Role of Heart Rate Reduction in the Prevention of Experimental Heart Failure
Comparison Between I_f-Channel Blockade and β-Receptor Blockade

Peter Moritz Becher, Diana Lindner, Kapka Miteva, Konstantinos Savvatis, Christin Zietsch, Bastian Schmack, Sophie Van Linthout, Dirk Westermann, Heinz-Peter Schultheiss, Carsten Tschöpe

See Editorial Commentary, pp 908–910

Abstract—To investigate whether heart rate reduction via I_f-channel blockade and β-receptor blockade prevents left ventricular (LV) dysfunction, we studied ivabradine and metoprolol in angiotensin II–induced heart failure. Cardiac dysfunction in C57BL/6J mice was induced by implantation of osmotic pumps for continuous subcutaneous dosing of angiotensin II (1.8 mg/kg per day SC) over a period of 3 weeks. Ivabradine (10 mg/kg per day) and metoprolol (90 mg/kg per day), which resulted in similar heart rate reduction, or placebo treatments were simultaneously started with infusion of angiotensin II. After 3 weeks, LV function was estimated by conductance catheter technique, cardiac remodeling assessed by estimation of cardiac hypertrophy, fibrosis, and inflammatory stress response by immunohistochemistry or PCR, respectively. Compared with controls, angiotensin II infusion resulted in hypertension in impaired systolic (LV contractility, stroke volume, end systolic elastance, afterload, index of arterial-ventricular coupling, and cardiac output; \( P < 0.05 \)) and diastolic (LV relaxation, LV end diastolic pressure, \( r \), and stiffness constant \( \beta \); \( P < 0.05 \)) LV function. This was associated with a significant increase in cardiac hypertrophy and fibrosis. Increased cardiac stress was also indicated by an increase in cardiac inflammation and apoptosis. Both ivabradine and metoprolol led to a similar reduction in heart rate. Metoprolol also reduced systolic blood pressure. Ivabradine led to a significant improvement in systolic and diastolic LV function (\( P < 0.05 \)). This was associated with less cardiac hypertrophy, fibrosis, inflammation, and cardiac apoptosis (\( P < 0.05 \)). Metoprolol treatment did not prevent the reduction in cardiac function and adverse remodeling, despite a reduction of the inflammatory stress response. Behind heart rate reduction, additional beneficial cardiac effects contribute to heart failure prevention with I_f-channel inhibition. (Hypertension. 2012;59:949-957.)

Key Words: angiotensin II ■ cardiac dysfunction ■ diastolic dysfunction ■ ivabradine ■ metoprolol ■ heart rate

Arterial hypertension is the most common chronic disease. It is the leading risk factor for death attributed to stroke, myocardial infarction, or end-stage renal failure.1,2 The current available classes of antihypertensive drugs reduce these risks. However, their role in the prevention of chronic heart failure (CHF) is still under investigation. It has been shown in the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial that the diuretic chlorthalidone but not the calcium channel blocker amlodipine or the angiotensin-converting enzyme inhibitor lisinopril effectively reduced the risk of heart failure.3 Several meta-analyses have shown that lowering blood pressure by 5 mmHg reduces the risk of CHF development by \( \approx 20\%.4–6 \) These studies have included those analyzing angiotensin-converting enzyme inhibitors, angiotensin II (Ang II) type 1 receptor blockers, calcium channel blockers, and β-blockers. The preventative effects of β-blockers have often been inferior to the other first-line antihypertensive drugs.7,8 This has called into question the effect of heart rate (HR) reduction in patients at risk for CHF. However, high resting HR is an independent risk factor in the prognosis of cardiovascular diseases.5,10–11 Furthermore, HR reduction is a critical and beneficial stage in the initial treatment of CHF8 and coronary artery disease with left ventricular (LV) dysfunction.13 It has been shown by the Ivabradine and Outcomes in Chronic Heart Failure investigators that selective HR reduction with ivabradine in combination with β-blockers re-
duces heart failure hospitalizations and cardiovascular death in systolic heart failure. In contrast to β-blockers, ivabradine is a highly specific and selective “funny” current I_{f}-channel blocking agent. It does not modify atrioventricular or intraventricular conduction or myocardial contractility in animals or humans. In addition, ivabradine and metoprolol provide equal reductions in angiotensin receptor expression, plasma aldosterone levels, and atrial natriuretic factor expression. Nevertheless, the role of HR reduction in the prevention of CHF, especially in hypertensive patients, remains unclear. The role of I_{f}-channel blockade versus β-blockade in treating these patients is still under debate. Therefore, we examined β-blockade with metoprolol versus ivabradine-induced I_{f}-channel blockade in a mouse model that develops mild arterial hypertension and CHF as a result of chronic renin-angiotensin-aldosterone system stimulation.

**Materials and Methods**

**Drugs and Dose Determination**

Ang II (1.8 mg/kg per day; Bachem, Weil am Rhein, Germany) or sterile ringer solution (Merck, Darmstadt, Germany) was continuously infused via osmotic pumps over a period of 3 weeks (model 2004, ALZET, Cupertino, CA). Experimental animal groups were randomly assigned and received ivabradine (10 mg/kg per day; Servier, Courbevoie, France) or metoprolol (90 mg/kg per day; Sigma, St Louis, MO) in 100 mL of drinking water over the same time period.

**Surgical Procedures and Hemodynamic Measurements**

Eight- to 10-week-old male C57BL/6J mice were randomly divided into 6 groups (n=8 per group). The experimental groups consisted of control, ivabradine, metoprolol, Ang II, Ang II-metoprolol, and Ang II-ivabradine. After 3 weeks, all of the animals were anesthetized (thiopental, 125 mg/g IP; Merial, Hallbergmoos, Germany), intubated, and artificially ventilated with a rodent ventilator type 7025 (Ugo Basile, Comerio VA, Italy). HR, systolic blood pressure, LV contractility (dP/dt_{max}), stroke volume, stroke work, end-systolic elastance (E_{se}, afterload; E_{es}/E_{a}, index of arterial-ventricular coupling). Data are expressed as mean±SEM.

**Table. Animal Characteristics and Hemodynamic Results 21 d After Treatment**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Ang II</th>
<th>Ang II-METO</th>
<th>Ang II-IVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>28.2±0.3</td>
<td>27.5±0.2</td>
<td>26.3±0.4</td>
<td>27.6±0.7</td>
</tr>
<tr>
<td>LV weight, mg</td>
<td>90±5</td>
<td>146±2†</td>
<td>129±2*</td>
<td>115±3†</td>
</tr>
<tr>
<td>LV weight/body wt ratio, mg/g</td>
<td>3.1±0.07</td>
<td>5.5±0.03‡</td>
<td>4.3±0.04‡</td>
<td>3.6±0.02‡</td>
</tr>
<tr>
<td>Global LV function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>547±13</td>
<td>538±14</td>
<td>485±13‡</td>
<td>479±20*</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>101.2±8</td>
<td>133.8±6‡</td>
<td>108.5±3‡</td>
<td>125±7†</td>
</tr>
<tr>
<td>Cardiac output, μL/min</td>
<td>14231±938</td>
<td>7823±573‡</td>
<td>8584±1248‡</td>
<td>10442±876†</td>
</tr>
<tr>
<td>Stroke volume, μL</td>
<td>25.1±0.7</td>
<td>15.7±0.3‡</td>
<td>17.7±0.8‡</td>
<td>21.8±0.6‡</td>
</tr>
<tr>
<td>Stroke work, μL/mm Hg</td>
<td>1871±28</td>
<td>1062±41‡</td>
<td>1245±63‡</td>
<td>1489±57†</td>
</tr>
<tr>
<td>E_{es}, mm Hg/μL</td>
<td>1.7±0.06</td>
<td>0.87±0.08‡</td>
<td>0.96±0.05†</td>
<td>1.5±0.03†</td>
</tr>
<tr>
<td>E_{a}, mm Hg/μL</td>
<td>4.2±0.2</td>
<td>6.6±0.4‡</td>
<td>5.4±0.4‡</td>
<td>4.4±0.3†</td>
</tr>
<tr>
<td>E_{es}/E_{a} index</td>
<td>0.4±0.04</td>
<td>0.13±0.07‡</td>
<td>0.17±0.05†</td>
<td>0.34±0.00†</td>
</tr>
<tr>
<td>Systolic LV function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV contractility, mm Hg/s</td>
<td>11730±642</td>
<td>7899±726‡</td>
<td>6626±417‡</td>
<td>8923±542†</td>
</tr>
<tr>
<td>Diastolic LV function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV diastolic relaxation, mm Hg/s</td>
<td>−6483±384</td>
<td>−5168±420‡</td>
<td>−3958±580‡</td>
<td>−5672±250*†</td>
</tr>
<tr>
<td>LV diastolic relaxation time, ms</td>
<td>9.7±0.3</td>
<td>11.9±0.9‡</td>
<td>12.4±0.7‡</td>
<td>9.9±0.2†</td>
</tr>
<tr>
<td>LV end diastolic pressure, mm Hg</td>
<td>1.9±0.2</td>
<td>2.8±0.5‡</td>
<td>2.3±0.6‡</td>
<td>1.6±0.2†</td>
</tr>
<tr>
<td>Stiffness constant β</td>
<td>0.04±0.005</td>
<td>0.15±0.003‡</td>
<td>0.11±0.006‡</td>
<td>0.07±0.005†</td>
</tr>
</tbody>
</table>

Data are from 8 mice per group. Ang II indicates angiotensin II; METO, metoprolol; IVA, ivabradine; LV, left ventricular; E_{es}, end-systolic elastance; E_{a}, afterload; E_{es}/E_{a}, index of arterial-ventricular coupling. *P<0.05 vs Ang II. †P<0.05 vs Ang II-METO. ‡P<0.05 vs control.

**Immunohistological Measurements**

As described previously, murine tissue of the left ventricle was embedded in Tissue-Tek (Dako), and immunohistochemistry was performed with specific antibodies directed against CD3 (Santa Cruz), CD4 (BD Biosciences), CD68 (Abcam), vascular cell adhesion molecule (VCAM; BD Biosciences), α-smooth muscle actin (SMA; Abcam), matrix metalloproteinase (MMP) 9 (Chemicon), tissue inhibitor of metalloproteinase (TIMP) 1 (Chemicon), collagen I (Chemicon), and collagen III (Chemicon). Quantification was performed by digital image analyses. In brief, the ratio between the...
heart tissue area and the specific chromogen-positive area was calculated (area fraction, percentage). The amount of infiltrating cells was calculated by measuring the number of cells per area of heart tissue (cells per millimeter squared).

**TUNEL Assay**
Apoptotic cells were detected in cryosections by end-labeling the fragmented DNA using the DeadEnd Colorimetric TUNEL System (Promega) according to the manufacturer’s instructions. TUNEL-positive cells were calculated as cells per area of heart tissue.25

**Cell Culture**
In vitro, the effects of ivabradine and metoprolol supplementation in murine HL-1 cardiomyocytes, indexed by caspase 3/7 activity, as well as HCN-2 and HCN-4 mRNA expression and in human cardiac fibroblasts detected by collagen synthesis, were analyzed (please see the online data supplement).

**Statistical Analyses**
Statistical analysis was performed using SPSS version 12.0. Data are expressed as the mean±SEM. Statistical differences were assessed by using the Kruskal-Wallis test in conjunction with the Mann-Whitney post hoc test. Bonferroni correction was applied to the data expressed as mean±SEM. *P<0.05 vs control; †P<0.05 vs Ang II-METO; #P<0.05 vs control. Ang II indicates angiotensin II; METO, metoprolol; IVA, ivabradine. □, control; ■, Ang II; □, Ang II-METO; ■, Ang II-IVA.

**Figure 1.** Cardiac cytokines. Relative mRNA expression of interleukin (IL) 1β, IL-6, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β in heart tissue 21 days after treatment. Data are from 8 mice per group and 1 data point per heart. Data expressed as mean±SEM. *P<0.05 vs Ang II; †P<0.05 vs Ang II-METO; #P<0.05 vs control. Ang II indicates angiotensin II; METO, metoprolol; IVA, ivabradine. □, control; ■, Ang II; □, Ang II-METO; ■, Ang II-IVA.

Chronic infusion of Ang II (1.8 mg/kg per day SC) resulted in impaired cardiac output and significant reduction of systolic function (dP/dt\text{max} -33%; stroke volume, -38%; stroke work, -44%; E\text{es} -59%; E\text{es}/E\text{a} -68%; cardiac output, -45%; P<0.05) and diastolic function (LV relaxation, -21%; LV end-diastolic pressure, -33%; τ, +19%; stiffness constant β, +62%; P<0.05), indicating a significant global cardiac dysfunction (Table). In contrast, ivabradine ameliorated systolic LV dysfunction (dP/dt\text{max} +22%; stroke volume, +38%; stroke work, +29%; E\text{es} +36%; E\text{es}/E\text{a} -33%; E\text{es}/E\text{a} +72%; cardiac output, +33%; P<0.05) and improved diastolic dysfunction (LV relaxation, +9.7%; LV end-diastolic pressure, -30%; τ, -20%; stiffness constant β, -46%; P<0.05) to values similar to the control values (Table).

**Cardiac Cytokine Activation**
We measured cytokine activation in hearts from those cytokines associated with the inflammatory immune response in CHF.28 Quantification of expression of the proinflammatory cytokines IL-1β, IL-6, tumor necrosis factor-α, and the profibrotic transforming growth factor-β revealed a 2.3-fold (P<0.05), 8.1-fold (P<0.05), 5.6-fold (P<0.05), and 1.4-fold (P<0.05) elevation after 3 weeks of Ang II treatment, respectively (Figure 1). Both metoprolol and ivabradine prevented the Ang II–driven increase in tumor necrosis factor-α and transforming growth factor-β expression. In addition, ivabradine treatment led to a significant reduction in IL-1β and IL-6 expression (P<0.05), whereas metoprolol and ivabradine alone did not affect any cytokine expression levels.

**Cardiac Immune Cell Infiltration**
Ang II induced a cardiac inflammatory response, as indicated by increases in CD3\text{+}, CD4\text{+}, and CD68\text{+} cell infiltration (Figure 2, representative images) and the expression of VCAM-1, whereas this response was reduced by ivabradine.
and metoprolol to values similar to the controls. Metoprolol and ivabradine alone did not show any effect on immune cell infiltration or VCAM-1 expression. However, the reduction of CD4-positive cell and VCAM-1 protein expressions in the myocardium was found to be even more reduced after ivabradine than after metoprolol treatment \( (P < 0.05) \).

**Cardiac Apoptosis**

As shown in Figure 2, Ang II–treated mice displayed a significantly increased amount of TUNEL-positive apoptotic cells when compared with controls (2.8-fold; \( P < 0.05 \)). In mice with metoprolol treatment, the amount of TUNEL-positive apoptotic cells was reduced; however, this did not reach statistical significance (\( P \) value not significant). In contrast, ivabradine-treated mice displayed a significant reduction of TUNEL-positive apoptotic cells when compared with the animal groups Ang II and Ang II-metoprolol \( (P < 0.05) \). Metoprolol and ivabradine alone did not show any effect on cardiac apoptosis.

**Cardiac Fibrosis and Remodeling**

Cardiac fibrosis, as determined by collagen I and III gene and protein expression; its ratio and \( \alpha \)-SMA protein content (Figures 3 and 4); and LV hypertrophy, indexed by LV weight/body weight ratio, were elevated 3 weeks after Ang II–induced cardiac dysfunction. This was significantly attenuated by both treatments, but the effect was great after ivabradine than after metoprolol (Table). In detail, Ang II treatment resulted in a 2.4- and 3.6-fold elevation of collagen I and III mRNA and in a 6.6- and 2.5-fold elevation of collagen I and III protein expression (Figures 3 and 4), respectively. These increased mRNA and protein expressions were reduced when animals were treated with ivabradine \( (P < 0.05) \). In addition, Ang II treatment led to significantly increased protein expressions of \( \alpha \)-SMA, MMP-9, and TIMP-1. Increased expressions of \( \alpha \)-SMA, MMP-9, and TIMP-1 were significantly downregulated after ivabradine treatment when compared with metoprolol \( (P < 0.05) \), whereas metoprolol and ivabradine alone did not affect cardiac fibrosis and remodeling.

**HCN Channel Expression and Cardiac Hypertrophy**

As shown in Figure 3 and 5A, Ang II–treated mice displayed significantly increased mRNA expression levels of HCN-2 (2.6-fold; \( P < 0.05 \)), HCN-4 (1.7-fold; \( P < 0.05 \), and atrial
natriuretic peptide (4.9-fold; \( P<0.05 \)) when compared with controls. In mice with metoprolol treatment, HCN-2, HCN-4, and atrial natriuretic peptide mRNA expression were reduced; however, this did not reach statistical significance (\( P \) value not significant). In contrast, ivabradine-treated mice displayed a significant reduction of cardiac HCN-2 and atrial natriuretic peptide mRNA expression (\( P<0.05 \)) when compared with the animal groups Ang II and Ang II-metoprolol, whereas metoprolol and ivabradine alone did not show any effect on cardiac HCN channel expression and cardiacyptrophy.

**Cell Culture Experiments**

Cardiomyocyte stimulation with Ang II induced apoptosis by 1.4-fold (Figure 6A; \( P<0.05 \)) in HL-1 cardiomyocytes when compared with controls. Treatment with ivabradine, as well as metoprolol, reduced the Ang II-induced apoptosis by 1.2-fold (\( P<0.05 \)) and 1.1-fold (\( P<0.05 \)), respectively. In addition, Ang II stimulation significantly increased HCN-2 (4.6-fold; \( P<0.05 \)) and HCN-4 (7.2-fold; \( P<0.05 \)) mRNA expression in HL-1 cardiomyocytes (Figure 5B). Increased expressions of HCN-2 and HCN-4 genes were significantly reduced after ivabradine (7.4-fold; \( P<0.05 \)) and metoprolol (8.5-fold; \( P<0.05 \)) treatment. Under basal conditions, ivabradine and metoprolol did not show any effects on apoptosis and HCN channel expression in cardiomyocytes.

Fibroblast stimulation with Ang II increased collagen synthesis (2.2-fold; \( P<0.05 \)) in human cardiac fibroblasts when compared with controls (Figure 6B). Treatment with ivabradine, as well as metoprolol, decreased the Ang II-induced collagen synthesis by 2.8-fold (Figure 6B; \( P<0.05 \)) and 2.2-fold (Figure 6B; \( P<0.05 \)). Under basal conditions, ivabradine and metoprolol did not show any effects on collagen production in fibroblasts.

**Discussion**

The salient finding of this study is that HR reduction is important for the prevention of heart failure after chronic stimulation of the renin-angiotensin-aldosterone system. Despite comparable HR reduction, \( I_f \)-channel blockade was more effective than \( \beta \)-blockade. This indicates that differences in the hemodynamic profile and direct antiapoptotic and antifibrotic effects modifying the cardiac inflammatory stress response are crucial for CHF prevention.

Chronic Ang II infusion led to the development of hypertension and systolic and diastolic LV dysfunction.\(^{21,29}\) Cardiac remodeling and stress were indicated by changes in cardiac hypertrophy, fibrosis, myocardial inflammatory response, and apoptosis, most likely by the increase in afterload and also by direct toxic effects induced by Ang II infusion.\(^{30,31}\) Comparable HR reduction led to an improvement in cardiac remodeling after ivabradine and metoprolol treatment. However, both drugs differed in the cardiac hemodynamic response, indicated by a reduction in afterload seen after ivabradine treatment.

In addition, different efficiency in modulating the inflammatory response, quality of extracellular matrix regulation by collagen turnover, cardiac apoposis, and LV hypertrophy was seen between both drugs. These findings are in line with Lin et al.,\(^{32}\) showing that the HCNs encoded mainly by the HCN-2 and HCN-4 genes in the heart play a significant role in ventricular hypertrophy. This may belong to additional involved mechanisms, leading to the result that ivabradine but not metoprolol led to a significant improvement in cardiac function. This finding was even more unexpected, because metoprolol also lowered blood pressure.

Ivabradine reduces HR by directly acting on the \( I_f \)-channel, which controls the electric pacemaker activity in the sinoatrial node.\(^{33}\) Recently, it was reported that HR reduction by ivabradine was able to reduce diastolic dysfunction and
ventricular-arterial coupling, in part by decreasing EA.42,43 In clinical studies, lowering HR was shown to improve coronary perfusion. This results in decreased cardiac LV wall stress,41 an effect that is important in mild hypercholesterolemia.34 It has been shown that ivabradine increases diastolic time35 and enhanced ventricular relaxation during peak exercise36 to a greater extent than β-blockers, which is in agreement with our findings. In contrast to β-blockers, ivabradine did not decrease the maximal rise in LV pressure with time (dP/dt_{max}) or alter postsystolic wall thickening.37 Both β-blockers propranolol38,39 and atenolol35,40 showed negative inotropic effects at rest and during adaptation of dP/dt_{max}, whereas ivabradine did not change dP/dt_{max} at rest and did not limit its increase during exercise. Similar to these findings, we found negative inotropic effects after metoprolol treatment but not after ivabradine treatment. Ivabradine is also known to decrease cardiac afterload and to improve coronary perfusion. This results in decreased cardiac LV wall stress,33 an effect that is important in mild hypertension and CHF. Afterload (E_A) is proportional to the product of the total peripheral resistance (TPR) and HR (E_A = TPR × HR), explaining its HR dependency. In clinical studies, lowering HR was shown to improve ventricular-arterial coupling, in part by decreasing E_A.42,43 Similarly, these hemodynamic profiles seen in our study probably play a role in the improvement of global LV function after ivabradine treatment. Afterload reduction and improved arterial-ventricular coupling are key hemodynamic factors that influence not only LV performance but also cardiac remodeling in the long term. This is in agreement with our finding showing that HR reduction after metoprolol had only a minimal effect on cardiac remodeling. Our hypothesis is supported by the additional reduction in the amount of cardiac collagen expression, especially after ivabradine treatment. Although both drugs influenced extracellular matrix regulation in our model, the effects of metoprolol were less, showing only a reduction of collagen III mRNA expression without a significant change in the collagen I/III ratio. In contrast, ivabradine treatment led to improved changes in collagen mRNA expression, collagen I/III ratio, and collagen I protein expression to values similar to those of the control group. The most important finding with respect to extracellular matrix regulation is the fact that only ivabradine treatment led to a significant reduction in the number of α-SMA-myofibroblasts and to an improvement of the MMP/TIMP system regulation by normalizing MMP-9 and TIMP-1 protein levels. During connective tissue remodeling, fibroblasts are activated and can transform to α-SMA-expressing myofibroblasts.44 The myofibroblasts synthesize...
and form a newly created extracellular matrix, leading to accumulation of cardiac collagen. Abnormal persistence of the myofibroblast is a hallmark of fibrotic diseases in other organs, as well as the heart. Modulating this may be a therapeutic option for these diseases.45,46 This is known to be an important trigger for diastolic dysfunction and increased LV stiffness and is in agreement with our finding that ivabradine but not metoprolol improved diastolic LV function. Myofibroblasts are not only collagen-producing cells, they are also inflammatory supporter cells.47 A reduction in myofibroblasts influences the cardiac inflammatory stress response. We found a reduction in immune cell infiltration and cytokine activation after ivabradine and metoprolol treatment. Ivabradine treatment displayed additional anti-inflammatory and antiapoptotic actions with significantly reduced CD4-positive immune cell infiltration, VCAM-1 protein content, and number of myocardial TUNEL-positive cells in the myocardium. This is in line with reports that ivabradine can inhibit chemokine-induced migration of CD4-positive lymphocytes.48 Interestingly, ivabradine treatment led to a slightly more prominent reduced expression of the cytokines IL-1β and IL-6, which are known to be strong

**Figure 5.** Hyperpolarization-activated channel (HCN) expression in vivo, in vitro, and cardiac hypertrophy. Relative mRNA expression of the HCN-2 and HCN-4 in heart tissue 21 days after treatment. Data are from 8 mice per group and 1 data point per heart. Data are expressed as mean±SEM. *P<0.05 vs Ang II; †P<0.05 vs Ang II-METO; #P<0.05 vs control. Ang II indicates angiotensin II; METO, metoprolol; IVA, ivabradine. □, control; ■, Ang II; □, Ang II-METO; □, Ang II-IVA. Effects of ivabradine and metoprolol on Ang II–altered HCN-2 and HCN-4 expression in murine HL-1 cardiomyocytes. Data are from 6 values per group and 1 data point per heart. Data are expressed as mean±SEM. ***P<0.001.

**Figure 6.** Cell culture: apoptosis and collagen synthesis. Effects of ivabradine and metoprolol on Ang II–altered apoptosis in HL-1 cardiomyocytes and collagen synthesis in human cardiac fibroblasts. Data are from 6 values per group and 1 data point per heart. Data are expressed as mean±SEM. *P<0.05; ***P<0.001. Ang II indicates angiotensin II; METO, metoprolol; IVA, ivabradine.
predictive factors in cardiovascular diseases and may contribute to the additional effects after ivabradine treatment. These findings describe a reduction in cardiac inflammatory stress response. It is interesting to speculate whether differences in the hemodynamic profile can explain the described effects in cardiac stress response and remodeling in toto or whether additional drug specific effects are involved as described recently in a model of regional myocardial ischemia and reperfusion by Heusch et al. In agreement with the hypothesis that hemodynamic and HR-independent mechanisms contribute to the cardiobeneficial effects of ivabradine treatment, we could show that ivabradine exerts direct anti- fibrinogenic and antiapoptotic effects in vitro after Ang II stimulation.

Because we found an increased LV expression of the I_F channels HCN-2 and HCN-4 after Ang II stimulus and downregulation after ivabradine and metoprolol treatment, we suggest that, in the in vivo situation, LV HCN channels are targets of ivabradine, independent of the I_F-channel expression in the sino-atrial node.

In conclusion, HR reduction by metoprolol does not effectively prevent CHF in Ang II–induced hypertension and end organ damage. It can, however, be prevented when cardiac load is optimized and sino-atrial node and LV HCN channels are inhibited by ivabradine. In cases of more severe hypertension, it remains to be seen which of the factors, blood pressure reduction, HR reduction, or afterload reduction, are the most important.

Perspectives

The clinical evidence that high resting HR is not only a risk marker but also a risk factor in hypertension is meager. Our experimental data show that HR is a risk marker and a risk factor for CHF in a model of Ang II–induced hypertension and end organ damage. Development of CHF can be improved to a greater extent after I_F-channel blockade than after β-blockade. However, in the clinical situation, both drugs have their advantages and disadvantages. The antiarrhythmic and antihypertensive effects after β-blockade are often useful and necessary, whereas the negative inotropic effects, an increase in afterload and other adverse effects, are unwanted and lead to a low compliance. Further studies are necessary to prove the hypothesis that additional pleiotropic mechanisms contribute to the cardiobeneficial effects seen after ivabradine treatment. A well-timed combination of β-blockade and I_F-channel blockade to reduce adverse effects and to enforce prevention of CHF could be a new alternative therapeutic option in hypertensive patients with high resting HR, a concept that has to be further investigated and proven in clinical outcome studies.

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Disclosures

None.

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Role of heart rate reduction in the prevention of experimental heart failure:
Comparison between If-channel blockade and β-receptor blockade.

Supplementary data

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Short title: If-channel blockade leads to prevention of heart failure.

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Expanded material and methods

Drugs and dose determination
Angiotensin II (1.8 mg/kg/day/s.c.; Bachem, Weil am Rhein, Germany) and sterile ringer solution (Merck, Darmstadt, Germany) were prepared and continuously infused via osmotic pumps over a period of three weeks (Alzet, model 2004, Cupertino CA, USA). Experimental animal groups were randomly assigned and received ivabradine (10 mg/kg/day; Servier, Courbevoie, France) or metoprolol (90 mg/kg/day; Sigma, St. Louis, Missouri, USA) in 100 ml drinking water over the same time period. In a pilot study we investigated the effects of different doses of the drugs ivabradine and metoprolol in HR reduction. In agreement with other studies [1-2], the chosen dose of ivabradine and metoprolol showed a significant and comparable HR reduction of 14% in the control animals without changes in systolic and diastolic LV performance.

Surgical procedures and hemodynamic measurements
Eight to ten-week-old male C57BL/6J mice were randomly divided into 6 groups (n = 8 per group). The experimental groups consisted of control, IVA, METO, ANGII, ANGII-METO, ANGII-IVA. After three weeks, all animals were anesthetized (thiopental 125 mg/g i.p.; Merial, Hallbergmoos, Germany), intubated, and artificially ventilated with a rodent ventilator type 7025 (Ugo Basile, Comerio VA, Italy). A 1.2F microconductance pressure catheter (ARIA SPR-719; Millar Instruments Inc, Houston-Texas, USA) was positioned in the LV via the right carotid artery for continuous registration of LV pressure-volume loops in closed-chest animals [3]. Calibration of the recorded volume signal was obtained by the hypertonic (10%) saline wash-in technique [4]. All measurements were performed while ventilation was momentarily turned off. Indices of systolic and diastolic cardiac performance were derived from LV pressure-volume data obtained both at steady state and during transient preload reduction by occlusion. Global myocardial performance and systolic function were quantified by heart rate, systolic blood pressure (SBP), LV contractility (peak rate of rise in LV pressure − dP/dtmax), stroke volume (SV), stroke work (SW), end systolic elastance (Ees), afterload (Ea), index of arterial-ventricular coupling (Ees/Ea) and cardiac output (CO). Diastolic performance was measured by LV relaxation (dP/dtmin), LV relaxation time (Tau) and LV end diastolic pressure (LVEDP). After hemodynamic characterization, hearts and lungs of sacrificed animals were removed and weight and weight ratios were determined. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US NIH (NIH Publication No. 85-23, revised 1996).

RNA isolation
Total RNA was extracted from LV sections by the TRIzol method (Invitrogen, Carlsbad, CA) as described previously [2]. Further purification of TRIzol isolated tissue RNA or RNA isolation from cell culture samples the RNeasy Mini kit (Qiagen, Hilden, Germany) was used. 1µg RNA was reverse transcribed using the High Capacity cDNA kit (Applied Biosystems, Darmstadt, Germany).

TaqMan low-density arrays of heart tissue
The relative quantification of target genes was performed using the comparative Ct method with RQ plates on an Applied Biosystems 7900HT system [4-6]. Wells (n = 384) of TaqMan Arrays have been preloaded with TaqMan gene expression assays (two replicates per assay). Then, 1µg of total RNA converted to cDNA were mixed with an equal volume of TaqMan
Universal PCR Master Mix and loaded into the fill reservoirs of the TaqMan Arrays. The real-time PCR was performed according to the manufacturer’s instructions. Each TaqMan gene expression assay contains a forward and reverse primer as well as a specific FAM-labelled probe for each of the target genes selected (Col1A1 (Mm00483888-m1), Col3A1 (Mm00802331_m1), interleukin (IL)-1β (Mm00514993_m1), IL-6 (Mm00446190_m1), tumor necrosis factor (TNF)-α (Mm00443258_m1), transforming growth factor (TGF)-β (Mm00441724_m1), the hyperpolarization-activated channel (HCN)-2 (Mm00469838_m1, Hs00606903_m1) and HCN-4 (Mm01176086_m1, Hs00975492_m1). 18S RNA was incorporated into our customized TaqMan Low-Density Array as internal standard.

**Immunohistological measurements**

As described previously [2, 6-7], immunostaining in LV tissue was carried out with specific antibodies (Abs) using commercially available kits. Avidin-biotin complex (ABC) staining method was used with specific primary goat anti-CD3 antibody (Ab) (Santa Cruz; 1:75) in conjunction with ABC-anti-goat-Kit (Vector Labs). Specific primary rat anti-CD4 (1:50; BD Biosciences), rat anti-CD68 (1:350; Abcam) and vascular cell adhesion protein-I (VCAM-1) (1:50; BD Biosciences) were used in conjunction with biotinylated rabbit anti-rat Ab (1:200; Dako) and ABC-Standard-Kit. In addition, EnVision staining method was used with specific primary rabbit anti-smooth muscle actin (α-SMA) (1:200; Abcam), tissue inhibitor of matrix metalloproteinase (TIMP-1), rabbit anti-collagen I (1:500; Chemicon) and rabbit anti-collagen III (1:200; Chemicon) Abs were used in conjunction with secondary biotinylated HRP-conjugated anti-rabbit Ab (EnVision, Dako). Quantification was performed by digital image analyses [2]. In brief, the ratio between the heart tissue area and the specific chromogen-positive area was calculated (area fraction, in percentage). The amount of infiltrated cells was calculated by measuring the number of cells per area of heart tissue (cells/mm²).

**Cell culture**

Murine HL-1 cells (10,000 and 300,000 per 96-well and 6-well, respectively) were cultured in Claycomb medium (SAFC Biosciences, Kansas, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 100µM norepinephrine (Sigma, Steinheim, Germany) and 2mM glutamine. After 24 h of culture, medium was changed and cells were stimulated with or without 5µM of angiotensin II in the presence or absence of 20ng/ml of ivabradine or 3µM of metoprolol for 4h in Claycomb medium supplemented with 0.01% FBS, 1% penicillin/streptomycin, 100µM norepinephrine and 2mM glutamine. Then, caspase 3/7 activity was measured in the 96-well plate and the cells plated in the 6-wells were collected for mRNA isolation. Human cardiac fibroblasts (Cell Applications, Inc. San Diego, USA) (10,000 and 135,000 per 96-well and 6-well, respectively) were cultured in Lung/Cardiac Fibroblasts Basal Medium (Cell Applications, Inc. San Diego, USA), 10% FBS and 1% penicillin/streptomycin. After 24h of culture, medium was changed and cells were stimulated with or without 5µM of angiotensin II in the presence or absence of 20ng/ml of ivabradine or 3µM metoprolol for 24h. Then, the cells in the 96-well plate were fixed overnight in methanol at room temperature and the cells plated in the 6-wells were collected for RNA isolation.

**Caspase 3/7 activity assay**

Caspase 3/7 activity was measured with a caspase-Glo 3/7 assay kit (Promega) according to the manufacturer’s protocol. In brief, 100µL caspase-Glo 3/7 reagent containing caspase 3/7 buffer and the proluminescent caspase 3/7 substrate were added to each sample. After 1h of incubation at room temperature, the luminescence of each sample was measured in a
microplate-reading luminometer (Mithras LB 940, Berthold Technologies GmbH & Co KG, Germany).

Sirius Red staining
After overnight fixation in methanol, the cells were washed with PBS and incubated in 0.1% Direct Red 80 (Sirius Red; Sigma-Aldrich, St. Louis, MO, USA) staining solution at room temperature for 1h. After a second wash with PBS, the Sirius red staining was eluted in 0.1N sodium hydroxide at room temperature for 60min on a rocking platform. The optical density of Sirius Red, representative for the accumulation of collagen accumulation was measured at 540 nm with a VersaMax microplate reader (Molecular Device GmbH, Munich, Germany).

References: