ACE2 Activation Promotes Antithrombotic Activity

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The aim of the present study was to test the hypothesis that the activation of the angiotensin-converting enzyme (ACE2)/angiotensin-(1-7)/Mas receptor axis by use of a novel ACE2 activator (XNT) would protect against thrombosis. Thrombi were induced in the vena cava of spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats, and ACE2 and ACE activity in the thrombus was determined. Real-time thrombus formation was viewed through intravital microscopy of vessels in nude mice. Thrombus weight was 40% greater in the SHR (4.99 ± 0.39 versus 7.04 ± 0.66 mg). This weight increase was associated with a 20% decrease in ACE2 activity in the thrombus. In contrast, there were no differences between the WKY and SHR in ACE2 protein and ACE activity in the thrombus. ACE2 activation in the thrombus, ACE2 inhibition (DX600; 0.1 µmol/L/kg) increased thrombus weight by 30% and XNT treatment (10 mg/kg) resulted in a 30% attenuation of thrombus formation in the SHR. Moreover, XNT reduced platelet attachment to injured vessels, reduced thrombus size, and prolonged the time for complete vessel occlusion in mice. Thus, a decrease in thrombus ACE2 activity is associated with increased thrombus formation in SHR. Furthermore, ACE2 activation attenuates thrombus formation and reduces platelet attachment to vessels. These results suggest that ACE2 could be a novel target for the treatment of thrombo- genic diseases.

INTRODUCTION

Thrombogenic events, such as ischemic stroke, pulmonary embolism, deep venous thrombosis, mesenteric ischemia and acute coronary syndrome, are major complications of certain pathological conditions, such as hypertension, atherosclerosis, and diabetes mellitus (1). Despite the many therapeutic advances and increasingly effective drug treatments, thrombogenic events remain the major cause of morbidity and mortality worldwide (2). Significant efforts have been made to identify novel drug treatments.

The renin-angiotensin system (RAS) is a pivotal regulator of cardiovascular function. Increased activity of the vasoconstrictive and proliferative axis (angiotensin [Ang] II/angiotensin-converting enzyme [ACE]/AT1 receptor) has been reported to be associated with a higher risk of acute thrombosis through the destabilizing of atherosclerotic plaque and enhancing of platelet activity and coagulation (3). In the classical concept, the RAS is made up of a series of enzyme reactions that culminate in the generation of Ang II, the main peptide of this system, by ACE. Ang II has many adverse cardiovascular effects when acting through the AT1 receptor. However, this simplistic concept of a linear RAS cascade has given way to a more complex concept since the discovery of ACE2, which is an ACE homologue (4,5).

Unlike ACE, ACE2 catalyzes the conversion of Ang II to the heptapeptide Ang-(1-7) (6). Ang-(1-7) has been reported to be counterregulatory to Ang II actions, promoting many beneficial cardiovascular effects through the interaction with its own receptor Mas (7). Therefore, a novel concept of two opposing axes regulating the activity of the RAS has emerged: the ACE/Ang II/AT1 axis is involved in hypertensive, proliferative, vasoconstrictive, hypertrophic and fibrotic actions; whereas the counterregulatory ACE2/Ang-(1-7)/Mas axis is involved in vasoprotective actions of the RAS (5,8).

Given these recent advances, it may be hypothesized that the ACE2/Ang-(1-7)/Mas axis is involved in the hemostatic process, and its activation produces antithrombotic activity. The aim of the present study was to evaluate the role of ACE2 in thrombus formation in normotensive and hypertensive rats and investigate the effects on thrombus formation of a novel ACE2 activator (XNT) (9).
MATERIALS AND METHODS

Animals

All procedures involving animals were carried out in compliance with approved International Animal Care and Use Committee protocols and University of Florida regulations. Male Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) 12- to 13-wks-old (body weight: 280 to 310 g) were purchased from the Charles River Laboratories (Wilmington, MA, USA). Nude male mice 9- to 10-wks-old were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA).

Thrombosis Model in Rats

Thrombus formation was induced in the abdominal vena cava of SHR and WKY using a ferric chloride (FeCl₃) solution, as described elsewhere (10). Briefly, after anesthesia (ketamine, xylazine and acepromazine at 30, 6, and 1 mg/kg, respectively), the jugular vein was cannulated for drug administration. Subsequently, the abdominal vena cava was exposed via a midline abdominal incision and was carefully separated from the surrounding tissues. Either XNT at 10 mg/kg, the ACE2 inhibitor DX600 at 0.1 μmol/L/kg, or vehicle (saline) was administered 5 min before thrombus induction. The XNT and DX600 doses were based on previous studies (9,11). Filter paper (3 × 5 mm) steeped in a 5% FeCl₃ solution was topically applied to the vena cava 5 mm below the left renal vein. The paper was removed after 3 min and the abdomen closed with suture. After 30 min, the thrombus formed was carefully removed, weighed and frozen at –80°C. The thrombus was then carefully removed, weighed and frozen at –80°C. The thrombus was then removed after 3 min.

Determination of ACE and ACE2 Activity

ACE and ACE2 activity was determined in the thrombi obtained from the WKY and SHR by using their respective fluorogenic peptide substrates (ACE substrate, fluorogenic peptide VI [FPS VI], Mca RPPGFSAFK(Dnp)-OH, catalog. ID: ES005; ACE2 substrate, fluorogenic peptide VI [FPS VI], Mca-YVADAPK(Dnp)-OH, catalog. ID: ES007, where Mca is 7-(methoxycoumarin-4-yl) acetyl and Dnp is 2,4-dinitrophenyl; R&D Systems, Minneapolis, MN, USA), as described elsewhere (12). The reaction buffer (75 mmol/L Tris/HCl [pH 7.5], 1 mol/L NaCl and 0.5 mmol/L ZnCl₂) was added to the thrombus and sonicated; the protein content was determined by use of a standard Bradford assay. The enzymes removed the C-terminal dinitrophenyl moiety that quenches the inherent fluorescence of the 7-methoxycoumarin group, resulting in an increase in fluorescence at excitation and emission spectra of 328 and 392 nm, respectively. Enzyme activity was determined using a Spectra Max Gemini EM Fluorescence Reader (Molecular Devices, Sunnyvale, CA, USA). All assays were performed at least in triplicate and samples were read every min for at least 120 min immediately after the addition of fluorogenic peptide substrates at 37°C. Enzyme activity was corrected to the background and the data were normalized considering untreated WKY as having 100% ACE2 activity.

Western Blotting for ACE2

Sixty micrograms of protein extracted from thrombi of WKY rats and SHR were run on a 12% SDS-PAGE gel. The proteins were then transferred onto a nitrocellulose membrane. The membrane was probed with the rabbit polyclonal antibody against ACE2 (sc-20998 [Santa Cruz Biotechnology, Santa Cruz, CA, USA]) 1 μg/mL in 1% milk/Tris-buffered saline-Tween solution) overnight at 4°C. Membrane was washed three times for 10 min in Tris-buffered saline-Tween solution and incubated with antirabbit IgG–horseradish peroxidase–conjugated secondary antibody (1:5000) for 1 h at room temperature. After the final washes, the membrane was incubated with chemiluminescent agent for 1 min and then exposed to a film to visualize the protein bands. β-actin was used as a loading control.

Intravital Microscopy

Real-time thrombus formation was visualized using a mouse dorsal skinfold chamber and intravitral microscopy in nude mice, as described elsewhere (13,14). Briefly, the mice were anaesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally and the jugular vein was cannulated for intravenous access. A titanium window chamber was surgically implanted in the dorsal skin flap of the mice. From the extended double layer of the dorsal skin, the top layer was removed to visualize the vasculature on the opposite side of the skin flap in a circular area of about 6 mm². Veins with a diameter of 80 to 100 μm were chosen for visualization of localized thrombus formation via intravitral microscopy. The cannulated jugular vein was used to treat the mice with intravenous carboxyfluorescein diacetate succinimidyl ester (Invitrogen, Carlsbad, CA, USA; cat# CI157), which is a nonfluorescent precursor taken up into platelets and partly into leukocytes, which forms a stable fluorochrome denominated carboxyfluorescein succinimidyl ester (CFSE) in the cells. CFSE-labeled platelets flowing through the mouse vasculature and the subsequent vascular injury–induced thrombus formation were visualized through multifunctional intravitral fluorescence microscopy. A Zeiss AxioImager microscope (Carl Zeiss, Thornwood, NY, USA) served as the basic platform. Fluorescently labeled platelets flowing through the mouse vasculature were imaged at a magnification of 10× using the EC Plan-Neofluar long-working distance objective (Carl Zeiss). An ANDOR iXon electron multiplying CCD (EMCCD) camera (ANDOR Technology, South Windsor, CT, USA) was used to capture streaming videos of the fluorescently labeled platelets in the microvasculature at a frame rate of about 30 Hz. A Zeiss FluoroArc mercury lamp was used as the light source for the fluorescent imaging. Fluorescent platelets were viewed using an FITC filter set (Carl Zeiss; filter set

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The appropriate vessel region in the mouse vasculature was selected for thrombus induction based on vessel size (80 to 100 µm) and proper network functionality. Either XNT at 5 mg/kg or vehicle (saline) was administered 5 min before thrombus induction. Vascular injury and thrombus formation were induced by a precise topical application of filter paper (3 mm²) soaked in 5% FeCl₃ solution on the selected vessel location. After 2 min, the paper was removed and the vessels were washed with saline at 37°C. Streaming videos of 20-s duration were obtained for the treatment area every min. The microvessels were viewed approximately 30 min after FeCl₃ application, and during this period, an evaluation was performed of the time necessary to stop the blood flow as a result of the total occlusion of the vessel and the thrombus size over time.

### Statistical Analysis

All data are expressed as mean ± SEM. Comparisons between experimental and control groups were analyzed using one- or two-way ANOVA followed by the Bonferroni posttest or Student t test. Differences were considered significant at a P ≤ 0.05.

### RESULTS

#### Higher Thrombus Formation in SHR Is Associated with Lower ACE2 Activity

As expected, FeCl₃-induced thrombus weight was 40% higher in the SHR compared with the WKY (4.99 ±0.39 versus 7.04 ±0.66 mg in SHR) (Figure 1A). This result was associated with a 20% reduction in ACE2 activity in the thrombi from the SHR and WKY (Figure 1B). There were no differences in ACE2 protein levels in the thrombi from the SHR and WKY (Figure 1C, D). These data reveal that the greater thrombus formation in the SHR was associated with lower ACE2 activity, with no significant change in ACE2 protein. In contrast with ACE2, there were no significant differences in ACE activity in the thrombi between the SHR and WKY (Figure 2A). However, the ratio between ACE2 and ACE activity was significantly decreased in the SHR (Figure 2B). To further evaluate the participation of ACE2 in the thrombogenic process, the animals were treated with a specific ACE2 inhibitor (DX600) 5 min before thrombus induction by FeCl₃. Intravenous administration of this inhibitor produced an increase in thrombus weight by 46% in the SHR and 37% in the WKY (Figure 3A). Although ACE2 activity in thrombi from the SHR treated with DX600 decreased, the difference did not achieve statistical significance (Figure 3C).
Effects of ACE2 Activation on Antithrombotic Activity

To address the effect of ACE2 activation on thrombus formation, we used two different models. The effect of XNT, a small-molecule ACE2 activator (9), on thrombus weight was evaluated first, using large vessels in rats. Second, platelet adhesion and thrombus formation were viewed in real time using mouse microvessels. XNT administered through the jugular vein 5 min before thrombus induction resulted in a decrease in thrombus weight (34.7% in the SHR and 32.3% in the WKY) (Figure 3B). This effect was accompanied by an increase in ACE2 activity in the thrombi in the SHR (Figure 3C). Moreover, treatment with XNT reduced platelet attachment to vessels and thrombus formation in mice, as observed under real-time intravital microscopy (Figure 4A). Thrombus area was reduced by 60%, whereas time for thrombus formation was prolonged by 45% in XNT-treated mice (Figure 4B, C).

DISCUSSION

The most significant findings of the present study are that ACE2 plays an important role in thrombogenic events and its activation by the small-molecule ACE2 activator XNT has significant antithrombogenic effects. The role of ACE2 in the pathophysiology of cardiovascular diseases is currently under intense investigation. The involvement of this enzyme in cardiac contractile function, hypertension, atherosclerosis and other cardiovascular diseases has recently been demonstrated (15–17). The present study offers evidence that ACE2 is involved in the hemostatic process and exhibits a protective action against thrombosis. We observed that a decrease in ACE2 activity in the thrombi, but not in ACE2 protein, is associated with an increase in thrombus formation in the SHR. In keeping with this finding, the pharmacological activation of...
ACE2 using XNT produced a significant reduction in thrombus formation in the SHR to a level similar to that found in the WKY. XNT is a small-molecule ACE2 activator that was recently discovered based on the crystal structure of ACE2 and a virtual screening strategy (9). This compound enhances ACE2 activity, causes a reduction in arterial blood pressure and an impressive reversal of cardiac and renal fibrosis in SHR, and also prevents pulmonary hypertension (9,18). It is important to note that the increase in thrombus weight caused by DX600 administration in SHR (46%) and in WKY (37%) was not statistically different (P > 0.05). One could argue that the inhibition of ACE2 by DX600 should have greater impact on thrombus formation in WKY rats than in SHR, considering that ACE2 activity is reduced in SHR. However, because the balance between the two counterregulatory axes of the RAS is shifted toward the ACE/Ang II/AT1R branch in hypertensive rats, it is pertinent to speculate that the responses to pharmacological manipulations of the ACE2/Ang-(1-7)/Mas axis can produce distinct responses in hypertensive compared with normotensive animals.

The beneficial effects of ACE2 activation are primarily due to the shifting of the balance of the RAS from the vasoconstrictive fibrotic axis to the vasodilator branch. This hypothesis is supported by recent data (18). Although neither Ang II nor Ang-(1-7) levels in the thrombi were determined in the present investigation, a large number of studies have shown that Ang II is a prothrombotic agent (19,20). Ang II is associated with an increase in the production and secretion of plasminogen activator-inhibitor type 1 from endothelial and smooth muscle cells and an augmentation of tissue factor expression, thereby enhancing the activity of the coagulation system (19,20). Moreover, it is well established from previous studies that platelets express the AT1 receptor and its activation by Ang II potentiates platelet activation and aggregation (21–23). On the other hand, Ang-(1-7) has been described as an antithrombotic peptide (24). Kucharewicz et al. (24,25) have shown that intravenous infusion of Ang-(1-7) produces a potent reduction in thrombus formation in renovascular hypertensive rats. Furthermore, these authors also found that the intrinsic antithrombotic effects of the antihypertensive drugs captopril (ACE inhibitor) and losartan (AT1 receptor blocker) were attenuated by the selective Ang-(1-7) receptor antagonist A-779 (24,25). More recently, it was demonstrated that the Ang-(1-7) receptor Mas is present in platelets and the interaction between Ang-(1-7) and Mas on platelets promotes nitric oxide (NO) production, which is a major antiplatelet agent (26). Moreover, the activation of endothelial Mas by Ang-(1-7) has been reported to increase NO and prostacyclin synthesis (27). Both of these mediators are important antithrombotic agents in the hemostasis process (1,28). Because ACE2 can catalyze the conversion of Ang II to Ang-(1-7), it is possible that the significant antithrombotic effect observed with XNT treatment could be a result of a decrease in the bioavailability of the prothrombotic peptide Ang II, followed by an increase in the formation of the antithrombotic peptide Ang-(1-7). Unfortunately, no Mas antagonists were used in the present study. However, an experimental model of pulmonary hypertension demonstrated that the protective effects evoked by XNT were completely abolished by the coadministration of A-779 (18). This important finding should be taken into consideration in future experiments.

The present study employed two different in vivo models of thrombosis induced by FeCl3 to demonstrate the antithrombotic effects of XNT: thrombus formation in the abdominal vena cava in a rat model and in microveins of dorsal mouse skin. According to Kucharewicz et al. (25), an advantage of using the above mentioned models is the ability to separate antithrombotic and hypotensive effects of the compounds studied, because the thrombus forming inside the veins is unlikely to be affected by changes in arterial blood pressure (25). Because the relationship between changes in arterial and venous blood pressure is negligible, alterations in arterial blood pressure should not interfere in thrombus formation in veins (25). Thus, it was possible to exclude the influence of the hypotensive effect of XNT (9) from its antithrombotic action. Moreover, the acute intravenous administration of XNT at 10 mg/kg has been found to cause a decrease in blood pressure in SHR and WKY rats; however, this effect is significantly lower in WKY compared with hypertensive rats (21.3 ±8.2 mmHg in WKY versus 71.0 ±9.0 mmHg in SHR) (9). Despite this difference, the antithrombotic effect of XNT was similar in both strains (32.3% reduction in thrombus weight in the WKY versus 34.7% reduction in thrombus weight in the SHR). These findings again suggest that the antithrombotic effect of XNT is independent from changes in blood pressure. Moreover, intravital microscopy allowed the real-time visualization of the participation of platelets in the thrombus formation process.

In summary, the results of this study demonstrated that a decrease in ACE2 activity in thrombi was associated with an increase in thrombus formation in SHR. Furthermore, the pharmacological activation of ACE2 attenuated platelet attachment to vessels and thrombus formation. These results clearly suggest that XNT could be a potential lead compound for the treatment of thrombogenic diseases.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.
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