Renin-Angiotensin-Aldosterone-System

Compound 21 Induces Vasorelaxation via an Endothelium- and Angiotensin II Type 2 Receptor-Independent Mechanism

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See Editorial Commentary, pp 616–617

Abstract—Angiotensin II type 2 (AT2) receptor stimulation has been linked to vasodilation. Yet, AT2 receptor-independent hypertension and hypotension (or no effect on blood pressure) have been observed in vivo after application of the AT2 receptor agonist compound 21 (C21). We, therefore, studied its effects in vitro, using preparations known to display AT2 receptor-mediated responses. Hearts of Wistar rats, spontaneously hypertensive rats (SHRs), C57Bl/6 mice, and AT2 receptor knockout mice were perfused according to Langendorff. Mesenteric and iliac arteries of these animals, as well as coronary microarteries from human donor hearts, were mounted in Mulvany myographs. In the coronary vascular bed of Wistar rats, C57Bl/6 mice, and AT2 receptor knockout mice, C21 induced constriction followed by dilation. SHR hearts displayed enhanced constriction and no dilation. Irbesartan (angiotensin II type 1 receptor blocker) abolished the constriction and enhanced or (in SHRs) reintroduced dilation, and PD123319 (AT2 receptor blocker) did not block the latter. C21 relaxed preconstricted vessels of all species, and this did not depend on angiotensin II receptors, the endothelium, or the NO-guanylyl cyclase-cGMP pathway. C21 constricted SHR iliac arteries but none of the other vessels, and irbesartan prevented this. C21 shifted the concentration-response curves to U46619 (thromboxane A2 analog) and phenylephrine (α-adrenoceptor agonist) but not ionomycin (calcium ionophore) to the right. In conclusion, C21 did not cause AT2 receptor-mediated vasodilation. Yet, it did induce vasodilation by blocking calcium transport into the cell and constriction via angiotensin II type 1 receptor stimulation. The latter effect is enhanced in SHRs. These data may explain the varying effects of C21 on blood pressure in vivo. (Hypertension. 2012;60:722-729.)

Key Words: angiotensin II ■ compound 21 ■ AT2 receptor ■ vasorelaxation ■ coronary circulation ■ mouse ■ rat ■ Langendorff model

Stimulation of the angiotensin (Ang) II type 2 (AT2) receptor mediates vasorelaxant, natriuretic, growth-suppressing, and antifibrotic effects. As such, it seems to counteract Ang II type 1 (AT1) receptor-mediated effects. However, opposite findings have been reported as well, and according to some studies, Ang II type 2 (AT2) receptor effects mimic those of the AT1 receptor (eg, inducing vasoconstriction and hypertrophy). Our knowledge on AT2 receptor function is largely based on the use of the AT2 receptor antagonist PD123319, AT2 receptor-deficient (AT2R(−/−)) animals, and the peptidic AT2 receptor agonist CGP42112A. The use of the latter is hampered by its partial agonistic properties. In 2004, Wan et al reported the synthesis of compound 21 (C21), the first selective nonpeptide AT2 receptor agonist. C21 has an oral bioavailability of 20% to 30% and an estimated half-life of 4 hours in plasma. Administration of C21 in various cardiovascular disease models, including the postmyocardial infarction Wistar rat; the stroke-prone hypertensive rat and the 2-kidney, 1-clip hypertensive Sprague-Dawley (SD) rat, resulted in beneficial organ-protective effects.

The vasorelaxant properties of C21 are less straightforward. C21 lowered mean arterial pressure by ~25 mm Hg in anesthetized spontaneously hypertensive rats (SHRs) but not in SD rats. Remarkably, this response was not affected by AT2 receptor blockade, suggesting that it did not involve AT2 receptor stimulation. Bosnyak et al reported a vasodepressor response after administration of C21 (300 ng/kg per minute) in conscious SHRs (but not Wistar-Kyoto rats) on top of low-dose AT1 receptor antagonism, which could be blocked by the AT2 receptor antagonist PD123319. In the absence of AT1 receptor blockade, no blood pressure—lower-
ing effects were observed in stroke-prone hypertensive rats; 2-kidney, 1-clip hypertensive SD rats; post-myocardial infarction Wistar rats; and C57BL/6 mice.\textsuperscript{15–18,20} When infused over a short time period, 300 ng/kg per minute of C21 induced a modest rise in mean arterial pressure (\(\approx 4\) mm Hg) in male SD rats, which was not seen in combination with PD123319.\textsuperscript{21} A 3.3-fold higher dose increased mean arterial pressure by 20 mm Hg in male SHR in an AT\(_1\) receptor-dependent manner.\textsuperscript{19} Similarly, C21 transiently increased systolic blood pressure by \(\approx 20\) mm Hg in an AT\(_1\) receptor-dependent manner when given orally to stroke-prone hypertensive rats at a dose of 1 mg/kg per day.\textsuperscript{17} Yet, in the latter study, C21 did not lower blood pressure on top of AT\(_1\) receptor antagonism; in fact, C21 exerted no effect at all on blood pressure, possibly because the applied AT\(_1\) receptor blocker dose had already resulted in maximum blood pressure-lowering effects.

Taken together, these in vitro data demonstrate C21-induced relaxation via AT\(_2\) receptors or unknown mechanisms, as well as constriction via both AT\(_2\) and AT\(_1\) receptors, the latter requiring high doses. AT\(_1\) receptor blockade appeared a prerequisite to observe AT\(_2\) receptor-mediated hypertensive effects in vivo, but this is not a universal finding and may depend on the degree of AT\(_1\) receptor blockade.\textsuperscript{17,19,20} In vitro data on C21-induced relaxation are scarce, despite the wide range of in vitro studies supporting AT\(_2\)-induced relaxation in multiple vascular beds. No human data are available. Therefore, it was the aim of the present study to investigate C21-induced vasodilation/constriction in vitro, taking into consideration species- (including humans), pathology- (hypertension), and concentration-related effects and carefully considering its blockade by AT\(_1\) receptor antagonists or in AT\(_2\)-receptor-deficient (AT\(_2\)-R\(^{−/−}\)) mice. We made use of preparations that, in previous studies, displayed clear AT\(_2\)-receptor-mediated vasodilatation.\textsuperscript{2,14,12,22}

**Methods**

**Animal Studies**

Male Wistar rats (337±5 g; n=45), male SHRs (320±3 g; n=18), and male C57BL/6 mice (29±1 g; n=10) were obtained from Harlan. Male AT\(_1\)-R\(^{−/−}\) (28±1 g; n=10) bred on a C57BL/6 background were obtained from the animal facilities of the Charité (Campus Benjamin Franklin, Berlin, Germany). C57BL/6 and AT\(_1\)-R\(^{−/−}\) mice were genotyped to verify AT\(_1\) receptor expression. All of the experiments were performed under the regulation and permission of the animal care committee of the Erasmus MC.

Animals were anesthetized with pentobarbital (60 mg/kg IP). Rat hearts were excised and placed in ice-cold Tyrode buffer,\textsuperscript{22} whereas mouse hearts were placed in modified Krebs-Henseleit buffer,\textsuperscript{22} both gassed with 95% O\(_2\)/5% CO\(_2\). Subsequently, iliac and mesenteric arteries were removed and either used directly or after overnight storage in cold, oxygenated Krebs-Henseleit solution. Such storage does not affect responsiveness.\textsuperscript{24,25}

**Human Studies**

Human coronary microarteries were obtained from 5 heart-beating donors (2 men and 3 women, age 41±7 years) who died of noncardiac causes (2 cerebrovascular accident, 1 head trauma, 1 subarachnoid bleeding, and 1 suicide) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the heart valves for transplantation purposes. The study was approved by the ethics committee of the Erasmus MC. Human coronary microarteries were isolated and stored in Krebs-Henseleit, as described before.\textsuperscript{1}

**Langendorff Preparation**

Rat and mouse hearts were perfused according to Langendorff, as described previously.\textsuperscript{22,23} Gassed perfusion buffer was used to superfuse the mouse hearts to prevent temperature fluctuations. Coronary flow (CF) was measured with a flow probe (Transonic systems). After a stabilization period of 30 minutes, baseline values of CF were obtained. Next, bolus injections (100 \(\mu\)L) of perfusion buffer were applied 3 times to determine injection-induced changes in CF. Subsequently, concentration-response curves (CRCs) to C21 (kindly provided by Vicore Pharma) were constructed via bolus injections, in the absence or presence of the AT\(_1\) receptor antagonist irbesartan (provided by Sanofi-Synthelabo) or PD123319. Blockers were present in the perfusion buffer starting 15 minutes before the first bolus injection.

**Mulvany Myograph**

Human coronary microarteries (diameter, \(\approx 750\) \(\mu\)m), rat iliac arteries (diameter, \(\approx 800\) \(\mu\)m), rat mesenteric arteries (diameter, \(\approx 150\) \(\mu\)m), mouse iliac arteries (diameter, \(\approx 350\) \(\mu\)m), and mouse abdominal aortas (diameter, \(\approx 550\) \(\mu\)m) were cut into ring segments of \(\approx 2\)-mm length. In some rat iliac artery segments, the endothelium was removed by gently rolling the vessel after insertion of the tip of small-angled forceps into the lumen. Segments were mounted in a Mulvany myograph with separated 6-\(\mu\)L organ baths containing gassed Krebs-Henseleit buffer at 37°C, as described previously, and tension was normalized to 90% of the estimated diameter at 100 mm Hg of effective transmural pressure. After a 30-minute stabilization period, the maximal contractile response was determined by exposing the vessels to 100 mM/L of KCl. Thereafter, vessels were preincubated for 30 minutes in fresh buffer in the absence or presence of 1 \(\mu\)mol/L of irbesartan, 1 \(\mu\)mol/L of PD123319, 100 \(\mu\)mol/L of \(N^2\)-nitro-L-arginine methyl ester (NAME), 10 \(\mu\)mol/L of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 200 \(\mu\)mol/L of hydroxocobalamin, 10 \(\mu\)mol/L of Y27632, or 0.1 to 100 \(\mu\)mol/L of C21, and CRCs were constructed to C21, phenylephrine, U46619, or 4-aminopyrine. C21-induced relaxation was studied after preconstriction with U46619 (10–100 \(\mu\)mol/L) or 30 \(\mu\)mol/L of KCl.

**AT\(_1\) Receptor Binding Studies**

HEK 293 cells stably expressing rat AT\(_1\) receptors under genetic selection (kindly provided by Dr. W.G. Thomas, University of Queensland, Brisbane, Queensland, Australia) were grown in DMEM supplemented with 10% FCS, 100 U/mL of penicillin/streptomycin, and 200 \(\mu\)g/mL of geneticin. For binding studies, the cells were trypsinized and seeded in 48-well plates (Corning) at a density of 5\(\times\)10\(^4\) cells per well. Cells were allowed to attach for 48 hours. The plates were then placed on ice and washed once with ice-cold Hanks’ balanced salt solution, followed by another wash with cold Hanks’ balanced salt solution supplemented with 0.1% BSA (binding buffer). After removal of binding buffer, C21, vehicle, or PD123319 (to determine nonspecific binding) was added in 100 \(\mu\)L of cold binding buffer and allowed to incubate for 20 minutes. Next, 50 \(\mu\)L of binding buffer containing 25000 cpm of \(^{125}\)I-Ang II was added. After 4 hours of incubation, binding buffer was removed, and wells were washed twice with Hanks’ balanced salt solution. Subsequently, cells were lysed with 0.1 mol/L of NaOH, and radioactivity was counted in a gamma counter.

**Spectral Analysis**

To determine the molecular interaction between C21 and hydroxocobalamin, solutions containing hydroxocobalamin (200 \(\mu\)mol/L) and C21 (0.1–1.0 \(\mu\)mol/L) or NaCl (0.1–1.0 \(\mu\)mol/L; negative control) were prepared. Absorption spectra (300–560 nm) were determined using a UV mini-1240 spectrophotometer (Shimadzu). The pH values of individual solutions were measured afterward and were within the range of 6.7 to 7.0.
was performed by 1- or 2-way ANOVA, followed by post hoc evaluation according to Bonferroni. \( P<0.05 \) was considered significant.

**Results**

**AT\(_2\) Receptor Binding Studies**

C21 concentration-dependently prevented \(^{125}\)I-Ang II binding to AT\(_2\) receptor-transfected HEK-293 cells (inhibition constant, 1.02±0.14 mmol/L; \( n=3 \); Figure 1). In addition, 1 \( \mu \)mol/L of PD123319 displaced \(^{125}\)I-Ang II binding to these cells by 96.9±1.6% (\( n=4 \)).

**Langendorff Preparation**

At concentrations >1 \( \mu \)mol/L (in the injection fluid), C21 induced a biphasic response in the coronary circulation of the Wistar rat (\( n=4 \)), a CF decrease (constrictor phase) of maximally 14±3% (Figure 2A) followed by a CF increase (relaxant phase) of maximally 32±10% (Figure 2B). Irbesartan and PD123319 (\( n=4-5 \)) abolished (\( P<0.05 \)) the CF decrease and enhanced the CF increase (\( P<0.05 \)).

In SHRs, the constrictor effects of C21 were greatly enhanced (\( E_{\text{max}} \)= 48±4%; \( P<0.05 \) versus Wistar rat; Figure 2C), whereas its relaxant effects were abolished (\( n=7 \); Figure 2D). Irbesartan (\( n=4 \)) fully abolished the C21-induced coro-
nary constriction \(P<0.05\) in SHRs and allowed the return of the relaxant response to C21 \(P<0.05\). PD123319 \((n=4)\) partially reduced the constrictor response \(P<0.05\) but did not induce relaxation. Results in C57BL/6 and AT2R−/− mice \((n=4; \text{Figure 2E and 2F})\) mimicked those in Wistar rats.

**Mulgany Myograph**

C21 concentration-dependently relaxed preconstricted human coronary microarteries \(pE_{C50}, 4.8 \pm 0.3; E_{\text{max}}, 83 \pm 4.2\%; n=7; \text{Figure 3A}\), Wistar rat iliac arteries \(pE_{C50}, 5.6 \pm 0.2; E_{\text{max}}, 93.2 \pm 4.0\%; n=7; \text{Figure 3B}\), Wistar rat mesenteric arteries \(pE_{C50}, 5.8 \pm 0.3; E_{\text{max}}, 93.8 \pm 6.6\%; n=3; \text{Figure 3C}\), and SHR mesenteric arteries \(pE_{C50}, 6.6 \pm 0.2; E_{\text{max}}, 93.2 \pm 2.6\%; n=2; \text{Figure 3D}\). C21 did not constrict human coronary microarteries \((n=2; \text{data not shown})\) or Wistar rat iliac arteries \((n=4; \text{Figure 3E})\). It did, however, constrict SHR iliac arteries \((n=4; \text{Figure 3F})\), and both irbesartan \((n=4)\) and PD123319 \((n=3)\) blocked this constrictor effect \(\text{Figure 3F}\).

To study the mechanism underlying the C21-induced vasorelaxation, we focused on AT receptors, the NO pathway, and calcium entry. The NO scavenger hydroxocobalamin shifted the C21 CRCs in Wistar rat iliac arteries \(pE_{C50}, 4.7 \pm 0.2; E_{\text{max}}, 85.5 \pm 8.4\%; n=4; \text{Figure 3B}\) and rat mesenteric arteries \(pE_{C50}, 5.1 \pm 0.2; E_{\text{max}}, 89.5 \pm 7.0\%; n=3; \text{Figure 3C}\) ~5- to 10-fold to the right \(P<0.05\), whereas irbesartan, PD123319, the endothelial NO synthase inhibitor l-NMMA, or the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ), or hydroxocobalamin (HC). E and F show the C21-induced constrictor responses (or absence thereof) in iliac arteries of Wistar rats and SHRs. Data are mean±SEM of \(n=6\) to 10 and have been expressed as a percentage of the maximum contraction-induced KCl or U46619.

**Figure 3.** Effect of compound 21 (C21) in KCl-preconstricted human coronary microarteries (A), U46619-preconstricted Wistar rat iliac arteries (B), Wistar rat mesenteric arteries (C), and spontaneously hypertensive rat (SHR) mesenteric arteries (D), as well as in U46619-preconstricted iliac arteries of C57BL/6 (G) and AT2 receptor-deficient (AT2R−/−) mice (H), in the absence or presence of irbesartan, PD123319, N\(^{\text{\u00b5}}\)-nitro-L-arginine methyl ester (l-NMMA), 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ), or hydroxocobalamin (HC). E and F show the C21-induced constrictor responses (or absence thereof) in iliac arteries of Wistar rats and SHRs. Data are mean±SEM of \(n=6\) to 10 and have been expressed as a percentage of the maximum contraction-induced KCl or U46619.
and 100 μmol/L, respectively) shifted the U46619 CRCs ~8, ~25, and >80-fold to the right (P<0.05 for all), and hydroxocobalamin but not endothelium removal, irbesartan, PD123319, L-NAME, or 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ), hydroxocobalamin (HC), or Y27632 in iliac (Figure 4 through 4D). As expected, Y27635 did suppress the ionomycin-induced contraction of 100 μmol/L of C21 or water did not reveal any absorption peaks within this wavelength range.

**Spectral Analysis**

Spectral analysis (300–560 nm) of solutions containing 200 μmol/L of hydroxocobalamin demonstrated a peak absorption at a wavelength of 349.5 nm. In the presence of 100 μmol/L and 1 mmol/L of C21, the peak absorption was shifted to 354.5 and 358.0 nm, respectively, whereas this was unaffected in the presence of equimolar NaCl concentrations (Figure 5). Spectral analysis of solutions containing 100 μmol/L of C21 or water did not reveal any absorption peaks within this wavelength range.

**Discussion**

The present study does not reveal C21-induced, AT<sub>2</sub> receptor-mediated vasodilation in any of the models tested, despite the fact that such vasodilation has been demonstrated previously in these models.1,2,11,12,22 Simultaneously, we were able to confirm that C21 binds with high affinity to AT<sub>2</sub> receptors.14,27 Yet, C21 did induce relaxant, as well as constrictor effects, in full agreement with the diversity of C21 effects on blood pressure in a wide range of models, ranging from decreases to increases of ~25 mm Hg.15,19,21 Our study now shows at what concentrations these effects occur and provides the mechanisms that potentially underlie these phenomena. Remarkably, the constrictor effects appeared to be enhanced under pathological conditions, because they were best observed in the coronary vascular bed and iliac artery of the SHR. Irbesartan blocked these constrictor effects, suggesting that they were AT<sub>1</sub> receptor mediated. This agrees with the well-established AT<sub>1</sub> receptor upregulation in SHRs.26 Clearly, therefore, C21 is capable of stimulating AT<sub>1</sub> receptors, as has also been suggested based on in vivo studies.19,20

Interestingly, in both Wistar rats and SHRs, PD123319 partially blocked the coronary constrictor effects of C21 (Figure 2), and partial blockade was also observed in C21-constricted iliac arteries of the SHR. Moreover, in male SD rats, Hilliard et al.21 reported inhibition of a C21-induced rise in mean arterial pressure by PD123319. A unifying explanation of these findings is the existence of AT<sub>1</sub>/AT<sub>2</sub> receptor heterodimers, coupling to net dilatory/constrictor effects, depending on their ratio and/or location. Alternatively, it should be considered that PD123319, at the applied concentration of 1 μmol/L, exerted a modest degree of AT<sub>1</sub> receptor blockade in our studies.

The biphasic coronary effects of C21 in Wistar rats were mimicked in C57BL/6 mice. In SHRs, after the enhanced coronary constrictor response to C21, a dilator phase was virtually absent. Blocking the initial constrictor effect with irbesartan enhanced the subsequently occurring vasodilation in Wistar rats and reintroduced coronary vasodilation in
PD123319, if anything, blocked vasoconstriction and enhanced coronary dilation. In addition, in a previous study we determined.19 Remarkably, this study observed larger effects at 1 to those observed here. However, this study stopped its CRCs vessels reported C21-induced relaxations that are comparable previous study investigating C21-induced effects in rodent SHR coronary vascular bed.11 Thus, a non-AT receptor-AT2 receptors. Complete relaxation of preconstricted vessels molar range, that is, well above the nanomolar affinity for aorta than in the mouse aorta or rat mesenteric artery (\(10^{-7}\) to \(10^{-4}\)) in humans. Concentrations of 1 \(\mu\)mol/L were sufficient to shift the constrictor curves to 10-fold to the right. Most in vitro studies investigating the effects of C21 applied concentrations of \(10^{-8}\) to \(10^{-5}\) \(\mu\)mol/L.14,19,29 The single (to the best of our knowledge) previous study investigating C21-induced effects in rodent vessels reported C21-induced relaxations that are comparable to those observed here. However, this study stopped its CRCs at 1 \(\mu\)mol/L of C21, and, thus, no \(E_{\text{max}}\) or pEC_{50} could be determined.19 Remarkably, this study observed larger effects (\(25\%\) to \(30\%\) relaxation at 1 \(\mu\)mol/L of C21) in the SHR aorta than in the mouse aorta or rat mesenteric artery (\(10\%\) to \(20\%\) relaxation).19 This is unexpected because of the AT2 receptor stimulation.

Figure 5. Compound 21 (C21)-induced alteration of the absorption spectrum of hydroxocobalamin (HC). Please note that NaCl has no such effect.

SHRs. At first sight, this supports an unmasking of AT2 receptor-mediated coronary vasodilation. However, PD123319 did not block the C21-induced coronary vasodilation in Wistar rats, and a similar vasodilation occurred in AT2R-/- mice. Moreover, as discussed above, PD123319, if anything, blocked vasoconstriction and enhanced coronary dilation. In addition, in a previous study we were unable to detect AT2 receptor-mediated vasodilation in the SHR coronary vascular bed.11 Thus, a non-AT receptor-dependent mechanism must underlie the coronary relaxant effect of C21.

Indeed, all of the responses in isolated vessels, including human coronary microarteries, in our study support a C21-induced, AT2 receptor-independent vasorelaxation. The concentrations at which this effect occurred were in the micromolar range, that is, well above the nanomolar affinity for AT2 receptors. Complete relaxation of preconstricted vessels required a C21 concentration of \(10^{-7}\) to \(10^{-6}\) \(\mu\)mol/L in rodents and 10 to 100 \(\mu\)mol/L in humans. Concentrations of 1 \(\mu\)mol/L were sufficient to shift the constrictor curves to U46619 \(10^{-5}\) to the right. Most in vitro studies investigating the effects of C21 applied concentrations of \(10^{-8}\) to \(10^{-5}\) \(\mu\)mol/L.14,19,29 The single (to the best of our knowledge) previous study investigating C21-induced effects in rodent vessels reported C21-induced relaxations that are comparable to those observed here. However, this study stopped its CRCs at 1 \(\mu\)mol/L of C21, and, thus, no \(E_{\text{max}}\) or pEC_{50} could be determined.19 Remarkably, this study observed larger effects (\(25\%\) to \(30\%\) relaxation at 1 \(\mu\)mol/L of C21) in the SHR aorta than in the mouse aorta or rat mesenteric artery (\(10\%\) to \(20\%\) relaxation).19 This is unexpected because of the AT2 receptor stimulation. C21 did not block the RhoA-Rho kinase pathway, which has been reported previously to underlie AT2 receptor-mediated vasodilation.32

Perspectives

Despite overwhelming data supporting AT2 receptor-mediated vasodilation,3,6,33-35 for instance, in the preparations investigated in these studies,2,11,12 we were unable to demonstrate such vasodilation in response to the AT2 receptor agonist C21 in rat, mouse, and human vessels. Yet, our current study does support C21-induced vasorelaxation, albeit in an AT receptor-independent manner, possibly involving blockade of Ca^{2+} transport into the cell. Simultaneously, C21 is capable of activating AT1 receptors, thereby causing vasoconstriction. Taken together, this combination of both relaxant and constrictor effects can help explaining the hypertensive and hypotensive effects of C21 in vivo, including even the absence of such effects.15-17,20,36 It rules out the application of C21 as an antihypertensive agent. Clearly, studies observing organ-protective effects confirm that the relaxant effects of C21 in the SHR aorta truly involved AT2 receptor stimulation. In vivo, C21 has been infused at doses ranging from 0.05 to 5.00 µg/kg per minute or was applied IP or orally at doses ranging from 0.3 to 10.0 mg/kg per day.14-21,30 Given its distribution volume of 3 times total body water, its half life of \(\approx 4\) hours, and a bioavailability of \(\approx 30\%\),14 this is expected to result in C21 plasma levels ranging from 0.1 to 5.0 \(\mu\)mol/L, that is, well within the range applied here. Such levels (up to \(10000\)-fold above the reported inhibition constant for the AT2 receptor) are also in agreement with the fact that C21 induced AT1 receptor-mediated effects in vivo, because its inhibition constant for AT1 receptors is \(10000\) times above that for AT2 receptors.

C21-induced relaxation occurred in an endothelium-independent manner and could be blocked by the NO scavenger hydroxocobalamin but not the NO synthase inhibitor L-NAME or the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (Figures 3 and 4). This initially suggested a role for nonendothelial NO synthase–derived NO-like factors like S-nitrosothiols.31 However, an alternative explanation is that the cobalt group of hydroxocobalamin inactivated C21 through interaction with its imidazole ring. Indeed, spectral analysis subsequently confirmed this concept. Thus, most likely, the effect of hydroxocobalamin is attributed to its capacity to bind/inactivate C21 and does not involve NO scavenging.

C21 concentration-dependently shifted the CRCs to both the thromboxane A2 agonist U46619 and the α-adrenoceptor agonist phenylephrine to the right and fully relaxed U46619- and KCl-preconstricted vessels, which demonstrates that its relaxant effects are not related to a specific receptor. Importantly, C21 did not alter the constrictor response to the calcium ionophore ionomycin. This raises the possibility that C21, instead of directly interfering with contractile (Ca^{2+}-dependent) responses, blocks calcium transport into the cell, thus preventing responses that depend on extracellular calcium, like vasoconstriction. C21 did not block the RhoA-Rho kinase pathway, which has been reported previously to underlie AT2 receptor-mediated vasodilation.32

Therefore, C21 effectively blocks calcium transport into the cell, thus preventing responses that depend on extracellular calcium, like vasoconstriction. C21 did not block the RhoA-Rho kinase pathway, which has been reported previously to underlie AT2 receptor-mediated vasodilation.32
of chronic C21 application should now carefully determine to what degree these effects truly depend on AT₂ receptor activation, by using either selective AT₂ receptor antagonists or by simultaneously studying the effects in AT₂ receptor knockout models. To guarantee AT₂ receptor selectivity, it appears that C21 infusion rates should stay well below 0.5 µg/kg per minute.

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Disclosures
None.

References
What Is New?
- The AT$_2$ receptor-selective compound C21 induces vasodilation by blocking calcium transport into the cell and constriction via AT$_1$ receptor stimulation.
- C21 did not relax human and rodent blood vessels via AT$_2$ receptor stimulation.

What Is Relevant?
- Non-AT$_2$ receptor-mediated effects should be taken into account when applying C21 in vivo.

Summary
Although AT$_2$ receptor stimulation is generally linked to vasodilation, the AT$_2$ receptor-selective compound C21 was unable to relax preconstricted human and rodent vessels in an AT$_2$ receptor-dependent manner. Instead it either induced relaxation by blocking calcium transport or constriction via AT$_1$ receptor stimulation. The latter effect was enhanced under pathological conditions.
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