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

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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 ionomycine (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,1–6 natriuretic,7 growth-suppressing,8 and antifibrotic8 effects. As such, it seems to counteract Ang II type 1 (AT1) receptor-mediated effects.10 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 vasoconstriction11,12 and hypertrophy13). 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 al14 reported the synthesis of compound 21 (C21), the first selective nonpeptidic 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 rat15; the stroke-prone hypertensive rat16,17; and the 2-kidney, 1-clip hypertensive Sprague-Dawley (SD) rat18 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.14 Remarkably, this response was not affected by AT2 receptor blockade, suggesting that it did not involve AT2 receptor stimulation. Bosnyak et al19 reported a vasodilator 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-
the Erasmus MC. Human coronary microarteries were isolated and stored in Krebs-Henseleit, as described before.1

### Langendorff Preparation

Rat and mouse hearts were perfused according to Langendorff, as described previously.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 μL) 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 AT1 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, ~750 μm), rat iliac arteries (diameter, ~800 μm), rat mesenteric arteries (diameter, ~150 μm), mouse iliac arteries (diameter, ~350 μm), and mouse abdominal aortas (diameter, ~550 μm) were cut into ring segments of ~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-mL 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 mmol/L of KCl. Thereafter, vessels were preincubated for 30 minutes in fresh buffer in the absence or presence of 1 μmol/L of irbesartan, 1 μmol/L of PD123319, 100 μmol/L of 1H-1,2,4-oxadiazolo[4,3-a]quinolin-1-one, 200 μmol/L of hydroxocobalamin, 10 μmol/L of Y27632, or 0.1 to 100.0 μmol/L of C21, and CRCs were constructed to C21, phenylephrine, U46619, or ionomycin. C21-induced relaxation was studied after preconstriction with U46619 (10–100 mmol/L) or 30 mmol/L of KCl.

### AT1 Receptor Binding Studies

HEK 293 cells stably expressing rat AT1 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 μg/mL of geneticin. For binding studies, the cells were trypsinized and seeded in 48-well plates (Corning) at a density of 5 × 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 Tyrode buffer,22 whereas mouse hearts were placed in modified Krebs-Henseleit buffer,22 both gassed with 95% O2/5% CO2. 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.24,25

### Spectral Analysis

To determine the molecular interaction between C21 and hydroxocobalamin, solutions containing hydroxocobalamin (200 μmol/L) and C21 (0.1–1.0 mmol/L) or NaCl (0.1–1.0 mmol/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.
Data Analysis

Data obtained with the Langendorff preparation were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments) and Labchart software (AD Instruments). After a manual selection of the desired signals preinjection and postinjection, data were analyzed using Matlab (Mathworks Inc) and Labchart. CRCs were analyzed as described before using GraphPad Prism 3.01 (Graph Pad Software Inc) to determine the maximum effect (E_max) and pEC50 (=-logEC50) values. Statistical analysis was performed by 1- or 2-way ANOVA, followed by post hoc evaluation according to Bonferroni. P<0.05 was considered significant.

Results

AT2 Receptor Binding Studies

C21 concentration-dependently prevented 125I-Ang II binding to AT2 receptor-transfected HEK-293 cells (inhibition constant, 1.02±0.14 mmol/L; n=3; Figure 1). In addition, 1 μmol/L of PD123319 displaced 125I-Ang II binding to these cells by 96.9±1.6% (n=4).

Langendorff Preparation

At concentrations >1 μmol/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_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 constriction response (P<0.05) but did not induce relaxation. Results in C57BL/6 and AT2R Δ/Δ mice (n=4; Figure 2E and 2F) mimicked those in Wistar rats.

Mulgany Myograph
C21 concentration-dependently relaxed preconstricted human coronary microarteries (pEC50, 4.8±0.3; Emax, 83±4.2%; n=7; Figure 3A), Wistar rat iliac arteries (pEC50, 5.6±0.2; Emax, 93.2±4.0%; n=7; Figure 3B), Wistar rat mesenteric arteries (pEC50, 5.8±0.3; Emax, 93.8±6.6%; n=3; Figure 3C), and SHR mesenteric arteries (pEC50, 6.6±0.2; Emax, 93.2±2.6%; n=2; Figure 3D). C21 did not constrict human coronary microarteries (n=2; data not shown) or Wistar rat iliac arteries (n=4; Figure 3E). It did, however, constrict SHR iliac arteries (n=4; Figure 3F), and both irbesartan (n=4) and PD123319 (n=3) blocked this constrictor effect (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 (pEC50, 4.7±0.2; Emax, 85.5±8.4%; n=4; Figure 3B) and rat mesenteric arteries (pEC50, 5.1±0.2; Emax, 89.5±7.0%; n=3; Figure 3C) 5- to 10-fold to the right (P<0.05), whereas irbesartan, PD123319, the endothelial NO synthase inhibitor L-NAME, or the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ) did not affect the C21 responses in SHR iliac arteries (n=4; Figure 3D).

C21 relaxed preconstricted mouse iliac arteries (pEC50, 5.7±0.1; Emax, 97.8±1.4; n=8; Figure 3G) and mouse abdominal aortas (pEC50, 5.7±0.1; Emax, 98.4±0.3%; n=9; data not shown). Like in the rat, hydroxocobalamin but not L-NAME shifted the C21 CRCs in these arteries 4- to 10-fold to the right (P<0.05). Results in iliac arteries (n=6–10; Figure 3H) and abdominal aortas of AT2R Δ/Δ mice (n=5–10; data not shown) were identical to those in C57BL/6 mice.

U46619 concentration-dependently constricted rat iliac arteries (n=21; Figure 4A). C21 (at a concentration of 1, 10,
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 4E). In contrast, 10 μmol/L of C21 did not affect contractions induced by the calcium ionophore ionomycin either alone or on top the RhoA-kinase inhibitor Y27635 (n=7; Figure 4F). As expected, Y27635 did suppress the ionomycin-induced contraction (n=7; P<0.05; Figure 4F).

**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, AT2 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 AT2 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 AT1 receptor mediated. This agrees with the well-established AT1 receptor upregulation in SHRs.26 Clearly, therefore, C21 is capable of stimulating AT1 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 al21 reported inhibition of a C21-induced rise in mean arterial pressure by PD123319. A unifying explanation of these findings is the existence of AT1/AT2 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 AT1 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
were unable to detect AT2 receptor-mediated vasodilation in enhanced coronary dilation. In addition, in a previous study we determined.19 Remarkably, this study observed larger effects at 1 to 20% relaxation.19 This is unexpected because of the AT2 receptor phenotype shift reported for SHRs, allowing AT2 receptors to induce constriction instead of relaxation.11,12

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 vasodilatation 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.13 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 to 100 μmol/L in rodents and 10 to 100 μmol/L in humans. Concentrations of 1 μmol/L were sufficient to shift the constrictor curves to U46619 10-fold to the right. Most in vitro studies investigating the effects of C21 applied concentrations of 0.1 μmol/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 μmol/L of C21, and, thus, no Emax or pEC50 could be determined.19 Remarkably, this study observed larger effects (∼25% to 30% relaxation at 1 μmol/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 phenotype shift reported for SHRs, allowing AT2 receptors to induce constriction instead of relaxation.11,12

Unfortunately, no studies with PD123319 were performed to 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.14,16–21,30 Given its distribution volume of 3 times total body water, its half life of ∼4 hours, and a bioavailability of ∼30%,14 this is expected to result in C21 plasma levels ranging from 0.1 to 5.0 μmol/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 (Ca2+-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

**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 Ca2+ 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
of chronic C21 application should now carefully determine to what degree these effects truly depend on AT$_2$ receptor activation, by using either selective AT$_2$ receptor antagonists or by simultaneously studying the effects in AT$_2$ receptor knockout models. To guarantee AT$_2$ 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**


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**Novelty and Significance**

**What Is New?**
- The AT₂ receptor-selective compound C21 induces vasodilation by blocking calcium transport into the cell and constriction via AT₁ receptor stimulation.
- C21 did not relax human and rodent blood vessels via AT₂ receptor stimulation.

**What Is Relevant?**
- Non-AT₂ receptor-mediated effects should be taken into account when applying C21 in vivo.

**Summary**

Although AT₂ receptor stimulation is generally linked to vasodilation, the AT₂ receptor-selective compound C21 was unable to relax preconstricted human and rodent vessels in an AT₂ receptor-dependent manner. Instead, it either induced relaxation by blocking calcium transport or constriction via AT₁ receptor stimulation. The latter effect was enhanced under pathological conditions.
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