Angiotensin II Type 2 Receptor Stimulation

A Novel Option of Therapeutic Interference With the Renin-Angiotensin System in Myocardial Infarction?

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Background—This study is the first to examine the effect of direct angiotensin II type 2 (AT$_2$) receptor stimulation on postinfarct cardiac function with the use of the novel nonpeptide AT$_2$ receptor agonist compound 21 (C21).

Methods and Results—Myocardial infarction (MI) was induced in Wistar rats by permanent ligation of the left coronary artery. Treatment with C21 (0.01, 0.03, 0.3 mg/kg per day IP) was started 24 hours after MI and was continued until euthanasia (7 days after MI). Infarct size was assessed by magnetic resonance imaging, and hemodynamic measurements were performed via transthoracic Doppler echocardiography and intracardiac Millar catheter. Cardiac tissues were analyzed for inflammation and apoptosis markers with immunoblotting and real-time reverse transcription polymerase chain reaction. C21 significantly improved systolic and diastolic ventricular function. Scar size was smallest in the C21-treated rats. In regard to underlying mechanisms, C21 diminished MI-induced Fas-ligand and caspase-3 expression in the peri-infarct zone, indicating an antiapoptotic effect. Phosphorylation of the p44/42 and p38 mitogen-activated protein kinases, both involved in the regulation of cell survival, was strongly reduced after MI but almost completely rescued by C21 treatment. Furthermore, C21 decreased MI-induced serum monocyte chemoattractant protein-1 and myeloperoxidase as well as cardiac interleukin-6, interleukin-1β, and interleukin-2 expression, suggesting an antiinflammatory effect.

Conclusions—Direct AT$_2$ receptor stimulation may be a novel therapeutic approach to improve post-MI systolic and diastolic function by ant apoptotic and anti inflammatory mechanisms. (Circulation. 2008;118:2523-2532.)

Key Words: angiotensin ■ angiotensin AT2 receptor ■ myocardial infarction ■ pharmacology

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A number of animal studies and the Valsartan in Acute Myocardial Infarction (VALIANT) trial have shown that AT$_1$R blockers improve survival and cardiac function after myocardial infarction (MI). It has been speculated that the AT$_2$R contributes to this effect because AT$_2$R blockade leads to an increased synthesis of angiotensin II, which in turn stimulates the unopposed AT$_1$Rs. However, results of animal...
studies directly addressing the role of the AT₂R in MI are again controversial: Although most studies claim that the AT₂R improves postinfarct cardiac function, others report either no effect on the outcome or even deterioration.

The inconsistency of data about AT₂R actions may be due in part to the fact that detection and assignment of AT₂R-mediated effects has always been difficult. AT₂R-mediated effects had to be examined either by treatment of cells or animals with angiotensin II under concomitant AT₁R blockade, which resulted in complex experimental protocols, or they were examined in genetically altered animals either overexpressing or lacking the AT₂R. The only currently available pharmacological AT₂R antagonist, PD123319, lacks selectivity when applied at higher doses and has thus given rise to contradictory findings, especially when applied in vivo. An experimental tool to directly stimulate the AT₂R under in vivo conditions was lacking until now.

In 2004, the synthesis of the first nonpeptide AT₂R agonist (compound 21 [C21]) was published, a compound that allows mediated effects to arise to contradictory findings, especially when applied in vivo. An experimental tool to directly stimulate the AT₂R under in vivo conditions was lacking until now.

In 2004, the synthesis of the first nonpeptide AT₂R agonist (compound 21 [C21]) was published, a compound that allows investigators to specifically and selectively stimulate the AT₂R in vitro and in vivo settings. With a Kₘ of 0.4 nM/L for the AT₂R and a Kₘ > 10 µM/L for the AT₁R, this substance possesses high selectivity for the AT₂R.

The present study is the first to examine the role of the AT₂R in post-MI cardiac function by direct AT₂R stimulation in vivo with the use of the nonpeptide AT₂R agonist C21. MI was induced in normotensive Wistar rats by permanent ligation of the anterior descending artery. After a 1-week treatment period, scar volume was assessed by magnetic resonance imaging, and hemodynamic function was assessed by echocardiography and Millar catheter. In a first attempt to address the underlying mechanisms of C21 actions, markers of inflammation and apoptosis were determined in cardiac tissue and plasma. To obtain a first qualitative estimate of the effectiveness of C21, we included a group of animals treated with the AT₁R antagonist candesartan as a reference drug.

Methods

Animals
Male normotensive Wistar rats (weight, 200 to 220 g; Harlan Winkelman, Borchen, Germany) were kept in a specific pathogen–free barrier under standardized conditions with respect to temperature and humidity and were housed on a 12-hour light/12-hour dark cycle in groups of 5 with food and water ad libitum. Animal housing, care, and applications of experimental procedures complied with the Guide for the Care and Use of Laboratory Animals of the State Government of Berlin, Germany.

Myocardial Infarction
Rats were anesthetized with ketamine/xylazine (Sigma Aldrich Chemie, Steinheim, Germany) 80 mg/10 mg/kg IP, intubated, and ventilated with a small-animal ventilator (Starling Ideal Ventilator, Harvard Apparatus) with room air at a rate of 75 cycles per minutes and a tidal volume of 300 µL. A left lateral thoracotomy was performed, and a suture was tightened around the proximal left anterior descending coronary artery. Sham-operated rats underwent the same surgical procedure with the exception of coronary ligature.

Experimental Protocol
Thoracic Doppler echocardiography was performed 24 hours after MI to assess post-MI baseline cardiac function. For better comparability of outcome between groups, rats were assigned to 2 different groups for later data analysis according to ejection fraction (EF): (1) rats with EF > 35% and (2) rats with EF ≤ 35%. All data presented in this study apply to animals with EF > 35% unless indicated otherwise. After being allocated to a certain category with respect to EF, animals were further randomly assigned to the following treatments: (1) vehicle; (2) C21 (0.01; 0.03; 0.3 mg/kg per day; C21 was kindly provided by A. Hallberg, University of Uppsala, Uppsala, Sweden; C21 is now available from Vicore Pharma, Göteborg, Sweden); (3) C21+PD123319 (0.03 mg/kg C21; 3 mg/kg PD123319); (4) candesartan (0.1 mg/kg per day); or (5) C21+candesartan (0.03 mg/kg C21; 0.1 mg/kg per day candesartan). A low dose of candesartan (0.1 mg/kg per day) was chosen because in prior experiments of our group, this dose had shown beneficial functional and cellular effects after stroke without affecting blood pressure. Drugs were applied intraperiosteally once daily starting 24 hours after MI to allow for prior determination of EF under untreated conditions. After 7 days of treatment, animals were euthanized, and plasma and hearts were collected. Sham-operated animals served as controls. The number of animals per group was 12 at the time of euthanasia. To achieve this group size, a total of 298 rats underwent successful coronary occlusion.

Transthoracic Doppler Echocardiography
Transthoracic Doppler echocardiography (M-mode and Doppler measurements) was performed on days 1 and 7 after MI in anesthetized rats with the use of the high-resolution imaging system Vevo 770 (VisualSonics Inc, Toronto, Canada). For details, see the online-only Data Supplement.

Determination of Left Ventricular and Scar Volumes by Magnetic Resonance Imaging
Cardiac-triggered cine and scar magnetic resonance imaging was performed on a conventional clinical 3.0-T scanner (Philips Achieva CV 3.0 T, Best, Netherlands) equipped with a QuasarDual gradient system (80 mT/m; 200 mT/m per millisecond slew rate) and specifically designed software (release 2.5.1 with implementation of a small-animal software patch developed by GyroTools Ltd, Zurich, Switzerland). For details, see the online-only Data Supplement.

Analysis of Hemodynamic Parameters
On day 7 after MI, before euthanasia, rats were anesthetized with ketamine/xylazine (80 mg/10 mg/kg IP; Sigma). An APR-407 MicroTip pressure catheter transducer (Millar Instruments, Houston, Tex) was passed through the right carotid artery and inserted into the aorta for the recording of heart rate and arterial pressure under constant pressure monitoring. The catheter was then advanced into the left ventricle for measurement of cardiac parameters. The catheter was connected via PowerLab/4 U (ADInstruments, Spechbach, Germany) to a computer running MacLab (Chart 5 software). Data were recorded over 10 minutes, stored, and later analyzed with Chart software (Blood Pressure Module).

Enzyme-Linked Immunosorbent Assay, Quantitative Real-time Reverse Transcription Polymerase Chain Reaction, and Western Blot Analysis
Enzyme-linked immunosorbent assays for the determination of plasma monocyte-chemoattractant protein-1 and myeloperoxidase, real-time reverse transcription polymerase chain reactions for the measurement of interleukin (IL)-1β, IL-2, and IL-6 in cardiac tissue as well as H9C2 cells, and immunoblotting for the quantification of cardiac caspase-3, Fas ligand, phospho-p38 and phospho-p44/42 mitogen-activated protein kinases (MAPKs) were performed according to standard protocols. Details, primers, and antibodies can be found in the Methods section of the online-only Data Supplement.
Cell Culture Experiments
Neonatal rat cardiomyocytes (H9C2 cell line) were incubated for 10 hours in high-glucose Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and treated with 10 ng/mL recombinant tumor necrosis factor-α (TNF-α) to induce cytokine expression and mimic the post-MI inflammatory response in the heart. Incubation was performed with or without coincubation with 10−6 mol/L C21 or C21 plus 10−6 mol/L PD123319.

Statistical Analysis
Results were expressed as mean±SEM. Multiple comparisons were analyzed with 1-way ANOVA followed by the Bonferroni post hoc test. Two-group comparisons were analyzed by the 2-tailed Student unpaired t test for independent samples. Differences were considered statistically significant at the value of P<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Basal Parameters
Postinfarct mortality was 31%. Animal losses all occurred within the first 24 hours after MI before first echocardiography and before animals were assigned to certain treatment groups. No animals died on days 2 to 7 after MI. Mean EF was equal in all treatment groups at treatment start (Figure 1). Body and heart weight did not differ between treatment groups or between animals with or without MI (data not shown).

Hemodynamic Parameters Measured by Millar Catheter 7 Days After MI

Table 1. Hemodynamic Parameters Measured by Millar Catheter 7 Days After MI

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=12)</th>
<th>Vehicle (n=12)</th>
<th>C21 0.03 mg/kg (n=12)</th>
<th>C21 0.3 mg/kg (n=12)</th>
<th>Candesartan 0.1 mg/kg (n=12)</th>
<th>C21+PD123319 (n=12)</th>
<th>C21+Candesartan (n=12)</th>
</tr>
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<tbody>
<tr>
<td>VSP, mm Hg</td>
<td>129±5</td>
<td>115±3</td>
<td>124±4</td>
<td>112±5</td>
<td>115±6</td>
<td>117±7</td>
<td>112±5</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>7.5±0.6</td>
<td>22.9±1.5*</td>
<td>13±1.6</td>
<td></td>
<td></td>
<td>9.0±1.3</td>
<td></td>
</tr>
<tr>
<td>Contractility index, 1/s</td>
<td>106.2±7.7</td>
<td>67±2.9*</td>
<td>96±2.5§</td>
<td>97±3.8§</td>
<td>81±2.3¶</td>
<td>83±1.5†#</td>
<td>95.2±3.4§</td>
</tr>
<tr>
<td>dP/dt max, mm Hg/s</td>
<td>7532±321</td>
<td>3664±114*</td>
<td>6667±235§</td>
<td>6950±261§</td>
<td>5193±278¶</td>
<td>4964±2144‡</td>
<td>6280±3194¶</td>
</tr>
<tr>
<td>dP/dt min, mm Hg/s</td>
<td>6795±231</td>
<td>2967±191*</td>
<td>5129±277‡</td>
<td>5198±309§</td>
<td>3385±163*</td>
<td>3861±2144†</td>
<td>4805±3155‡</td>
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<tr>
<td>SBP, mm Hg</td>
<td>122±6</td>
<td>115±3</td>
<td>124±6</td>
<td>115±5</td>
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<td>114±6</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>86±2</td>
<td>83±3</td>
<td>86±3</td>
<td>81±4</td>
<td>80±6</td>
<td>84±3</td>
<td>84±5</td>
</tr>
<tr>
<td>Pulse pressure, mm Hg</td>
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<td>32±2</td>
<td>37±3</td>
<td>34±3</td>
<td>35±2</td>
<td>33±3</td>
<td>30±3</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>239±5</td>
<td>258±19</td>
<td>232±10</td>
<td>248±6</td>
<td>252±10</td>
<td>240±9</td>
<td>246±10</td>
</tr>
</tbody>
</table>

VSP indicates ventricular systolic pressure; SBP, systolic blood pressure; and DBP, diastolic blood pressure.

*P<0.005 vs sham; †P<0.005 vs sham; ‡P<0.05 vs sham; §P<0.0005 vs vehicle; ¶P<0.005 vs vehicle; ¶¶P<0.05 vs vehicle; #P<0.05 vs C21.

Figure 1. Change of EF in individual animals from treatment start (day 1) to end of treatment (day 7).
Transthoracic Doppler Echocardiography

Measurements by transthoracic Doppler echocardiography confirmed that no changes in heart rate could be found between groups (Table 2; Table II in the online-only Data Supplement).

MI caused a significant increase in systolic (LVIDs) and diastolic left ventricular inner diameter (LVIDd) and peak velocity of early filling wave (E), whereas fractional shortening, EF, peak velocity of late filling wave (A), and E-wave deceleration time (EDT) were significantly reduced (Table 2; Figure 3). Treatment with C21 (0.03 or 0.3 mg/kg body wt; only 0.03 mg/kg body wt in rats with EF ≤35%) or with candesartan (0.1 mg/kg body wt) led to a significant improvement of all of these parameters with the only exceptions being LVIDd and A in rats with EF ≤35%.

The pronounced effect of treatment with C21 on left ventricular wall kinesis and peak velocity of late filling wave is also clearly recognizable on the representative echocardiographic images shown in Figure 4A and 4B.

Table 2. Transthoracic Doppler Echocardiography Measurements 7 Days After MI

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=12)</th>
<th>Vehicle (n=12)</th>
<th>C21 0.03 mg/kg (n=12)</th>
<th>C21 0.3 mg/kg (n=12)</th>
<th>Candesartan 0.1 mg/kg (n=12)</th>
<th>C21+ PD123319 (n=12)</th>
<th>Candesartan (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDd, mm</td>
<td>7.1±1.4</td>
<td>8.7±0.2‡</td>
<td>7.4±0.11§</td>
<td>7.5±0.11§</td>
<td>7.8±0.09∥∥∥</td>
<td>8.1±0.3*</td>
<td>7.7±0.25∥∥∥</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>3.8±0.11</td>
<td>6.3±0.2‡</td>
<td>4.5±0.18§</td>
<td>4.3±0.12§</td>
<td>5.2±0.2∥∥∥</td>
<td>6.0±0.3‡</td>
<td>4.7±0.27∥∥∥</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>46.3±1.2</td>
<td>26.1±1.6‡</td>
<td>39.0±1.81§</td>
<td>35.1±1.7∥∥∥</td>
<td>34.4±1.6∥∥∥</td>
<td>22.6±1.5∥∥∥</td>
<td>34.2±3.1∥∥∥</td>
</tr>
<tr>
<td>EF, %</td>
<td>77±1.1</td>
<td>49±2.0‡</td>
<td>67±2.1§</td>
<td>63±2.2∥∥∥</td>
<td>61±2.3∥∥∥</td>
<td>43±2.4∥∥∥</td>
<td>62.3±3.7∥∥∥</td>
</tr>
<tr>
<td>E, mm/s</td>
<td>1099±27</td>
<td>1560±29‡</td>
<td>1197±27§</td>
<td>1040±26§</td>
<td>1219±42∥∥∥</td>
<td>1437±45∥∥∥</td>
<td>1289±33∥∥∥</td>
</tr>
<tr>
<td>A, mm/s</td>
<td>629±41</td>
<td>271±24‡</td>
<td>586±46§</td>
<td>517±31§</td>
<td>571±45∥∥∥</td>
<td>285±44∥∥∥</td>
<td>576±43∥∥∥</td>
</tr>
<tr>
<td>E/A</td>
<td>1.7±0.06</td>
<td>5.7±0.4‡</td>
<td>2±1.1§</td>
<td>2.2±0.08§</td>
<td>2.1±0.07∥∥∥</td>
<td>5.0±1.2∥∥∥</td>
<td>2.2±0.5∥∥∥</td>
</tr>
<tr>
<td>EDT, ms</td>
<td>26±0.8</td>
<td>15±0.5‡</td>
<td>22±1.1∥∥∥</td>
<td>20±0.5∥∥∥</td>
<td>21±1.5∥∥∥</td>
<td>16.5±0.7∥∥∥</td>
<td>20.8±0.8∥∥∥</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>372±14</td>
<td>385±9</td>
<td>374±7</td>
<td>380±13</td>
<td>378±10∥∥∥</td>
<td>394±11</td>
<td>388±10</td>
</tr>
</tbody>
</table>

*P<0.05 vs sham; †P<0.001 vs sham; ‡P<0.0001 vs sham; §P<0.0001 vs vehicle; ||P<0.001 vs vehicle; ¶P<0.05 vs vehicle; #P<0.0001 vs C21; **P<0.001 vs C21.
any synergistic or additional effects. Whereas Figure 3 illustrates differences in outcome between treatment groups, the impact of different treatment regimens (vehicle, C21, candesartan) on EF in individual animals is shown in Figure 1 (and Figure I in the online-only Data Supplement for animals with EF ≤35%).

The effect of C21 on postinfarct cardiac function was dose dependent. Although 0.01 mg/kg body wt was widely ineffective...
(data not shown), cardiac function significantly improved with 0.03 mg/kg body wt; further improvement was only slight, and did not occur in all cases, in animals treated with 0.3 mg/kg body wt (Tables 1 and 2).

Magnetic Resonance Imaging

Magnetic resonance imaging at day 7 after MI/sham revealed that in the C21- but not in the candesartan-treated animals, scar size was significantly smaller than in vehicle-treated rats (Table 3, Figure 4C). Furthermore, C21, the combination of C21 plus candesartan, and, to a lesser extent, candesartan alone significantly improved EF (Table 3).

Inflammation Markers

Inflammation markers were measured (1) in plasma, (2) in cardiac tissue ex vivo, and (3) in fetal cardiomyocytes in vitro, as follows.

1. Plasma monocyte chemotactic protein-1 (MCP-1) and myeloperoxidase were significantly increased in rats after MI (Figure 5). In rats treated with C21, a significant reduction in MCP-1 and myeloperoxidase levels could be noticed compared with vehicle-treated animals.

2. The proinflammatory cytokines IL-1β, IL-2, and IL-6 were determined by real-time reverse transcription polymerase chain reaction in the peri-infarct zone. Expression of all 3 cytokines was increased in hearts of infarcted, vehicle-treated animals (Figure 6). C21 treatment significantly reduced expression levels of IL-1β, IL-2, and IL-6 back to baseline levels or even below. In this experimental setting, C21 proved to be as effective as the AT1R antagonist candesartan. The effect of C21 could be inhibited by cotreatment of animals with the AT2R antagonist PD123319.

3. To show that the antiinflammatory effects of C21 were not secondary to any hemodynamic changes or to reduced infarct size, the antiinflammatory properties of C21 were corroborated in the fetal cardiomyocyte cell line H9C2. Cells were incubated for 10 hours with TNF-α (10 ng/mL) to mimic the inflammatory response after MI. TNF-α treatment led to a significant increase in IL-6 expression in these cells (Figure 6D). Cotreatment of cells with C21 (10⁻⁶ mol/L) significantly reduced TNF-α–induced IL-6 expression. This antiinflammatory effect of C21 was inhibited by the AT2R antagonist PD123319.

Apoptosis Marker

The apoptosis markers Fas ligand and caspase-3 were measured in cardiac tissue (peri-infarct zone) by immunoblotting (Figure 7). MI led to a significant increase in the expression of both markers. C21 and candesartan reduced this increased expression to a comparable extent. The effect of C21 was blocked by the AT2R antagonist PD123319.

p44/42 MAPK, p38 MAPK

One week after MI, the cardiac expression of phosphorylated p44/42 MAPK and p38 MAPK was reduced to undetectable levels in vehicle-treated rats (Figure 8). Whereas candesartan

<table>
<thead>
<tr>
<th>Table 3. Magnetic Resonance Tomography 7 Days After MI</th>
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<tr>
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<tr>
<td></td>
</tr>
<tr>
<td>EF, %</td>
</tr>
<tr>
<td>Scar volume, %</td>
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</table>

*P<0.0001 vs sham; †P<0.05 vs vehicle; ‡P<0.0005 vs vehicle.
treatment led to a slight increase in the expression of both MAPKs, C21 restored phospho-p44/42 and phospho-p38 MAPK expression back to levels seen in sham-operated, noninfarcted animals. This effect of C21 was almost completely blocked by cotreatment with the AT2R antagonist PD123319.

**Discussion**

Although it is unanimously accepted that stimulation of AT1Rs contributes to tissue damage, inflammation, and unfavorable tissue remodeling after MI, data about the role of the AT2R in this context are controversial. This is certainly owed to the fact that, until now, practicable and reliable tools to study AT2R actions by direct AT2R stimulation have not been available. Treatment with the natural ligand angiotensin II primarily elicits effects mediated by the dominating AT1R; the only available AT2R antagonist, PD123319, is nonselective at higher doses; and the only available AT2 agonist, CGP42112, is a peptide and furthermore has agonistic and

**Figure 5.** Myeloperoxidase (MPO) (A) and MCP-1 (B) levels in plasma estimated by enzyme-linked immunosorbent assay. MI led to a significant increase in myeloperoxidase and MCP-1 levels, which could be significantly reduced by C21 (0.03 mg/kg per day IP (MPO: ###P<0.001 vs vehicle, *P<0.01 vs C21; MCP-1: #P<0.05 vs vehicle, ***P<0.01 vs C21; n=6 per group).

**Figure 6.** Expression of IL-1β (A), IL-2 (B), and IL-6 (C) in the peri-infarct zone in rat hearts. MI caused a significant increase in the expression of all cytokines examined (sham vs MI/vehicle [V]: §P<0.05, §§P<0.01; n=6 per group). Elevated expression levels were significantly reduced either by candesartan (cand; 0.1 mg/kg per day IP) or by C21 (0.03 mg/kg per day IP) (vehicle vs Cand: §P<0.05, §§P<0.01; vehicle vs C21: §P<0.05, §§P<0.01; n=6 per group). The effect of C21 was inhibited by cotreatment with PD123319 (PD; 3 mg/kg per day IP) (C21 vs C21+PD: #P<0.05, ##P<0.01; n=6 per group). D, IL-6 expression in H9C2 neonatal rat cardiomyocytes. TNF-α-induced (10 ng/mL) IL-6 expression was significantly reduced by C21 (10−8 mol/L). The effect of C21 was abolished by cotreatment with PD123319 (PD; 10−8 mol/L) (1 of 3 independent experiments; n=3 per group; §§P<0.01, #P<0.01). rel. indicates relative.
antagonistic properties, which renders the interpretation of in vivo data difficult.

We present the first in vivo study using a specific and selective nonpeptide AT2R agonist, C21. MI was induced in normotensive Wistar rats by permanent ligation of the left anterior descending coronary artery. Subsequently, animals were treated with C21 for 1 week. Direct AT2R stimulation by C21 improved systolic and diastolic cardiac function after MI, coinciding with a smaller infarct scar in C21-treated animals compared with vehicle treatment. Whereas MI led to an impairment of all parameters measured by echocardiography (LVIDs, EF, fractional shortening for systolic function; LVIDd, E, A, E/A, EDT for diastolic function) or Millar catheter (contractility, dP/dtmax for systolic function; LVEDP, dP/dtmin for diastolic function), respectively, treatment with C21 improved all of these parameters significantly without exception. For most parameters, the effect of C21 could be inhibited by cotreatment with the established AT1R antagonist PD123319, thus providing good evidence that the effects seen by C21 treatment are indeed a result of specific AT2R stimulation.

Previous studies addressing the role of the AT2R in cardiac function and remodeling after MI were performed primarily in genetically altered mice either overexpressing or lacking AT2Rs.5 The vast majority of these studies report a beneficial effect of the presence of AT2Rs on cardiac function after MI. For instance, Bove et al and Yang et al observed improved postinfarct cardiac function in AT2R-overexpressing mice, with NO apparently being of pivotal importance.9–11 Several other studies used male AT2R+/− mice and reported a deteriorated outcome after MI compared with wild-type mice.12–15 Xu et al16 did not detect any differences between AT2R+/− and AT2R−/− mice 4 weeks after MI but detected an impaired response to AT1R blockers in AT2R−/− mice. Ichihara et al17 used male AT2R−/− mice as well, but, in contrast to the other studies using this model, postinfarct scar area was reduced in knockout mice. Nevertheless, mortality in AT2R−/− mice was higher than in wild-type mice as a result of an increased rate of cardiac ruptures within the first week after ligation. Between weeks 2 and 6 after MI, mortality rate was identical in AT2R−/− and wild-type mice.

Our study differs from those discussed above because these studies examined effects related to AT2R expression and not to specific receptor stimulation. Admittedly, there may be some endogenous postinfarct AT2R stimulation by elevated cardiac angiotensin II levels. Furthermore, the AT2R has been reported to possess constitutive intrinsic activity,18 and cardiac AT2R expression is increased after MI.19,20 Still, the intensity of such an endogenous stimulation is very unlikely to reach the intensity achieved by stimulation with a specific agonist at an appropriate, pharmacological dosage.

Apart from rats treated with different doses of C21, our study also included a group of animals treated with the AT1R blocker candesartan. We used an AT1R blocker as a reference drug (1) because of its proven efficacy in the improvement of postinfarct cardiac function as shown in animals and clini-

Figure 7. Western blot analysis of caspase-3 (A) and Fas-ligand (B) expression in the peri-infarct zone of rat hearts. Shown is 1 representative of 3 blots for each marker. Note the increase in caspase-3 and Fas-ligand expression after MI, which was suppressed by candesartan (Cand; 0.1 mg/kg per day IP) or C21 (0.03 mg/kg per day IP). Cotreatment with PD123319 (PD; 3 mg/kg per day) blocked the effect of C21. V indicates vehicle.

Figure 8. Western blot analysis of phospho-p38 MAPK (A) and phospho-p44/42 MAPK (B) expression in the peri-infarct zone of rat hearts. Shown is 1 representative of 3 blots for each marker. Note the complete loss of phospho-p38 and phospho-p44/42 MAPK expression after MI. Phospho-p38 and phospho-p44/42 MAPK expression was only partly restored by candesartan (Cand; 0.1 mg/kg per day IP), whereas C21 (0.03 mg/kg per day IP) led to an almost complete rescue of MAPK expression. Cotreatment with PD123319 (PD; 3 mg/kg per day) blocked the effect of C21. V indicates vehicle.
cally4 and (2) because it may interfere with the renin-angiotensin system at least in part by indirect AT2R stimulation. We did not intend to compare the efficacy but rather the quality of actions of both drugs because, at present, with only very limited data and experimental experience with C21 available, it is hard to define what an equally effective dose of C21 or candesartan would be. Nevertheless, in mild to moderate infarctions, both drugs restored a number of cardiac parameters back to levels in noninfarcted animals (ie, with regard to these parameters, both drugs elicited an equal maximal response) (E, A, E/A, EDT). In regard to those parameters, in which a maximal response was not achieved, thus allowing a “comparison” of C21 and candesartan actions (LVIDs, LVIDd, EDP, contractility, dP/dtmax), the improvement by C21 was more effective than with candesartan. This outcome applied to systolic and diastolic function and may point to a superiority of C21.

Outcome of animals treated with the combination of C21 plus candesartan was not superior to that of animals treated with C21 alone, as may have been expected. A likely reason for this finding could be that C21 monotherapy already elicited a maximal functional improvement, which did not admit any further improvement.

To examine the molecular mechanisms underlying the beneficial effect of C21 on post-MI cardiac function, we looked for a putative effect of this drug on the apoptotic and inflammatory response in the peri-infarct zone.

MI led to a significant increase in Fas-ligand and caspase-3 expression in the infarct border zone. C21 significantly reduced the elevated, MI-related Fas-ligand and caspase-3 expression, and this effect could be blocked by the specific AT2R antagonist PD123319. Because apoptosis is a major contributor in postinfarct cardiac remodeling, including the development of infarct expansion,21 prevention of cardiomyocyte apoptosis by C21 may be a major molecular mechanism by which AT2R stimulation preserves cardiac function after MI.

The MAPKs are involved in numerous cellular processes such as apoptosis, inflammation, proliferation, or hypertrophy. In our study, we found that the activity of p38 MAPK and p44/42 MAPK was reduced to undetectable levels 7 days after MI in vehicle-treated animals and that treatment with C21 was able to completely rescue p38 MAPK and p44/42 MAPK expression in infarcted hearts. This effect was blocked by PD123319, indicating again AT2R specificity of the C21 effect. The effect of candesartan was by far weaker than the effect of C21.

Although MAPKs have traditionally been regarded as mediators of such processes as proliferation, apoptosis, and inflammation, recent data provided evidence that MAPK activation can also result in beneficial effects directly opposed to the aforementioned effects, namely, antiproliferation, cell survival, and anti-inflammation.22 In our experimental setting, the rescue of p38 MAPK and p44/42 MAPK expression by C21 7 days after MI coincided with an improvement of cardiac function and a smaller scar size. Although this is an observation of coincidence, a causal relationship between MAPK activation and improved cardiac function is supported by a recent study by Tenhunen et al.,23 in which the authors were able to improve postinfarct cardiac function by rescuing p38 MAPK expression through local adenovirus-mediated p38 MAPK gene transfer. Functionally, MAPK rescue reduced fibrosis and apoptosis (the latter shown by terminal deoxynucleotidyl transferase dUTP nick end labeling staining and, as in our study, by a reduction of caspase-3). Thus, the increase in p38 and p44/42 MAPK expression by C21 may be part of its antiapoptotic effect after MI, which is also reflected by a decrease in caspase-3 and Fas ligand.

In the early phase after acute MI, necrosis of cardiac cells sets into motion an inflammatory response that ultimately promotes fibrosis, scar formation, and thereby heart failure. C21 suppressed this postinfarct inflammatory response, as shown by a significant decrease in cytokine (MCP-1, IL-1β, IL-2, IL-6) expression in plasma and the peri-infarct zone. Again, the effect of C21 could be blocked by the AT2R antagonist, indicating the AT2R specificity of this effect. Because it has been shown for other drugs that inhibition of the inflammatory response contributes to the preservation of cardiac function, it can be assumed that anti-inflammation may be another mechanism of action of AT2R stimulation by C21.

It must be pointed out that this study was performed in normotensive rats and that no changes in blood pressure or heart rate occurred over the time of the experiment either by C21 at all doses used or by candesartan, which was given in a non–blood pressure–lowering dose. Therefore, the effects on cardiac function and cytokine and apoptosis markers as well as MAPK expression are unlikely to be caused by treatment-related hemodynamic changes. This notion is further supported by the inhibitory effect of C21 on IL-6 expression in rat fetal cardiomyocytes in vitro (ie, in an experimental setting independent of any hemodynamic influences).

In sum, this is the first study to use a nonpeptide AT2R agonist for direct stimulation of the AT2R after MI in rats. Treatment with C21 over a period of 7 days led to a pronounced improvement of systolic and diastolic cardiac function coinciding with a smaller scar volume. Furthermore, C21 reduced apoptosis and acted in an anti-inflammatory manner, suggesting that these mechanisms are contributing to the beneficial effect of AT2R stimulation. AT2R stimulation may thus represent a potential future therapeutic option for the improvement of postinfarct cardiac function but also for the prevention of end-organ damage in further organ systems.

Limitations

Because in our study infarct size has only been measured at the end but not before treatment, we cannot exclude an unmeasured difference in infarct size between groups at baseline, which may have influenced the outcome of the study. However, this possibility has been minimized by grouping of animals according to EF before the start of treatment to keep differences in mean EF between groups minimal. This procedure is based on the observation by us and others that EF correlated well with infarct size (r²=0.671; P<0.0001).24 Moreover, assignment of animals to the respective treatment groups was performed by an investigator who was blinded to the outcome of EF measurement in individual animals (only knowing whether EF was >35% or ≤35%).
Sources of Funding  
Dr Grzesiak and C. Curato received a grant from the EU Marie Curie graduate program CARDIOVASC (MEST-CT-2005-020268).

Disclosures  
Drs Dahlöf and Unger have modest ownership interest in Vicore Pharma and received speaker fees. Dr Dahlöf also has a significant interest in Vita Nova, the owner of Vicore Pharma. Dr Steckelings received speaker fees from Vicore Pharma. The other authors report no conflicts.

References  

CLINICAL PERSPECTIVE

Interference with the renin-angiotensin system is a very common, well-established, and successful concept of treatment in cardiovascular disease. All currently available and approved renin-angiotensin system–interfering drugs (angiotensin-converting enzyme [ACE] inhibitors, angiotensin II type 1 [AT1] receptor [AT1R] blockers, renin inhibitors, aldosterone antagonists) aim at inhibition of the unfavorable effects of an overactivated renin-angiotensin system signaling via the AT1R. However, the renin-angiotensin system not only harbors the renin/ACE/AT1R-aldosterone axis, which, when overstimulated, is involved in a variety of pathological conditions ranging from hypertension to end-organ damage or local processes in myocardial infarction and stroke; in fact, it also possesses a tissue-protective axis involving ACE2, Ang2, and the AT2R receptor (AT2R), which seems to counteract detrimental effects mediated via the AT1R but also inflammation, hyperproliferation, or fibrosis in general. At present, no pharmacological tool is available to specifically stimulate this beneficial ACE2/Ang1–7/AT2R axis in vivo. The novel nonpeptide AT2 receptor compound 21 may be such a drug. The study presented here is the first to test the therapeutic potential of direct AT2R stimulation in a model of myocardial infarction. Compound 21 significantly improved cardiac function and resulted in a smaller scar size independent of any hemodynamic changes. Because compound 21 is a nonpeptide with sufficient bioavailability (>30%), it may be a suitable lead compound for the development of a drug for the blood pressure–independent treatment or prevention of end-organ damage.

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Angiotensin II Type 2 Receptor Stimulation: A Novel Option of Therapeutic Interference With the Renin-Angiotensin System in Myocardial Infarction?

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In the article by Kaschina et al, “Angiotensin II Type 2 Receptor Stimulation: A Novel Option of Therapeutic Interference With the Renin-Angiotensin System in Myocardial Infarction?” which appeared in the December 9, 2008 issue of the journal (Circulation. 2008;118:2523–2532), the authors have noted a correction to their disclosure statement.

In the published version, authors stated the following disclosures:

“Drs Dahlöf and Unger have modest ownership interest in Vicore Pharma and received speaker fees. Dr Steckelings received speaker fees from Vicore Pharma. The other authors report no conflicts.”

The disclosure statement should read:

“Drs Dahlöf and Unger have modest ownership interest in Vicore Pharma and received speaker fees. Dr Dahlöf also has a significant interest in Vita Nova, the owner of Vicore Pharma. Dr Steckelings received speaker fees from Vicore Pharma. The other authors report no conflicts.”

The online version of the article has been updated to reflect this correction. The authors regret the error.

DOI: 10.1161/CIR.0b013e3181e6c405
Transthoracic Doppler Echocardiography:

Rats were anesthetised with 1,5% to 2% isoflurane in oxygen through a face mask and placed on a heated platform in the supine position with all legs taped to ECG electrodes for heart rate monitoring. A high–resolution imaging system Vevo 770 (VisualSonics Inc., Toronto) equipped with a 25 MHz single-crystal transducer with a focal length of 15mm, a frame rate of 40 Hz and the maximum field of view of 2D imaging 21x21mm was used. All data were transferred to a computer for offline analysis. Three measurements per heart were determined, averaged, and statistically analysed.

**M-mode measurements:**

M-mode tracings were recorded from short-axis view at the level of the papillary muscles with two-dimensional image guidance through the anterior and posterior LV walls. LV internal dimensions were measured through the largest diameter of the ventricle, both in diastole and systole. FS was calculated according to the following formula

$$FS=(LVDd –LVDs)/LVDd \times 100\%$$

where LVDd is left ventricular diameter in diastole, and LVDs is left ventricular diameter in systole.

**Doppler measurements:**

Pulsed wave Doppler spectra of mitral inflow were recorded from the apical four-chamber view. The sample volume (0,5mm - the smallest available size) was placed at the tip of the mitral leaflets and adjusted to the position at which velocity was
maximal. All measurements were conducted in accordance with the recommendation of the American Society of Echocardiography.

**Determination of Left Ventricular and Scar Volumes by Magnetic Resonance Imaging**

Cardiac triggered cine and scar magnetic resonance imaging was performed on a conventional clinical 3.0 Tesla scanner (Philips Achieva CV 3.0T, Best, The Netherlands) equipped with a QuasarDual gradient system (80mT/m; 200mT/m/ms slew rate) and specifically designed software (release 2.5.1 with implementation of a small animal software patch developed by GyroTools Ltd., Zurich, Switzerland). All animals were examined in the supine position and placed in a solenoid radiofrequency coil used for signal detection. Cardiac synchronization was performed using four electrodes (vector-electrocardiogram) fixed to the anterior chest wall and scans were triggered on the R-wave of the electrocardiogram. The animals were anesthetized by inhalation of isoflurane via a nose cone at a 1.0%–1.5% volume mixed with oxygen at a rate of 2 liter/min.

For cine imaging, a gradient echo pulse sequence (TR/TE/flip angle/number of signal averages = 9.0 msec/4.2 msec/15°/5) was used for complete coverage of the heart in short axis geometry (spatial resolution 0.2 x 0.2 mm, slice thickness 1.5 mm, number of slices 9) with 30 phases/cardiac cycle acquired per slice.

Scar imaging was started 2 min after tail injection of gadolinium-DTPA (0.2 mmol/kg bodyweight) employing a 3-dimensional inversion-recovery sequence (TR/TE/flip angle/number of signal averages = 6.7 msec/3.3 msec/15°/2) with an individually adapted inversion prepulse delay (range 100 to 130 ms). In-plane spatial resolution of scar imaging was 0.15 x 0.15 mm with a slice thickness of 0.8 mm.
All scans were processed with dedicated image analysis software (Philips View Forum, release 5.1.1.1, Best, The Netherlands). For left ventricular volume measurements, the endocardial border of each short axis slice was planimetered manually at end diastole and end systole, and volumes were calculated by using the Simpson rule. End diastole was defined as the first frame in each cine sequence and end systole as the cine frame with the smallest left ventricular cavity area. For determination of scar volume, the brightly enhanced scar area was manually segmented in each slice and multiplied with slice thickness for calculation of scar volume.

Plasma monocyte-chemoattractant protein-1 (MCP-1) and myelo-peroxidase (MPO) ELISAs:

Plasma MCP-1 (Biosource International) and MPO (Immundiagnostic AG, Bensheim, Germany) levels were determined by commercially available ELISA kits according to the manufacturer’s instructions.

In brief, plasma samples for the detection of MCP-1 were diluted in standard diluent, and 50 µl samples added to each well of an anti-Rat MCP-1 Pre-coated 96-well Strip Plate followed by an one hour incubation at room temperature. Samples were then incubated for another hour with a biotinylated antibody reagent. After adding streptavidin, absorbance was measured on an ELISA plate reader (Bio-Rad, Benchmark Plus) at 450 nm against 550 nm. Plasma amounts of MCP-1 were calculated referring to a standard curve.

For the detection of MPO, diluted plasma samples were incubated for one hour in an anti-Rat MPO Pre-coated 96 well microtiter plate, and for an additional hour with a peroxidase-coupled antibody. Absorbance was measured at 550 nm against 620 nm as a reference, and plasma amounts of MPO calculated referring to a standard curve.
Quantitative real-time RT-PCR (qPCR)

Total RNA was isolated from cardiac tissue (peri-infarct zone) using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. RNA (1 µg) was reverse transcribed in 25 µl final volume for 1 hour at 37 °C using 100 U Superscript Reverse Transcriptase, 5.0 µg random primer, 0.5 mM 2'-deoxynucleotide 5' triphosphate, 20 U RNAsin, and 5 µl 5x reaction buffer. Relative quantification of gene expression was performed with the ABI 5700 sequence detection system for real-time polymerase chain reaction (PCR; TaqMan technology by PE Biosystems, Weiterstadt, Germany) using the standard curve method. PCRs were performed with the TaqMan Universal Master Mix and the TaqMan assay reagent for GAPDH in a total volume of 25µl. The sequences of primers and probe were the following:

Rat IL-1β:

Sense:  5'-gggTTgAATCTATACCTgTCCTgTgT-3'
Antisense:  5'-TTggTATTgTTTgggATCCA-3'
Probe:  5'-AAgACggCACACCCACCCTgCA-3'

Rat IL-2:

Sense:  5'-CCCCATgATgCTCACgTTTA-3'
Antisense:  5'-CATTTTCCAggCACACTgAAgATgT-3'
Probe:  5'-TgCCCAAgCAgCCACgAATTg-3'

Rat IL-6:

Sense:  5'-AgTgCATCATCgCTgTTCATACA-3'
Antisense:  5'-ATATgTTCTCAgggAgATCTTggAA-3'
Probe:  5'-TgAgAAAAgAgTTgTgCAATtggCAATTCTg-3'

Rat β-actin:
Sense: 5'-ATCgCTgACAggATgCAgAAg-3'
Antisense: 5'-CgCTCAggAggAgCAATgAT-3'
Probe: 5'-AgATTACTgCCCTggCTCCTAgCACCA-3'

Each qPCR reaction was performed in triplicate wells, using the following conditions: activation of Taq-Polymerase 5 min at 95°C, 15 s at 95°C, 15 s at 60°C, 30 s at 72°C through 40 cycles, followed by 15 s at 50°C.

Data represent the mean expression level ± standard deviation (standardized to beta-actin mRNA expression) calculated according to the ddC_T method of at least three independent measurements.

Western Blot Analysis

Tissue samples were homogenised in lysis buffer (50 mM Tris-HCl, 500 mM EDTA, 150 mM NaCl, 0.1% Triton X-100) supplemented with 100 µg/ml PMSF and protease-inhibitor-cocktail (Roche) followed by centrifugation. Protein concentrations were determined by the method of Bradford using BSA as a standard. Proteins (30 µg per line) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham). Membranes were blocked in 5% non-fat milk/TTBS, and incubated consecutively with primary antibody and horseradish peroxidase-conjugated secondary antibody (Dako). Immunoreactive proteins were detected using ECL-reagents (Amersham). The following primary antibodies were used: FasL (1:500; Cell Signaling Technology Inc.), caspase 3 (1:500; Cell Signaling Technology Inc.), phospho-p44/42 MAPK, phospho-p38 MAPK (Phospho-MAPK Family Antibody Sampler Kit, Cell Signaling Technology Inc., USA), p44/42 MAPK, and p38 MAPK (MAPK family Antibody Sampler, Cell Signaling Technology Inc., USA). Quantification of Western Blots was performed computer assisted using the NIH
image analysis system (Scion, Frederick, MD, USA). To demonstrate equal protein loading of the gel, membranes were reprobed with GAPDH Ab (Acris).
Table 1. Hemodynamic Parameters Measured by Millar Catheter 7 Days After MI in Animals with Severe Impairment of Cardiac Function (EF ≤ 35%)

<table>
<thead>
<tr>
<th>mg/kg</th>
<th>Sham n=12</th>
<th>Vehicle n=12</th>
<th>Comp 21 (0.03 mg/kg) n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSP [mmHg]</td>
<td>131±9</td>
<td>103±7#</td>
<td>112±11</td>
</tr>
<tr>
<td>LVEDP [mmHg]</td>
<td>7.5±0.6</td>
<td>24.5±1.7###</td>
<td>12.2±1.6**</td>
</tr>
<tr>
<td>Contractility Index [1/s]</td>
<td>106±2.7</td>
<td>57±4##</td>
<td>90±4**</td>
</tr>
<tr>
<td>maxdP/dt [mmHg/s]</td>
<td>7532±204</td>
<td>3884±230###</td>
<td>6463±356*</td>
</tr>
<tr>
<td>mindP/dt [mmHg/s]</td>
<td>6795±286</td>
<td>2941±323###</td>
<td>4414±294*</td>
</tr>
<tr>
<td>SBP [mmHg]</td>
<td>122±6</td>
<td>100±8</td>
<td>114±7</td>
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<tr>
<td>DBP [mmHg]</td>
<td>86±2</td>
<td>74±3</td>
<td>79±3</td>
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<tr>
<td>Pulse pressure [mmHg]</td>
<td>35±2</td>
<td>26±2</td>
<td>35±3</td>
</tr>
<tr>
<td>HR [bpm]</td>
<td>239±5</td>
<td>250±7</td>
<td>232±10</td>
</tr>
</tbody>
</table>

VSP- ventricular systolic pressure, LVEDP- left ventricular end diastolic pressure, Contr - contractility: (maxdP/dt)/P, maxdP/dt- max peak rate of LV pressure, mindP/dt - min peak rate of LV pressure, SBP- systolic blood pressure, DBP- diastolic blood pressure, HR- heart rate.

###p < 0.0001 vs Sham; ##p < 0.005 vs Sham; **p < 0.005 vs Vehicle ; *p < 0.05 vs Vehicle
<table>
<thead>
<tr>
<th>mg/kg)</th>
<th>Sham</th>
<th>Vehicle</th>
<th>Comp 21 (0.03 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=12</td>
<td>n=12</td>
<td>n=12</td>
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<tr>
<td>LVIDd [mm]</td>
<td>7.1±0.14</td>
<td>9.3±0.2 ###</td>
<td>9.0±0.2 ###</td>
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<tr>
<td>LVIDs [mm]</td>
<td>3.8±0.11</td>
<td>7.9±0.16 ###</td>
<td>7.0±0.3 ###</td>
</tr>
<tr>
<td>FS [%]</td>
<td>46.3±1.2</td>
<td>10.6±1.6 ###</td>
<td>20±1.5 ### **</td>
</tr>
<tr>
<td>EF [%]</td>
<td>77±1.1</td>
<td>22±3 ###</td>
<td>39±2.6 ### **</td>
</tr>
<tr>
<td>E [mm/s]</td>
<td>1099±27</td>
<td>1702±36 ###</td>
<td>1249±46 ***</td>
</tr>
<tr>
<td>A [mm/s]</td>
<td>629±41</td>
<td>195±19 ###</td>
<td>255±18 **</td>
</tr>
<tr>
<td>E/A</td>
<td>1.7±0.06</td>
<td>8.7±1 ###</td>
<td>4.9±0.9 **</td>
</tr>
<tr>
<td>EDT [ms]</td>
<td>26.4±0.6</td>
<td>12.0±1 ###</td>
<td>22±2 **</td>
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<tr>
<td>HR [bpm]</td>
<td>372±14</td>
<td>358±9</td>
<td>367±10</td>
</tr>
</tbody>
</table>

LVId- left ventricular internal dimension end diastole, LVIDs- left ventricular internal dimension end systole, FS- fractional shortening, EF- ejection fraction, E – peak velocity of early filling wave, A- peak velocity of late filling wave, EDT- E wave deceleration time, HR- heart rate. ### p < 0.0001 vs Sham; ***p< 0.0005 vs Vehicle ; **p < 0.005 vs Vehicle ; *p < 0.05 vs Vehicle
Fig. 1

Change of ejection fraction in individual animals with EF ≤ 35% from treatment start (day 1)