Angiotensin Type 2 Receptor Agonist Compound 21 Reduces Vascular Injury and Myocardial Fibrosis in Stroke-Prone Spontaneously Hypertensive Rats

Asia Rehman, Avshalom Leibowitz, Naoki Yamamoto, Yohann Rautureau, Pierre Paradis, Ernesto L. Schiffrin

Abstract—Angiotensin type 2 receptor–mediated effects of angiotensin II appear to counteract many of the effects mediated via the angiotensin type 1 receptor. Compound 21 (C21), a selective angiotensin type 2 receptor agonist, has demonstrated beneficial effects on cardiac function after myocardial infarction in rodents. We hypothesized that C21 alone or in combination with an angiotensin type 1 receptor antagonist would blunt the development of hypertension and vascular damage in stroke-prone spontaneously hypertensive rats. Six-week–old stroke-prone spontaneously hypertensive rats received C21 (1 mg/kg per day), the angiotensin type 1 receptor antagonist losartan (10 mg/kg per day), C21 plus losartan, or vehicle PO for 6 weeks. Systolic blood pressure was lower in losartan and C21-losartan combination groups (P<0.001). Endothelial-dependent relaxation was enhanced (P<0.001) in the C21-losartan combination group at lower acetylcholine concentrations. C21 or C21-losartan combination reduced vascular stiffness, aortic medial and myocardial interstitial collagen content, and aortic fibronectin (P<0.05). C21 and losartan decreased the expression of 2 genes associated with cardiac hypertrophy, myosin heavy chain-β (myh7) by 30 to 50%, and α-skeletal muscle actin by 30% to 35% (P<0.05). C21-losartan combination caused an additional 40% reduction in myh7 compared with C21 (P<0.01). Aortic superoxide generation was reduced equally by the 3 treatments (P<0.001). Monocyte/macrophage infiltration in the aorta and kidney (P<0.001) and T-lymphocyte infiltration in the renal cortex (P<0.05) were lowered similarly by the 3 treatments. These data suggest that C21 alone or in combination with losartan may improve endothelial function and vascular composition and mechanics by reducing oxidative stress, collagen content, fibronectin, and inflammatory cell infiltration in stroke-prone spontaneously hypertensive rats. (Hypertension. 2012;59:291-299.) ● Online Data Supplement

Key Words: hypertension ■ vascular remodeling ■ resistance arteries ■ angiotensin type 2 receptor

Chronic hypertension results in vascular remodeling and inflammation, endothelial dysfunction, and accelerated development of atherosclerosis.1-3 Of the many factors implicated in hypertensive vascular remodeling, angiotensin (Ang) II, the main effector hormone of the renin-Ang-aldosterone system, seems to be one of the most important.4 Ang II exerts its effects by binding to 2 membrane-bound, G protein–coupled receptors, Ang II type 1 (AT1R) and type 2 receptors (AT2R). Most well-known effects of Ang II that contribute to unfavorable vascular remodeling and consequent hypertensive complications are mediated via AT1R, whereas those exerted via AT2R are less well known and appear to counteract many of its effects exerted via AT1R.4,5 AT2R-mediated effects seem to exert vasculoprotective actions via vasodilation, NO production,6-8 and apoptosis and inhibition of growth and fibrosis.5,9,10 Beneficial vascular effects of AT1R blockade have been at least partially attributed to unopposed AT2R stimulation.7,11,12 Several studies have suggested beneficial vascular effects of AT1R. We reported previously that AT1R mediated down-regulation of vascular RhoA/Rho kinase/myosin light chain phosphorylation in Ang II and AT1R antagonist valsartan–treated or AT2R partial agonist CGP42112A–treated cultured vascular smooth muscle cells, suggesting a role for vascular AT1R in blood pressure (BP) lowering during chronic AT1R blockade.12 In vivo, Ang II, via AT2R, facilitates mesenteric artery vasodilation through NO synthase (NOS)/NO–mediated pathways and down-regulation of cGMP-dependent protein kinase. In hypertensive diabetic patients, long-term AT1R blockade was associated with increased AT2R expression and AT2R–mediated Ang II–induced vasodilation of small resistance arteries.13 Knockout of AT1R enhanced Ang

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II–induced BP elevation, whereas lentivirus-mediated AT2R overexpression in the heart blunted Ang II–induced cardiac hypertrophy, remodeling, and fibrosis in the absence of reduction of BP. In addition, expression of AT2R varies under physiological and pathophysiological conditions and poor selectivity (PD123319) or poor dose-dependent specificity (CGP42112) of available AT2R antagonists and agonists, respectively, have created further obstacles in elucidation of the role of AT2R. The availability of the first selective, nonpeptide AT2R agonist, compound 21 (C21), represents a major breakthrough, and several studies reporting effects of AT2R stimulation with this compound on cardiac, renal, and nervous tissue and inflammation have followed. Kaschina et al were the first to report a reduction in infarct size and improvement of postmyocardial infarction systolic and diastolic functions in a rodent model. In stroke-prone spontaneously hypertensive rats (SHRsp) fed a high-salt diet, C21 treatment prolonged survival and delayed the onset of renal and cerebral damage in a dose-dependent fashion and preserved renal structure. More recently, C21 inhibited proinflammatory cytokines, tumor necrosis factor–α–induced interleukin (IL)–6 levels, monocyte chemoattractant protein-1, and nuclear factor–κB through activation of protein phosphatases and stimulated synthesis of epoxyeicosatrienoic acids both in vitro and in vivo. In renovascular hypertension, C21 treatment reduced early renal inflammatory responses in a BP-independent manner. We, therefore, hypothesized that chronic AT2R stimulation with C21 administered orally could blunt the development of hypertension and hypertensive vascular damage in SHRsp through reduction of oxidative stress and inflammation.

**Results**

**Materials**

Additional materials and methods are mentioned in the online Data Supplement (please see http://hyper.ahajournals.org).

**Animals**

The study protocol was approved by the animal care committees of the Lady Davis Institute for Medical Research and of McGill University and was conducted according to the recommendations of the Canadian Council for Animal Care. Six-week–old male SHRsp were housed under constant temperature and humidity and exposed to 14-hour light/10-hour dark cycles. Rats underwent 1 week of training for intake of powdered chow mixed with water and measurement of systolic BP (SBP) by the tail-cuff method, followed by a 6-week treatment period. Rats received C21 (1 mg/kg per day PO) mixed with the food, losartan (10 mg/kg per day PO) in drinking water, a combination of C21 (1 mg/kg per day) and losartan (10 mg/kg per day), or vehicle. A 1 mg/kg per day dose of C21 was selected from preliminary experiments that showed no effect on vascular structure and function of 0.5 mg/kg per day of C21, whereas 3 mg/kg per day of C21 did not enhance beneficial effects of 1 mg/kg per day. Losartan was used to elicit unopposed Ang II effects on AT1R to enhance the AT1R stimulation with C21 in the combined treatment group. SBP was measured by the tail-cuff method using a MC 4000 BP analysis system (Hatters Instruments, Cary, NC) at baseline and weekly thereafter.

**Small Artery Endothelial Function and Vascular Mechanics**

Third-order branches of the mesenteric arterial tree (internal diameter between 180 and 250 μm) were dissected and mounted on a pressurized myograph, as described previously. Media thickness and lumen diameter were measured at increasing intraluminal pressures from 3 to 140 mm Hg. Media cross-sectional area, media: lumen ratio, and stress and strain were calculated as described previously.

**NADPH Oxidase Activity and Generation of Reactive Oxygen Species**

Reactive oxygen species generation was determined in aorta and mesenteric arteries by measuring NADPH oxidase activity by chemiluminescence using lucigenin and NADPH, as described previously. Vascular superoxide production was assessed on 5-μm cryosections of aorta with the superoxide-sensitive fluorescent dye dihydroethidium (2 μmol/L, Invitrogen Canada Inc, Burlington, Ontario, Canada) in dark conditions for 1 minute at 37°C. Fluorescence was visualized and captured with a fluorescence microscope with a CY3 filter. The intensity of the fluorescence over the total surface area was quantified with Image J software (National Institutes of Health, Bethesda, MD).

**Histology**

Sections (5 μm) of paraffin-embedded tissues were stained with Sirius red to determine aortic, coronary media, and perivascular, as well as interstitial myocardial collagen type I and III, content and analyzed by an image analysis system (Northern Eclipse 5.0, EMPIX Imaging Inc, Mississauga, Ontario, Canada). Collagen type I and III depositions were evaluated in the entire aortic sections, throughout the inner third (subendoocardial myocardium), middle third (myocardium), and the outer third (subepicardial myocardium) of the left ventricle using a 10× objective. From each of 3 nonconsecutive serial sections (which allowed convergence of results), ~3 fields in each region of the heart were recorded. Only intramyocardial coronary arteries that appeared circular on cross-section were analyzed. Coronary perivascular collagen was measured separately from images obtained with a 20× objective and was normalized to the media cross-sectional area of intramural coronary arteries. Collagen fraction was defined as the ratio of the stained area to the total tissue area and expressed as a percentage.

**Immunofluorescence**

Immunofluorescence microscopy was performed on 5-μm-thick aortic, cardiac, and renal frozen sections, as described previously. Rabbit anti-CD3 (1:200, Daku Canada, Burlington, ON, Canada), monoclonal mouse anti-CD68 (ED-1, 1:100, AbD Serotec, Raleigh, NC), and rabbit antifibronectin (1:40, Millipore, Temecula, CA) primary antibodies followed by Alexa Fluor 647 goat antirabbit IgG (1:200, Invitrogen, Eugene, OR), Alexa Fluor 488 goat antirabbit IgG (1:400), or Alexa Fluor 647 goat antimouse IgG (1:200) secondary antibodies were used. Images were captured using a fluorescence microscope with fluorescein isothiocyanate, Cy3, or Cy5 filters. Cell numbers or the intensity of the fluorescence over the total surface area was quantified as above. CD3+ and CD68+ cell number was determined in the renal cortex and medulla using 10 and 3 images, respectively, acquired with a 20× objective. CD68+ cell number was determined in aorta using 3 images acquired with a 40× objective. CD3+ and CD68+ cell numbers were determined using the single red (CD68+) or green (CD3+) images.

**Western Blot Analysis**

The expression of vascular cell adhesion molecule 1 in aorta was determined by Western blot, as described previously.
Quantitative RT-PCR
Total RNA was extracted from cardiac ventricles and the expression of myosin heavy chain-β (myh7), α-skeletal muscle actin (acta1), atrial natriuretic peptide (nppa), and the matrix metalloproteinases (MMPs) 2 and 9 (mmp2 and mmp9) were determined in cardiac ventricles by real-time quantitative RT-PCR. Details of protocols are in the online Data Supplement.

Data Analysis
Results are presented as mean ± SEM. Comparisons between multiple groups were performed by one-way ANOVA. Two-way ANOVA was used for comparison of SBP, the acetylcholine concentration-response curves, the sum-of-squares data from acetylcholine response curves in the presence or absence of Nω-nitro-L-arginine methyl ester, and interstitial myocardial collagen content. ANOVA was followed by Student-Newman-Keuls or Bonferroni post hoc tests. Stress-strain curves were compared with the F statistic. P < 0.05 was considered statistically significant.

Results
C21 Did Not Influence SBP
To determine the effect of C21 on SBP, baseline and weekly measurements were done. As expected, there was a steady increase in SBP in control SHRsp that reached a plateau after 5 weeks (Figure 1). C21 did not prevent this increase in SBP, whereas losartan prevented it. The C21-losartan combination did not show further reduction of SBP over losartan-treated rats.

Table 1. Body and Organ Weights of SHRsp

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ctrl</th>
<th>C21</th>
<th>Losartan</th>
<th>C21 + Losartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW 6 wk, g</td>
<td>116 ± 4.6</td>
<td>115.6 ± 5.4</td>
<td>120 ± 5.9</td>
<td>117 ± 6.7</td>
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<tr>
<td>BW, 12 wk, g</td>
<td>281 ± 6.3</td>
<td>267 ± 9.3</td>
<td>279.5 ± 7.2</td>
<td>278.1 ± 9.5</td>
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<tr>
<td>HW, g</td>
<td>0.92 ± 0.02</td>
<td>0.91 ± 0.03</td>
<td>0.83 ± 0.03*</td>
<td>0.83 ± 0.03*</td>
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<td>HW/LW, g/mm</td>
<td>0.25 ± 0.04</td>
<td>0.26 ± 0.10</td>
<td>0.23 ± 0.10</td>
<td>0.23 ± 0.10</td>
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<tr>
<td>KW, g</td>
<td>1.90 ± 0.04</td>
<td>1.88 ± 0.07</td>
<td>1.89 ± 0.05</td>
<td>1.91 ± 0.07</td>
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<tr>
<td>KW/LW, g/mm</td>
<td>0.53 ± 0.10</td>
<td>0.54 ± 0.20</td>
<td>0.53 ± 0.20</td>
<td>0.54 ± 0.20</td>
</tr>
<tr>
<td>LuW, g</td>
<td>1.06 ± 0.03</td>
<td>1.09 ± 0.05</td>
<td>1.04 ± 0.03</td>
<td>1.02 ± 0.05</td>
</tr>
<tr>
<td>LuW/LW, g/mm</td>
<td>0.29 ± 0.10</td>
<td>0.32 ± 0.20</td>
<td>0.29 ± 0.10</td>
<td>0.28 ± 0.20</td>
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<tr>
<td>LiW, g</td>
<td>10.10 ± 0.23</td>
<td>9.85 ± 0.37</td>
<td>10.25 ± 0.21</td>
<td>9.88 ± 0.38</td>
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<tr>
<td>LiW/LW, g/mm</td>
<td>2.81 ± 0.60</td>
<td>2.85 ± 0.10</td>
<td>2.87 ± 0.60</td>
<td>2.78 ± 0.90</td>
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<tr>
<td>SW, g</td>
<td>0.54 ± 0.02</td>
<td>0.52 ± 0.02</td>
<td>0.52 ± 0.02</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td>SW/LW, g/mm</td>
<td>0.15 ± 0.10</td>
<td>0.16 ± 0.04</td>
<td>0.15 ± 0.10</td>
<td>0.15 ± 0.10</td>
</tr>
</tbody>
</table>

Body weight (BW) and the weight of the heart (HW), kidney (KW), lung (LuW), liver (LiW), and spleen (SW) normalized by tibia length (TL) in stroke-prone spontaneous hypertensive rats (SHRsp) treated or not with vehicle (Ctrl). compound 21 (C21), losartan and C21 and losartan combination (C21 + losartan). Values are mean ± SEM. * P < 0.05 vs Ctrl and † P < 0.05 and †† P < 0.001 vs C21; n = 7.

Body and Organ Weights
SHRsp treated with C21-losartan combination or losartan alone had significantly lower heart weights (P < 0.05) compared with control rats and those receiving C21, whereas there was no significant difference in body, kidney, lungs, liver, and spleen weights or tibia length between treatment groups (Table 1). The expression of myh7, acta1, and nppa was determined by quantitative RT-PCR in cardiac ventricles to determine whether C21 has effects on expression of genes associated with cardiac hypertrophy. Myh7 expression was decreased 30% by C21 and 50% by losartan compared with control (Figure S1, available in the online Data Supplement). C21-losartan combination caused an additional 40% decrease in myh7 mRNA level compared with C21. Acta1 expression was decreased 30% by C21, 35% by losartan, and 60% by C21-losartan combination compared with control. Nppa showed a different pattern of expression. Its expression was not altered by C21 but decreased 75% by both losartan and C21-losartan combination.

C21 Improved Endothelium-Dependent Relaxation in Mesenteric Resistance Arteries in Combination With Losartan
Acetylcholine-induced endothelium-dependent relaxation was significantly increased at lower acetylcholine concentrations in C21-losartan combination treated rats compared with other groups, but there was no difference in response at higher concentrations (Figure 2A). When the acetylcholine concentration-response curve was repeated in the presence of the endothelial NOS (eNOS) inhibitor Nω-nitro-l-arginine methyl ester, vasodilatory responses to acetylcholine were abrogated in the control but not in the 3 treatment groups (Figure 2B). This suggests presence of an eNOS-independent vasorelaxation mechanism in these rats. Endothelium-independent relaxation responses to sodium nitroprusside were similar in all of the groups (Figure 2C).

C21 Reduced Mesenteric Artery Stiffness in SHRsp
Media:lumen ratio of mesenteric resistance arteries was unchanged by C21 but was significantly smaller in losartan-treated rats compared with C21 or control rats. No difference in this parameter was observed between C21-losartan combination versus losartan, C21, or control. The media cross-sectional area of mesenteric resistance arteries was unchanged with all of the treatments. C21 alone or in combination with losartan reduced mesenteric artery stiffness as indicated by the rightward displacement of the media stress/strain curve compared with control or losartan-treated rats (Figure 3). This was confirmed by comparing the stress/strain/strain curve compared with control or losartan-treated rats.
strain curves using the F statistic ($P<0.05$). The isobaric media stress at 45 mm Hg was significantly lower in losartan-treated rats compared with the C21, but no difference was seen in the other groups (Table 2).

**C21 Reduced Perivascular, Media, and Interstitial Myocardial Collagen Type I and III Content**

Aortic media type I/III collagen content was reduced by C21, C21-losartan combination, and losartan alone (Figure 4A and 4B). However, there was a significantly greater reduction in collagen content in the combination-treated rats compared with losartan alone. Coronary media collagen content was reduced by C21-losartan combination and losartan but not by C21 alone (Figure 4C and 4D), whereas C21 alone significantly reduced the coronary perivascular collagen:media cross-sectional area ratio (Figure 4C and 4D) compared with other groups. Interstitial myocardial collagen content was significantly reduced by all of the treatments, but this reduction was greater in the C21-losartan combination group compared with C21 (Figures 4E and S2). Subendocardial interstitial collagen content was reduced to the same extent in all of the groups, whereas the subepicardial and myocardial interstitial collagen content was decreased only in the C21-losartan combination group compared with the control. The expression of *mmp1*, *mmp2*, and *mmp9* was determined by quantitative RT-PCR in cardiac ventricles to determine the mechanism of reduction in interstitial collagen deposition. *Mmp1* expression could not be detected by quantitative RT-PCR, whereas expression of *mmp9* was low (detected with cDNA diluted 10 times at a cycle threshold of 32) and unchanged after any of the treatments. *Mmp2* was 10 times more abundant than *mmp9* (detected with cDNA diluted 100 times at a cycle threshold of 32) and also remained unchanged with the different treatments (Figure S1). The validity of the *mmp1* oligonucleotides was confirmed by detection of *mmp1* expression in the thymus (data not shown).

**C21 Reduced Fibronectin in SHRsp Aorta**

Fibronectin expression was determined in the aorta by immunofluorescence in SHRsp as a marker of vascular remodeling (Figure 5). Aortic fibronectin was significantly reduced by C21, losartan, and C21-losartan combination compared with control. There was no significant difference between treatment groups.

**C21 Effects on Vascular Oxidative Stress**

Vascular oxidative stress was measured in the aorta as superoxide ion production (Figure 6A) and as NADPH-oxidase activity in aorta (Figure 6B) and mesenteric arteries (Figure 6C). Aortic superoxide was reduced to a similar extent by the 3 treatments,

**Table 2. Vascular Morphological Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ctrl</th>
<th>C21</th>
<th>Losartan</th>
<th>C21+Losartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/L, %‡</td>
<td>3.95±0.37</td>
<td>4.45±0.51</td>
<td>2.95±0.29†</td>
<td>3.23±0.27</td>
</tr>
<tr>
<td>M/Cs, μm^‡</td>
<td>7499±767</td>
<td>7714±638</td>
<td>6461±731</td>
<td>6015±687</td>
</tr>
<tr>
<td>Stress, ×10^6</td>
<td>3.45±0.49</td>
<td>3.17±0.49</td>
<td>5.93±1.02‡</td>
<td>4.43±0.69‡</td>
</tr>
<tr>
<td>Strain, ∆D/D§</td>
<td>0.49±0.03</td>
<td>0.64±0.09</td>
<td>0.58±0.07</td>
<td>0.67±0.06</td>
</tr>
</tbody>
</table>

Media: lumens ratio (M/L), media cross-section area (M/Cs), stress, and strain were determined in stroke-prone spontaneous hypertensive rats treated or not with vehicle (Ctrl), compound 21 (C21), losartan, and C21 and losartan combination (C21+losartan). Data are mean±SEM. *P<0.05 vs Ctrl with n=7. †P<0.05 vs C21 with n=7. ‡Data were measured at 45 mm Hg. §Data were measured at 140 mm Hg.
whereas no significant difference was observed in vascular NADPH oxidase activity between treatment groups.

C21 Reduced Aortic and Renal Immune Cell Infiltration

Vascular cell adhesion molecule-1 expression in aorta measured using Western blot tended to be lower in C21, losartan, and C21-losartan combination-treated rats compared with controls (Figure 7). Anti-CD68 immunofluorescence revealed that C21, losartan, and C21-losartan combination decreased monocyte/macrophage infiltration by \(\approx 70\%\) in aorta, renal cortex, and medulla (Figure S3 through S5). Anti-CD3 immunofluorescence showed that the 3 treatments reduced similarly T-lymphocyte infiltration by \(\approx 50\%\) in the renal cortex, whereas C21 and C21-losartan combination tended to lower T-cell infiltration in the renal medulla (Figure S6 and S7).

Discussion

Various lines of evidence have suggested that AT\(_2\)R have beneficial vascular effects. Synthesis of the first selective, orally active nonpeptide AT\(_2\)R agonist, C21, provides an opportunity to study these effects.\(^{16}\) To our knowledge, the present study is the first to examine vascular effects of chronic, direct AT\(_2\)R stimulation with C21 in a genetic hypertensive model. The novel findings from this study include that, in SHRsp, C21 reduced mesenteric artery stiffness associated with a decrease in aortic media and pericoronary collagen type I/III and aortic oxidative stress and inflammatory cell infiltration and fibronectin. C21 induced antifibrotic and antihypertrophic effects on the heart. C21 reduced myocardial interstitial collagen type I/III and expression of cardiac fetal genes *myh7* and *acta1* in ventricles. These effects were BP independent and may be related to antioxidant and anti-inflammatory actions of AT\(_2\)R stimulation by C21.

Recent data on effects of AT\(_2\)R stimulation with C21 have shown improvement in cardiac function after myo-
cardiac infarction and delay in the onset of hypertensive renal and cerebral damage through AT2R-mediated anti-inflammatory effects. The findings from the present study are novel and extend the concept of AT2R-mediated cardiovascular end-organ protection by demonstrating reduction in aortic oxidative stress, inflammatory cell infiltration, and fibronectin expression, as well as collagen type I/III in the heart and vasculature and cardiac fetal genes myh7 and acta1 in the heart. As observed previously, C21 caused a reduction in monocyte/macrophage infiltration and a decrease in T-lymphocyte infiltration in the renal cortex. These findings suggest a potential approach for preventing hypertensive vascular complications through antifibrotic, anti-inflammatory, and antioxidant effects by chronic stimulation of AT2R with C21 and support previous data on the cardiovascular protective mechanisms mediated via AT2R stimulation.

BP reduction by direct AT2R stimulation could be expected considering that AT2R may contribute to vasorelaxation of isolated resistance arteries. However, C21 did not prevent the rise in SBP with age in SHRsp. This absence of an in vivo depressor effect of C21 was shown already by others and may depend on the predominance of AT1R-mediated vasoconstriction. This was not confirmed in the present study, because the reduction in BP by AT1R blockade with losartan administered was not altered by a concomitant treatment with C21 in the present study. However, it should be noted that the dose of losartan was not maximal, because a greater decrease in BP (45–100 mm Hg) has been observed with higher doses in SHRsp, and the rats were still hypertensive at the end of the experiment. Thus, there was not complete prevention of genetic hypertension. Our results are supported by other studies reporting lack of or only minimal AT1R-mediated effect on BP regulation in chronic studies in aged Wistar Kyoto rats and SHRsp. Data from an earlier study that documented BP lowering with a lower dose of C21 given intravenously to anesthetized SHRsp need to be interpreted with caution, because a stimulated renin-Ang system under anesthesia renders it more susceptible to pharmacological interruption. In the present study, the treatment is chronic, and C21 was administrated orally. Altogether, these results indicate that the beneficial effects of C21 were BP independent.

The vascular functional study showed that an enhancement of endothelium-dependent relaxation was observed only with

Figure 6. Compound 21 (C21) effects on vascular oxidative stress. Aortic superoxide (•O2−) generation measured with dihydroethidium (DHE) staining (A) and NADPH oxidase activity by lucigenin chemiluminescence in aorta (B) and mesenteric arteries (C) were determined in the same groups as Figure 1. Representative images of DHE staining (red) and elastin autofluorescence (green) are showed. Data are mean±SEM; *P<0.001 vs vehicle (Ctrl); n=5 for A; n=7 for B and C.

Figure 7. Immunoblots show vascular cell adhesion molecule (VCAM)-1 and β-actin expression (A) quantified in B. Groups are the same as in Figure 1. Data are mean±SEM; n=5.
the C21-losartan combination treatment, whereas the response was similar in the other treatment groups and controls. The maximum endothelium-dependent relaxation in untreated SHRsp was similar to the treated groups, which can be explained by the reported increased NO production and eNOS activity in younger SHRsp.\textsuperscript{29,30} The latter show endothelial dysfunction only at an older age as levels of reactive oxygen species rise resulting in reduced NO bioavailability. In the presence of reactive oxygen species, peroxynitrite generated from NO inactivates prostacyclin-mediated vasorelaxation and contributes further to endothelial dysfunction.\textsuperscript{29} Unaltered vasorelaxant responses after eNOS inhibition by exposure to the NOS inhibitor N\textsuperscript{\textdegree}-nitro-L-arginine methyl ester in the groups receiving combination therapy, C21, or losartan alone suggest a non-eNOS/NO-mediated pathway of vasorelaxation by direct (C21) or indirect (losartan) stimulation of AT\textsubscript{2}R. AT\textsubscript{2}R-mediated relaxation involves activation of the vascular kallikrein-kinin system, release of bradykinin, stimulation of NO-cGMP pathway-mediated release of cyclooxygenase products,\textsuperscript{31} and stimulation of the large-conductance, calcium-activated potassium channels.\textsuperscript{6}

C21 alone or combined with low-dose losartan reduced mesenteric artery stiffness as indicated by the rightward shift of media stress-strain relationship, independent of BP, the effect of which was not superior with the C21-losartan combination than with C21 alone. No further enhancement by combination with losartan suggested maximal functional improvement with C21 alone.\textsuperscript{19} Losartan did not reduce arterial stiffness, which may be attributed to the fact that we used a lower dose than in previous studies that improved vascular mechanics in SHRsp.\textsuperscript{7} Reduction in vascular stiffness can be explained by the decrease in extracellular matrix (ECM) components, media collagen type I and III, and total fibronectin. Increased vascular wall stiffness is a consequence of hypertensive vascular remodeling and contributes to vascular complications and end-organ damage. In SHR resistance arteries, increased wall stiffness is associated with increased collagen fraction and collagen:elastin ratio, expression of adhesion molecules, specifically integrins, and fibronectin,\textsuperscript{32–34} as well as vascular smooth muscle cell growth. Type I, III, and IV collagen gene expression is upregulated in vessels of SHRsp.\textsuperscript{35} Accumulation of ECM proteins is facilitated by diminished MMP activity that degrades ECM components, such as collagen and fibronectin, as demonstrated by reduced serum concentrations of MMP-1 in hypertensive patients and SHRsp.\textsuperscript{33} Antifibrotic effects of AT\textsubscript{2}R have been documented indirectly in arteries from AT\textsubscript{2}R null mice,\textsuperscript{36} SHRsp,\textsuperscript{35} aged Wistar Kyoto rats,\textsuperscript{28} and Ang II-infused Sprague-Dawley rats\textsuperscript{37} using AT\textsubscript{2}R blockade. Our findings are supported by C21-mediated reduction in renal fibrosis in SHRsp in a recent study.\textsuperscript{20} In this study, C21-induced decrease in interstitial collagen deposition could not be explained by an increase in the expression of MMPs. Mmp-1 mRNA was not detectable, and a change in the mmp-2 and mmp-9 mRNA expression was not observed in cardiac ventricles of SHRsp treated with C21, losartan, or the combination treatment. C21 could cause a decrease in tissue inhibitors of metalloproteinase resulting in increased MMP activity. Additional studies are required to confirm this hypothesis.

The cardiac antifibrotic effects of C21 were associated with a reduction in 2 fetal cardiac genes, myh7 and acta1, in the absence of reduction in nppa expression. These genes are highly expressed in embryonic ventricles, decreased in normal adult ventricular myocardium, and upregulated in cardiac hypertrophy and failure (reviewed in Paradis et al\textsuperscript{38}). Increase in myh7 and acta1 was associated with a decrease in cardiac function, whereas the increase in nppa was considered a compensatory mechanism to maintain tissue perfusion. C21 improved cardiac function after myocardial infarction,\textsuperscript{19} which could be associated, at least in part, with changes in gene expression. The lack of effect on nppa could be associated with a lack of BP effect, or AT\textsubscript{2}R signaling pathways might not regulate this gene. Further studies are required to determine the mechanisms of cardiac fetal gene regulation by AT\textsubscript{2}R.

C21 reduced various inflammatory markers in vivo, such as monocyte chemoattractant protein-1, IL-1β, IL-2, and IL-6 in normotensive rats\textsuperscript{19}; urinary inflammatory markers in SHRsp\textsuperscript{32}; and renal tumor necrosis factor-α, IL-6, and transforming growth factor-β1 in renovascular hypertension\textsuperscript{32}; as well as tumor necrosis factor-α and nuclear factor-κB in primary cultured human and murine dermal fibroblasts.\textsuperscript{21} Furthermore, C21 has been shown to decrease monocyte/macrophage infiltration in the kidney of SHRsp fed a high-salt diet.\textsuperscript{20} The present study confirmed the anti-inflammatory effects of C21 by demonstrating a reduction in aortic oxidative stress, as well as a decrease in monocyte/macrophage infiltration in aorta and in renal cortex and medulla, and T-lymphocyte infiltration in the renal cortex. However, reductions in aortic vascular cell adhesion molecule-1 expression in our study failed to achieve statistical significance, perhaps because of differences in the model studied, dosage, and route of administration of C21. C21 did not influence vascular NADPH oxidase activity but decreased vascular superoxide generation. NADPH oxidase activity is not the only source of vascular oxidative stress in SHRsp, and reactive oxygen species may originate in mitochondria, xanthine oxidase, or uncoupled NOS and other sources. SHRsp in this study were relatively younger and in an earlier phase of established hypertension than in other studies. It is possible that oxidative stress in these animals is not markedly increased, as also suggested by the absence of endothelial dysfunction in the untreated group.

In conclusion, we report the vascular effects of direct stimulation of AT\textsubscript{2}R with C21 in hypertension and demonstrate that C21 exerts beneficial BP-independent mechanical and structural actions on vessels from hypertensive rats. These beneficial vascular effects of C21 are not dependent on concomitant AT\textsubscript{1}R blockade and may have important clinical implications during long-term antihypertensive therapy. Reduction in stiffness of resistance arteries by C21 is a finding of key importance clinically because it participates in mechanisms contributing to the development of end-organ damage.\textsuperscript{39} Limitations to this study include lack of reversal of AT\textsubscript{2}R-mediated effects of C21 by using the AT\textsubscript{2}R blocker...
PD123319, which would have been informative, but because of the chosen route of drug administration and prohibitive cost of PD123319 for a chronic study, this could not be performed. However, the specificity of C21 for AT2R has been addressed in other in vivo studies. Acute perfusion of C21 in the striatum-induced decrease in the release of 3,4-dihydroxyphenylacetic acid, the major metabolite of dopamine, was prevented by PD123319.40 Subacute intracerebroventricular infusion of C21 causing a decrease in BP was prevented by PD12319.41 PD123319 prevented chronic C21 treatment delay in the appearance of brain abnormalities and extension of SHRsp survival when fed high sodium.30 Determination of ECM components, such as collagen and fibronectin, was done in aorta but not mesenteric arteries because of a lack of sufficient tissue from the mesenteric vasculature. A technique that could have been used is flow cytometry, which would have provided additional support to infiltrating cell count done by immunofluorescence. Further studies, particularly in humans, are required to confirm these results and establish the role of direct AT2R stimulation with C21 or a similar molecule as an alternative or complementary therapeutic approach in the long-term management of hypertension and possibly other forms of cardiovascular disease.

**Perspectives**

Abnormalities of endothelial or smooth muscle cell adhesion molecules and ECM in the vasculature may contribute to structural, mechanical, and functional changes that reduce the lumen size of small arteries and arterioles in hypertension. C21 by interfering with these mechanisms may serve as an effective therapy contributing to the prevention of end-organ damage in hypertension. Reduction in arterial stiffness is shown for the first time by direct stimulation of AT2R. This finding is of importance because increased stiffness can contribute to increased peripheral vascular resistance and BP. As well, through changes in wave reflection, it may affect central BP and participate in vascular complications. AT2R stimulation with C21 in combination with AT1R blockade may represent a novel approach for the treatment of hypertension and prevention of vascular complications and target organ damage.

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**Disclosures**

Compound 21 used in this study was a gift from Vicore Pharma.

**References**


Angiotensin Type 2 Receptor Agonist Compound 21 Reduces Vascular Injury and Myocardial Fibrosis in Stroke-Prone Spontaneously Hypertensive Rats
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Angiotensin type 2 receptor agonist compound 21 reduces vascular injury and myocardial fibrosis in stroke-prone spontaneously hypertensive rats

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Running title: Vascular effects of Compound 21 (C21)

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Expanded Methods

Quantitative RT-PCR

The mRNA expression levels of myosin heavy chain β (myh7), α skeletal muscle actin (acta1), atrial natriuretic peptide (nppa) and the matrix metalloproteinases 2 and 9 (mmp2 and mmp9) were determined in cardiac ventricles by real-time quantitative reverse transcriptase (RT) PCR (qRT-PCR). RNA was isolated with the mirVana kit according to the company’s recommendations (Life Technologies, Carlsbad, CA, USA) using a Polytron PT 1600 E homogenizer (Brinkmann Instruments, Mississauga, ON, Canada). One microgram of total RNA was RT with Quantitect RT kit (Qiagen). QPCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) with the Mx3005P real-time PCR cycler (Agilent Technologies, Mississauga, ON, Canada). QPCR results were normalized to the expression level of ribosomal small protein 16 (S16) and expressed as relative change. Primers were designed to have a melting temperature (Tm) of 60°C and a 3’ GC clamp using Primer3. Primers described in supplemental Table 1 were used with an annealing temperature of 58°C.
References

### Supplemental Table S1: Oligonucleotides used in qRT-PCR assays

<table>
<thead>
<tr>
<th>Genes</th>
<th>Oligonucleotides</th>
<th>Oligonucleotides</th>
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<tbody>
<tr>
<td></td>
<td>Forward</td>
<td>Reverse</td>
</tr>
<tr>
<td>Myh7</td>
<td>5’-CTCTACAAGAGGCCACCAG-3’</td>
<td>5’-CAATCCTGGCGTTGAGTG-3’</td>
</tr>
<tr>
<td>Acta1</td>
<td>5’-CACTTCCTACCCTCGGCAC-3’</td>
<td>5’-AGGTGTGGTGGCCAGATCTTC-3’</td>
</tr>
<tr>
<td>Nppa</td>
<td>5’-CCGATAGATCTGCCCTCTTG-3’</td>
<td>5’-TCCAGGAGGATTCACCAC-3’</td>
</tr>
<tr>
<td>Mmp2</td>
<td>5’-TGACGATGAGCTGTGGACTC-3’</td>
<td>5’-GGTCATAATCCTCGGTGGTG-3’</td>
</tr>
<tr>
<td>Mmp9</td>
<td>5’-TCACCTTCCCCTACCTTC-3’</td>
<td>5’-CGGCTCGAGTAGGACAGAAG-3’</td>
</tr>
<tr>
<td>Rsps16</td>
<td>5’-TCTGGGCAAGGAGAGATTTG-3’</td>
<td>5’-CCGCCAAACTTCTGGATTCC-3’</td>
</tr>
</tbody>
</table>

The ribosomal small protein 16 (rsPS16) was chosen as a house keeping molecule for relative quantification.
Figure S1. C21, losartan and/or C21-losartan combination reduced the expression of genes associated with cardiac hypertrophy without altering the expression of two matrix metalloproteinases. The mRNA levels of myosin heavy chain β (myh7), α skeletal muscle actin (acta1), atrial natriuretic peptide (nppa), the matrix metalloproteinases 2 and 9 (mmp2 and mmp9) and ribosomal small protein 16 (rpS16) were determined by quantitative reverse transcriptase PCR in the ventricles of SHRsp treated or not with vehicle (Ctrl), C21, losartan (Los) and C21-losartan combination (C21+Los). QPCR results were normalized to the expression level of rpS16 and expressed as relative change. Data are means ± SEM, *P<0.05 and **P<0.01 vs. Ctrl and †P<0.01 vs. C21, n = 4-6.
Figure S2. C21-losartan combination was the most efficient treatment to reduce interstitial collagen content in the left ventricle of SHRsp. Left ventricular interstitial collagen content was determined by Sirius red staining of ventricular sections of SHRsp treated or not with vehicle (Ctrl), C21, losartan (Los) and C21-losartan combination (C21+Los). Representative images of Sirius red stained sections of left ventricle subendocardial, myocardial and subepicardial regions are showed. Data are means ± SEM, *P<0.05 and **P<0.001 vs. Ctrl and †P<0.05 vs. C21, n = 6.
Figure S3. C21, losartan and/or C21-losartan combination reduced monocyte/macrophage infiltration in the aorta. Immunofluorescence was used to determine monocyte/macrophage infiltration using an anti-CD68 antibody (in red) in aorta of SHRsp treated or not with vehicle (Ctrl), C21, losartan (Los) and C21-losartan combination (C21+Los). Elastin autofluorescence and nuclear stain DAPI are shown in green and blue, respectively. Representative red, green and blue merged images are shown. Insert presents a magnification of the Ctrl red image showing clearly CD68\(^+\) cells. Arrowheads point at CD68\(^+\) cells. Yellow arrowheads are reproduced in the insert. Data are means ± SEM, *P<0.001 vs. Ctrl, n = 5.
Figure S4. C21, losartan and/or C21-losartan combination reduced monocyte/macrophage infiltration in the renal cortex. Immunofluorescence was used to determine monocyte/macrophage infiltration using an anti-CD68 antibody (in red) in renal cortex of SHRsp treated or not with vehicle (Ctrl), C21, losartan (Los) and C21-losartan combination (C21+Los). Nuclear stain DAPI is shown in blue. Representative red and blue merged images are shown. Insert presents a magnification of the Los red image showing clearly CD68⁺ cells. Arrowheads point at CD68⁺ cells. Yellow arrowheads are reproduced in the insert. Data are means ± SEM, *P<0.001 vs. Ctrl, n = 5-6.
Figure S5. C21, losartan and/or C21-losartan combination reduced monocyte/macrophage infiltration in the renal medulla. Immunofluorescence was used to determine monocyte/macrophage infiltration using an anti-CD68 antibody (in red) in renal medulla of SHRsp treated or not with vehicle (Ctrl), C21, losartan (Los) and C21-losartan combination (C21+Los). Nuclear stain DAPI is shown in blue. Representative red and blue merged images are shown. Insert presents a magnification of the C21 red image showing clearly CD68⁺ cells. Arrowheads point at CD68⁺ cells. Yellow arrowheads are reproduced in the insert. Data are means ± SEM, *P<0.001 vs. Ctrl, n = 5-6.
Figure S6. C21, losartan and/or C21-losartan combination reduced T lymphocyte infiltration in the renal cortex. Immunofluorescence was used to determine T lymphocyte infiltration using an anti-CD3 antibody (in green) in renal cortex of SHRsP treated or not with vehicle (Ctrl), C21, losartan (Los) and C21-losartan combination (C21+Los). Nuclear stain DAPI is shown in blue. Representative green and blue merged images are shown. Insert presents a magnification of the C21 + Los green image showing clearly CD3$^+$ cells. Arrowheads point at CD3$^+$ cells. Yellow arrowheads are reproduced in the insert. Data are means ± SEM, *$P<0.05$ vs. Ctrl, n = 5-6.
Figure S7. C21, losartan and/or C21-losartan combination reduced T lymphocyte infiltration in the renal medulla. Immunofluorescence was used to determine T lymphocyte infiltration using an anti-CD3 antibody (in green) in renal medulla of SHRsp treated or not with vehicle (Ctrl), C21, losartan (Los) and C21-losartan combination (C21+Los). Nuclear stain DAPI is shown in blue. Representative green and blue merged images are shown. Insert presents a magnification of the Ctrl green image showing clearly CD3$^+$ cells. Arrowheads point at CD3$^+$ cells. Yellow arrowheads are reproduced in the insert. Data are means ± SEM, n = 5-6.