ACE2-deficient mice (ACE2 KO; breeding pairs donated by Drs S. Gurley and T. Coffman, Duke University, Durham, NC) on C57BL6 and FVB genetic background were used to identify the pattern of potential XNT and rACE2-induced changes in BP, Ang peptides, and enzyme activities.

\textbf{In Vivo Studies}

To study the acute effect of XNT on systolic BP (SBP) and Ang II degradation, mice were anesthetized with an intraperitoneal ketamine injection (200 mg/kg of body weight) as previously described. Thirty minutes before anesthesia, WT mice were pretreated with an intraperitoneal injection of either sterile vehicle (VHC) or XNT (18 mg/kg). Immediately after inducing anesthesia, mice were placed on a heating platform for 10 minutes. SBP was measured noninvasively every 30 seconds for a period of 25 minutes by determining the tail blood volume with a volume–pressure recording sensor and an occlusion tail-cuff using a computerized system (COBA System; Kent Scientific). This volume–pressure recording system has been validated and provides a high correlation with telemetry and direct arterial BP measurements.\textsuperscript{36} After 5 minutes of baseline SBP recording, acute hypertension in anesthetized mice was induced with an intraperitoneal bolus of Ang II (0.2 mg/kg), and SBP was monitored for the remaining 20 minutes. In additional experiments, Ang II (0.2 mg/kg) was infused together with an ACE2 inhibitor (MLN-4760; 1 mg/kg; Millennium Pharmaceuticals) after XNT was infused 30 minutes earlier, as described above. In separate experiments, ACE2 KO mice, pretreated with vehicle or XNT, were injected with Ang II (0.2 mg/kg) as described above for the WT mice.

\textbf{Effect of XNT and DIZE on the Enzyme Activity of ACE2 and Aminopeptidase A}

Kidney and serum ACE2 activities were determined after incubation with the intramolecularly quenched synthetic ACE2-specific substrate Mca-APK-Dnp (Anaspec). Measurements were performed in black microtiter plates with a total volume of 100 μL as described previously.\textsuperset{20,37}

To study the effect of XNT and DIZE on the enzyme activity of purified ACE2, these compounds were added in quadruplicate at 10^{-4} to 10^{-10} mol/L (end concentrations) to the black microtiter plate wells containing mouse rACE2 (200 ng/mL). XNT and DIZE were added in duplicate at the same concentrations to human rACE2 (100 ng/mL; R&D Systems). Kinetic curves were followed for a period of 1 hour. Quadruplicate wells containing assay buffer alone constituted a reference control.

The in vivo effect of XNT and DIZE on the enzyme activity of human recombinant aminopeptidase A (APA; R&D Systems) was studied using synthetic-specific substrate H-Glu-AMC (Bachem Americas). Measurements were performed in black microtiter plates with a total volume of 100 μL. XNT and DIZE were added in concentrations from 10^{-4} to 10^{-8} to the buffer (50 mM L HEPES, 150 mM NaCl, 0.025 mM L ZnCl_2, 0.5% Triton-X-100, pH 7.4) and the recombinant protein. Fluorescence was measured continuously for 1 hour in 26 cycles using an FL-X800 microplate fluorescence reader (BIOTEK Instruments Inc, Winooski, VT) at 380 nm excitation and 460 nm emission wavelength for APA activity.

\textbf{Measurements of Plasma Ang II and Ang (1–7)}

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\textbf{Measurements of Ang II Degradation In Vitro}

Ang II was incubated at 37°C with either 200 or 800 ng/mL rACE2 in the presence of XNT or DIZE for 4 hours. Samples were collected at 0.5, 1, 2, and 4 hours and diluted in EDTA-containing II EIA Buffer (SPIBio; Cayman Chemical, Ann Arbor, MI) to stop the reaction. The samples were stored at -80°C. The quantity of Ang II in the samples was determined via enzyme immunoassay kit (SPIBio) as per the manufacturer’s instructions.

\textbf{Mass Spectrometry}

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\textbf{Effect of XNT on Hypertension Acutely Induced by Ang II Infusion in WT Mice}

The effect of XNT on BP was examined in studies with C57BL6 WT mice after a bolus of Ang II to induce acute hypertension. BP was monitored continuously for ≥25 minutes every 30 seconds (Figure 1). In WT mice pretreated with XNT 30 minutes before Ang II infusion (n=11), baseline SBP was not significantly different from mice pretreated with vehicle (n=11; 116±3.8 versus 111±4.4 mm Hg, respectively). The administration of a bolus of Ang II to PBS-pretreated mice resulted in a rapid increase in SBP in both groups. The recovery in the XNT group was markedly faster than in PBS-pretreated controls (slope, −3.26±0.2 versus −1.6±0.2 mm Hg/min; P<0.01; Figure 1). The difference in BP between the 2 groups persisted throughout the continuous monitoring for 20 minutes (Figure 1). At 5 minutes, BP has nearly completely normalized in the XNT group, whereas in the vehicle-treated group, it had only started to decline.

\textbf{Effect of XNT on Serum and Kidney ACE2 Activity and Ang II and Ang (1–7) Levels After Ang II Infusion}

In a separate group of experiments, a different set of WT mice was treated exactly the same way as described in Figure 1.
but with the purpose of euthanizing them at 5 minutes after the Ang II bolus. This time point was chosen because previous work by our laboratory revealed a marked fall in Ang II–induced hypertension as a result of rapid degradation of this peptide.

Serum and kidney ACE2 activity in mice infused with XNT or control was measured 5 minutes after an Ang II bolus. At the time of euthanization, animals had been exposed to XNT for a total of 35 minutes. Serum ACE2 activity is low in mice, whereas in the kidney, ACE2 activity is high.1,11 As shown in Figure 2, there were no significant differences in either kidney (upper panel) or serum (lower panel) ACE2 activity between XNT-treated and control animals. In fact, a slight decrease was noted after XNT infusion in both serum and kidney enzyme activity although this effect was not statistically significant.

In the same mice, the levels of plasma Ang II, measured 5 minutes after Ang II infusion, were not significantly different between vehicle and XNT group (0.771±0.129 versus 0.715±0.326 pmol/mL, respectively). Likewise, the plasma levels of Ang (1–7) were not significantly different between controls and XNT-treated mice (0.729±0.080 versus 1.163±0.371 pmol/mL, respectively; Figure 3).

**Effect of XNT Plus MLN4760 on Hypertension Acutely Induced by Ang II Infusion in WT Mice**

In a different set of experiments, C57BL6 WT mice received either vehicle (PBS; n=5) or XNT (n=5) as in the experiments described in Figure 1. Concurrently, another group was given XNT plus MLN-4760, a specific ACE2 inhibitor (n=8; Figure 4, upper panel), to establish whether the effect of XNT on BP could be prevented by MLN-4760. The BP recovery was faster in XNT-pretreated animals than in vehicle controls (slope, −3.12±0.4 versus −1.47±0.5 mm Hg/min). In XNT plus MLN-4760–treated mice, the recovery was not significantly different from XNT-treated animals (−2.88±0.4 versus −3.12±0.4 mm Hg/min, respectively). Both the XNT-treated group and the XNT plus MLN4760–treated group had a much faster recovery compared with controls (P<0.05), suggesting that XNT accelerates the BP recovery by a mechanism that is ACE2-independent.

**Effect of XNT on Hypertension Acutely Induced by Ang II Infusion in ACE2 KO Mice**

Studies in WT mice using the ACE2 inhibitor MLN-4760 clearly suggested that the effect of XNT on BP was ACE2-independent. To verify this finding, the effect of XNT on Ang II-induced hypertension was examined in ACE2-deficient mice (ACE2 KO) using the same infusion protocol as in WT mice. Baseline SBP was not different between XNT- (n=14) and vehicle-infused (n=11) animals (120.7±3.9 versus 122.2±4.8 mm Hg, respectively). The recovery in the XNT group was faster than in PBS-pretreated controls (slope, −3.09±0.30 mm Hg/min in XNT group versus −1.28±0.22 mm Hg/min in controls; P<0.001; Figure 4, lower panel). Moreover, the difference in BP persisted between the 2 groups throughout the 20 minutes of continuous monitoring (Figure 4, lower panel). SBP in the XNT-infused mice almost normalized <5 minutes after the Ang II bolus, whereas the BP of vehicle-infused mice had only begun to decline. These findings were essentially the same as those seen in WT mice (compare Figures 1 and 4, upper panel) and prompted us to conduct further experiments in vitro and ex vivo to examine ACE2 activity and Ang levels.

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**Figure 2.** Kidney (top) and serum angiotensin (Ang)-converting enzyme 2 (ACE2) activity (bottom) 5 minutes after the administration of Ang II bolus to wild-type mice pretreated with either vehicle (VHC; n=7) or XNT (n=7) in a similar manner as described in Figure 1. RFU indicates relative fluorescence unit.

**Figure 3.** Plasma angiotensin (Ang) II (top) and Ang (1–7) (bottom) measured 5 minutes after intraperitoneal bolus of Ang II in XNT or vehicle-pretreated wild-type mice (same as in Figure 2).
In Vitro Effect of XNT and DIZE on ACE2 Activity

To evaluate the effect of XNT and DIZE on ACE2 activity, fluorescence formation from hydrolysis of the ACE2-specific fluorogenic substrate, Mca-APK-Dnp, was monitored continuously in the presence of mouse rACE2. Several concentrations of XNT (10^{-4} to 10^{-10} mol/L) had no significant effect on Mca-APK-Dnp-mediated fluorescence formation conferred by recombinant mouse ACE2 in a time-dependent manner (Figure 6, upper left panel). At high concentrations (10^{-4} mol/L), XNT reduced the fluorescence and, therefore, ACE2 activity to 51.6% of controls.

DIZE, at the same concentrations as XNT, also had no significant effect on Mca-APK-Dnp hydrolysis by mouse rACE2 (Figure 6, lower left panel). At the highest concentration (10^{-4} mol/L) of DIZE, the fluorescence formation was reduced by 74.5%.

To examine the potential differences between human and mouse ACE2, the same experiments were performed with human rACE2. The addition of XNT had no significant effect on the enzyme activity of human rACE2 (Figure 6, upper right panel). DIZE also had no stimulatory effect on fluorescence formation mediated by human rACE2 and, at a highest concentration, inhibited substrate fluorescence to 43.6% of controls (Figure 6, lower right panel).

Effect of XNT and DIZE on Mouse and Rat Kidney ACE2 Activity Ex Vivo

In kidney lysates from WT mice (n=4) and ACE2 KO mice (n=4), ACE2 activity was measured for 1 hour when different concentrations of XNT and DIZE (10^{-4} to 10^{-10} mol/L) were added ex vivo. MLN-4760, used for specificity control, nearly completely inhibited ACE2 activity (Figure 7). XNT at various concentrations had no stimulatory effect on fluorescence formation from ACE2 from WT kidneys. In fact, at the highest concentration, XNT reduced the fluorescence by 27.1% (Figure 7, upper left panel).

DIZE at various concentrations also had no stimulatory effect on Mca-APK-Dnp–mediated fluorescence formation and, at the highest concentration, had a marked inhibitory effect, reducing fluorescence by 76.8% (Figure 7, lower left panel), which is consistent with the in vitro studies using rACE2.
The same experiment was conducted in rat kidney lysates. At concentrations of $10^{-5}$ to $10^{-10}$ mol/L, XNT did not alter fluorescence formation significantly. At the highest concentration ($10^{-4}$ mol/L), XNT decreased fluorescence by 31.2% (Figure 7, upper right panel). The addition of the highest concentration of DIZE caused an even stronger decline (Figure 7, lower right panel), reducing fluorescence by 41.8%, whereas lower concentrations ($10^{-5}$ to $10^{-10}$ mol/L) had no significant effect.

Ang Peptides Ex Vivo After Addition of XNT and Recombinant Murine ACE2

To further verify our findings in vivo and in vitro, we used a recently described ex vivo assay that permits a highly sensitive measurement of multiple Ang peptides concurrently in response to enzymes that affect Ang II metabolism and their inhibitors.20,38

Ang peptide analysis revealed no shift in the distribution of Ang peptides between samples from XNT and VHC group (Figure 8). By contrast, rACE2, used as a positive control, induced the expected shift toward Ang (1–7) formation and subsequent accumulation of its metabolite, Ang (1–5) (Figure 8).

In Vitro Effect of XNT and DIZE on the Enzymatic Activity of APA

A potential effect of XNT on APA, an enzyme capable of degrading Ang II to Ang (2–8) by cleaving the N-terminal amino acid aspartate, was also investigated. Various concentrations of XNT had no stimulatory effect on H-Glu-AMC (an APA substrate)-derived fluorescence generation, which increased linearly over time as a result of exposure to recombinant APA. In fact, at the highest concentrations, XNT reduced fluorescence formation by 43.6% (Figure 9, upper panel). DIZE at various concentrations also had no effect on the fluorescence of H-Glu-AMC substrate and, at high concentrations, had a marked inhibitory effect, reducing fluorescence by 71.5% (Figure 9, lower panel).

Discussion

Amplification of ACE2 enzymatic activity has substantial therapeutic potential, as this enzyme effectively degrades Ang II to Ang (1–7) and therefore could provide another approach to modify the RAS in addition to well-established drugs, such as ACE inhibitors and AT, R antagonists.6,11,20,39 Promising advances in this field were made after XNT and DIZE were identified via structure-based virtual screening as potential ACE2 activators.22,23 In subsequent studies, it was not directly examined whether the observed biological actions of these compounds were associated with an increase in ACE2 activity and an enhanced conversion of Ang II to Ang (1–7).26–35,40 Rather, it was assumed that the presumed mechanism of action was because of the activation of ACE2, based on the original reports by Hernandez Prada et al22 and Kulemina et al.23

In this present study, we investigated whether or not XNT and DIZE are effective activators of ACE2. Moreover, we used a model of acute Ang II–induced hypertension as a paradigm to investigate the previously reported effect of XNT on BP.22 In these acute studies, XNT markedly accelerated the recovery from Ang II–induced hypertension in a manner that resembled the effect of rACE2, but to a lesser extent (Figure 1). The dose of XNT (18 mg/kg) was in the same range as the dose that was proved most potent in previous studies (10 mg/
The plasma ACE2 activity after Ang II infusion, however, remained low in both XNT- and vehicle-infused mice. Moreover, after the infusion of Ang II, the plasma levels of Ang II, the substrate of ACE2 and Ang (1–7), the peptide generated by the cleavage of Ang II by ACE2, were not affected by the presence of XNT. These findings altogether suggested that the effect of XNT on Ang II–induced hypertension could not be because of the activation of ACE2. This possibility was substantiated in experiments with ACE2 KO mice where XNT also elicited enhanced recovery from Ang II–induced hypertension in a manner similar to the recovery in WT mice (compare Figures 1 and 4A). The BP-lowering effect of XNT in ACE2 KO mice, therefore, provides irrefutable evidence that this action of XNT cannot be because of ACE2 activation.

It was recently reported, however, that XNT and DIZE may cause a significant increase in ACE2 mRNA. This would suggest that these compounds upregulate ACE2 gene expression, which could contribute to their mechanism of action. We think that such an effect would not be able to explain the rapid effect of XNT on BP that we found in WT mice after Ang II infusion. Moreover, an increase in mRNA for ACE2 cannot happen in ACE2 KO mice. Because XNT markedly reduced Ang II–dependent hypertension in this model, we conclude that this action of XNT is ACE2-independent. Recent reports suggested that XNT induces vascular relaxation via an Ang (1–7)–independent mechanism because the administration of a Mas receptor antagonist did not alter the vasorelaxant responses to XNT.

We also considered the possibility that XNT could trigger Ang II dissipation by a mechanism that could be ACE2-independent. For this purpose, mass spectrometry was used to see if XNT had an effect on Ang II dissipation in an ex vivo plasma model. Murine rACE2, used as a positive control, nearly completely diminished Ang II and increased Ang (1–7) and subsequently Ang (1–5). Whereas, XNT had no effect whatsoever because Ang II, Ang (1–7), and Ang (1–5) levels were not altered as compared with control levels. We confirmed these findings through in vitro experiments, where incubation of Ang II in the presence of rACE2 did not result in accelerated Ang II dissipation by either XNT or DIZE.

Additionally, we evaluated whether the addition of different concentrations (10−10 to 10−4 mol/L) of XNT or DIZE ex vivo could alter ACE2 activity in the kidney, an organ with very high levels of ACE2 expression and activity. To address potential species differences in Ang II metabolism, we conducted these ex vivo experiments in both mouse and rat kidneys. Both molecules had no activating impact on endogenous mouse and rat ACE2 enzymatic activity at any concentration applied (Figure 7). In fact, ACE2 enzyme activity was inhibited by both compounds at the highest concentrations. The inhibitory effect was more prominent after DIZE addition. We also examined in vitro the effect of these compounds on APA in order to rule out that XNT triggers alternative pathways of Ang II dissipation. Neither XNT nor DIZE was capable of enhancing APA activity. Instead, at the highest concentration (10−4) of XNT and DIZE, APA activity was inhibited substantially. This inhibitory effect of both XNT and DIZE at high concentrations likely reflects a nonspecific effect and does not seem to be relevant to the in vivo situation, where such high concentrations would never be achieved.
In vitro experiments to evaluate whether or not XNT or DIZE could alter the enzyme activity of highly purified mouse and human rACE2 revealed no stimulatory effect. In fact, at high concentrations, both XNT and DIZE inhibited ACE2 activity, which is consistent with our ex vivo studies on kidney ACE2 activity (Figure 7). These findings are in sharp contrast with an earlier study showing about a 2-fold increase in ACE2 activity by XNT using a similar in vitro approach.\(^2^2\) We cannot find an explanation for the discrepancy between our in vitro studies and the report by Hernandez Prada et al.\(^2^2\) In both studies, human rACE2 was used to examine the effect of XNT in vitro. Overall, our data show that there is no stimulatory effect of XNT on ACE2 activity ex vivo in plasma or kidney tissue. In vivo XNT also has no effect on plasma or kidney ACE2 activity. Moreover, the lack of a significant difference in Ang II and Ang (1–7) plasma levels after XNT further negates any significant stimulatory effect of this compound on ACE2 activity. Finally, there was no effect of XNT on Ang II peptides measured using a sensitive ex vivo plasma system, which is in sharp contrast to the profound effect of murine recombinant ACE2, which rapidly dissipated Ang II through the formation of Ang (1–7) and subsequent degradation to Ang (1–5) (Figure 8).

In conclusion, the present studies show a complete lack of stimulatory effect of XNT and DIZE on ACE2 activity. The biological effects of these agents reported\(^2^2,2^3,2^5–3^3\) are, therefore, the result of mechanisms that remain to be elucidated, and should not be attributed to ACE2 activation. ACE2 remains a promising therapeutic target, and it is of utmost importance to continue the search for compounds that activate this enzyme.

**Perspectives**

ACE2 activators could provide a promising treatment option for a vast array of pathological conditions, such as hypertension, pulmonary hypertension, cardiovascular, and diabetic kidney disease. To date, only 2 compounds, XNT and DIZE, have been used as ACE2 activators with remarkable outcomes.\(^2^2,2^6,2^9–3^1,3^3\) The present study shows that XNT is
effective in the treatment of acute Ang II–induced hypertension in WT mice, even when ACE2 is inhibited pharmacologically, and in mice with genetic ablation of ACE2. The latter finding demonstrates that the mechanism of action of XNT is ACE2-independent. Our findings further show that XNT and DIZE are not activators of ACE2 enzymatic activity ex vivo and in vitro. Given the importance of ACE2 as a therapeutic target, renewed efforts should be made to search for compounds capable of amplifying ACE2 activity, particularly at the tissue level where this enzyme is present in substantial amounts. Plasma levels of this enzyme are low, and therapeutic approaches may be much more effective by the direct administration of rACE2.

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Disclosures
None.

References

ACE2-Independent Action of ACE2 Activators


What Is New?
- This study reports that compounds used as ACE2 activators, such as XNT and DIZE, lack a stimulatory effect on ACE2 activity ex vivo, in vivo, and in vitro.
- It was shown for the first time that XNT exerts its hypotensive effects via an angiotensin (Ang)-converting enzyme 2 (ACE2)- and Ang II–independent pathway.

What Is Relevant?
- Given the potential importance of ACE2 as a therapeutic target, there is a need to identify compounds that are capable of activating ACE2.

Novelty and Significance

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<th>What Is New?</th>
<th>Summary</th>
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<td>- This study reports that compounds used as ACE2 activators, such as XNT and DIZE, lack a stimulatory effect on ACE2 activity ex vivo, in vivo, and in vitro.</td>
<td>Reported ACE2 activator XNT greatly attenuated Ang II- induced acute hypertension not only in wild-type mice but also in an ACE2 knockout line, demonstrating an ACE2 independent mechanism of action. In further ex vivo and in vitro studies, neither XNT nor DIZE were capable of increasing ACE2 enzymatic activity. Both compounds had no effect on Ang II dissipation or Ang (1–7) formation.</td>
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<td>- It was shown for the first time that XNT exerts its hypotensive effects via an angiotensin (Ang)-converting enzyme 2 (ACE2)- and Ang II–independent pathway.</td>
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ACE2-INDEPENDENT ACTION OF PRESUMED ACE2 ACTIVATORS;

STUDIES IN VIVO, EX VIVO AND IN VITRO

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EXPANDED MATERIALS, METHODS

Animal models

The mice were housed in cages (12:12-h light-dark cycle) with ad libitum access to chow and water. The Institutional Animal Care and Use Committee approved all procedures adhering to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Measurements of Plasma Ang II and Ang-(1-7)

Blood samples were collected via cardiac puncture and kept in tubes on ice containing ethylenediamine tetraacetic acid (25 mmol/L), \(O\)-phenanthroline (0.44 mmol/L), pepstatin A (0.12 mmol/L), and \(P\)-hydroxymercuribenzoic acid (1 mmol/L) and then centrifuged (3000 g). The plasma was saved and stored at \(-80^\circ C\) until further processing. Ang peptides were extracted from plasma using reverse-phase phenyl silica columns (100 mg; HYPERSEP PH, Thermo Scientific) as per the manufacturer's instructions. The quantity of Ang II in the extract was determined using an enzyme immunoassay kit (SPIBio, Cayman Chemical, Ann Arbor, MI), as per the manufacturer's instructions. Results were reported in picomoles per milliliter of plasma. Ang (1-7) levels in the extract were also measured via an enzyme immunoassay kit (Peninsula Laboratories, Bachem, San Carlos, CA).

Mass spectrometry

In blood plasma, all of the RAS enzymes, except renin, are present in excess compared with their substrates. Therefore, 100 pg/mL of recombinant renin (Sigma) was added to isolated plasma, as described recently\(^1,2\). After 10 minutes of incubation at \(37^\circ C\) with XNT, protease inhibitor mixture was added to stop the reaction, and samples were subjected to liquid chromatography-tandem mass spectrometry analysis (Attoquant, Vienna, Austria). Plasma samples were spiked with 100 pg/mL of stable isotope labeled internal standards and subjected to solid-phase extraction using Sep-Pak cartridges (Waters), as described recently\(^1\). After elution and solvent evaporation, samples were reconstituted in 50 \(\mu\)L of 50.0% acetonitrile/0.1% formic acid and subjected to liquid chromatography-tandem mass spectrometry analysis using a reversed phase analytic column (Luna C18, Phenomenex) with a gradient ranging from 10.0% acetonitrile/0.1% formic acid to 70.0% acetonitrile/0.1% formic acid in 9 minutes. The eluate was analyzed in line with a QTRAP-4000 mass spectrometer (AB Scieix) operated in the MRM mode using dwell times of 25 ms at a cone voltage of 4000 volts and a source temperature of 300°C. For each peptide and corresponding internal standards, 2 different mass transitions were measured\(^1\). Ang peptide concentrations were calculated by relating endogenous peptide signals to internal standard signals provided that integrated signals achieved a signal:noise ratio >10. The quantification limits for individual peptides were found to range between 1 and 5 pg/mL of undiluted plasma. Ten Ang peptides were simultaneously evaluated by this method: Ang I, Ang-(1-9), Ang II, Ang III, Ang IV, Ang-(1-7), Ang-(1-5), Ang-(2-7), Ang-(3-7), and Ang-(2-10).
References
