Celiprolol, A Vasodilatory β-Blocker, Inhibits Pressure Overload–Induced Cardiac Hypertrophy and Prevents the Transition to Heart Failure via Nitric Oxide–Dependent Mechanisms in Mice

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Background—The blockade of β-adrenergic receptors reduces both mortality and morbidity in patients with chronic heart failure, but the cellular mechanism remains unclear. Celiprolol, a selective β₁-blocker, was reported to stimulate the expression of endothelial NO synthase (eNOS) in the heart, and NO levels have been demonstrated to be related to myocardial hypertrophy and heart failure. Thus, we aimed to clarify whether celiprolol attenuates both myocardial hypertrophy and heart failure via the NO-signal pathway.

Methods and Results—In rat neonatal cardiac myocytes, celiprolol inhibited protein synthesis stimulated by either isoproterenol or phenylephrine, which was partially suppressed by N G-nitro-L-arginine methyl ester (L-NAME). Four weeks after transverse aortic constriction (TAC) in C57BL/6 male mice, the ratio of heart weight to body weight (mg/g) (8.70 ± 0.42 in TAC, 6.61 ± 0.44 with celiprolol 100 mg · kg⁻¹·d⁻¹ PO, P < 0.01) and the ratio of lung weight to body weight (mg/g) (10.27 ± 1.08 in TAC, 7.11 ± 0.70 with celiprolol 100 mg · kg⁻¹·d⁻¹ PO, P < 0.05) were lower and LV fractional shortening was higher in the celiprolol-treated groups than in the TAC group. All of these improvements were blunted by L-NAME. Celiprolol treatment significantly increased myocardial eNOS and activated phosphorylation of eNOS. Myocardial mRNA levels of natriuretic peptide precursor type B and protein inhibitor of NO synthase, which were increased in the TAC mice, were decreased in the celiprolol-treated mice.

Conclusions—These findings indicated that celiprolol attenuates cardiac myocyte hypertrophy both in vitro and in vivo and halts the process leading from hypertrophy to heart failure. These effects are mediated by a selective β₁-adrenergic receptor blockade and NO-dependent pathway. (Circulation. 2004;110:692-699.)

Key Words: receptors, adrenergic, beta ▪ heart failure ▪ hypertrophy ▪ nitric oxide
Figure 1. Results of cardiac myocyte culture. A, Protein synthesis was inhibited by celiprolol (celi) at concentrations ranging from $10^{-4}$ to $10^{-7}$ mol/L in a dose-independent manner, and this concentration range did not affect normal myocytes. *$P<0.01$ vs Control. B, Celiprolol ($10^{-6}$ mol/L) inhibited protein synthesis stimulated by PE ($10^{-4}$ mol/L), and this effect was partially abolished by cotreatment with L-NAME ($10^{-6}$ mol/L). *$P<0.01$ vs PE, $S^P<0.05$ vs PE+Celi. C, Cell size was calculated from 200 cells in every treatment group. Increase in cell size caused by PE was reduced by treatment with celiprolol ($10^{-6}$ mol/L), and L-NAME diminished effect of celiprolol. *$P<0.01$ vs PE, $S^P<0.05$ vs PE+Celi. D, Representative images of cardiac myocytes stained with rhodamine phalloidin and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Concentrations for all agents are same as in B.

Figure 2. Celiprolol improves heart remodeling. HW/BW ratio (A) and myocyte cross-sectional area (B) were decreased significantly in TAC mice treated with celiprolol 100 mg · kg$^{-1}$ · d$^{-1}$ (Celi low) or 200 mg · kg$^{-1}$ · d$^{-1}$ (Celi high) in comparison with untreated TAC mice. L-NAME (100 mg · kg$^{-1}$ · d$^{-1}$) alone did not increase degree of myocyte hypertrophy under conditions of pressure overload. However, it partially abolished antihypertrophic effect of celiprolol ($100$ mg · kg$^{-1}$ · d$^{-1}$). Similar results on myocardial fibrosis (C) and perivascular fibrosis (D) were also noted. Numbers of mice in Sham, TAC, TAC+Celi low, TAC+L-NAME, and TAC+Celi+L-NAME groups are 10, 19, 11, 6, and 5, respectively.
Hemodynamic and Echocardiographic Results

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Transstenosis Gradient Pressure, mm Hg</th>
<th>Heart Rate, bpm</th>
<th>Tail-Cuff Systolic Blood Pressure, mm Hg</th>
<th>LV End-Systolic Dimension, mm</th>
<th>LV End-Diastolic Dimension, mm</th>
<th>LV Diastolic Posterior Wall Thickness, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (10)</td>
<td>24±0.3*</td>
<td>5±1</td>
<td>640±21</td>
<td>116±2.9*</td>
<td>3.07±0.06*</td>
<td>0.65±0.02*</td>
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<tr>
<td>TAC (8)</td>
<td>22.6±0.5</td>
<td>55±5</td>
<td>647±11</td>
<td>100±3.7</td>
<td>3.37±0.17</td>
<td>1.01±0.06</td>
<td></td>
</tr>
<tr>
<td>TAC+Celi (11)</td>
<td>22.4±0.7</td>
<td>52±4</td>
<td>604±17*</td>
<td>108±3.1</td>
<td>3.04±0.11*</td>
<td>0.79±0.03*</td>
<td></td>
</tr>
<tr>
<td>TAC+Celi+L-NAME (6)</td>
<td>21.2±0.6</td>
<td>50±3</td>
<td>652±11</td>
<td>108±3.5</td>
<td>3.31±0.20</td>
<td>0.99±0.05</td>
<td></td>
</tr>
<tr>
<td>TAC+Celi+L-NAME (5)</td>
<td>21.9±0.5</td>
<td>54±3</td>
<td>616±13*</td>
<td>106±2.1</td>
<td>3.15±0.12</td>
<td>0.93±0.02</td>
<td></td>
</tr>
</tbody>
</table>

TAC indicates transverse aortic constriction; Celi, celiprolol 100 mg · kg⁻¹ · d⁻¹ PO. The number of mice in each group is indicated in parentheses.

*P<0.05 vs TAC.

confirm whether celiprolol-induced attenuation of cardiac hypertrophy is blunted by N⁶-nitro-L-arginine methyl ester (L-NAME). Moreover, to delineate the role of NO in cardiac hypertrophy and failure, we measured cardiac hypertrophy, pulmonary congestion and plasma NO levels in a time course and analyzed the correlation among them.

Methods

Cell Culture

Rat neonatal ventricular myocytes were isolated and cultured as we described previously.14,15 Cardiomyocytes were exposed to either phenylephrine (PE) (10⁻⁴ mol/L), or isoproterenol (10⁻⁶ mol/L or other concentrations) for 24 hours in the presence or absence of celiprolol (Nippon Shinyaku Co Ltd), and the extent of increase in [³H]leucine uptake was examined. To test whether the inhibitory effect of celiprolol on protein synthesis induced by PE was mediated via release of NO, we used L-NAME in an attempt to suppress this effect.

TAC Model and Protocols

All procedures were in accordance with institutional guidelines for animal research. Mice (C57BL/6, male, 8 to 9 weeks old, weighing 19 to 23 g) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (25 mg/kg, intraperitoneal injection). The TAC model was created as we described previously.14,15

We treated the mice with saline (TAC group) or celiprolol at 100 mg · kg⁻¹ · d⁻¹ (PO, Celi low group) and 200 mg · kg⁻¹ · d⁻¹ (PO, Celi high group). L-NAME at 100 mg · kg⁻¹ · d⁻¹ in drinking water and L-NAME plus celiprolol 100 mg · kg⁻¹ · d⁻¹ PO were also used. No difference was found among all the experimental groups in age and body weight before surgery. Two to 3 mice in each group were used to measure the transstenosis pressure gradient. Tail-cuff blood pressure and heart rate were measured (BP-90A, Softron). Mice were euthanized at different time points after TAC, and morphometric analysis was performed. Cell surface area and myocardial and perivascular fibrosis measurements were performed using 3 hearts in each group as described previously.15,16

Echocardiographic Assessment

Transthoracic echocardiography was performed with a Sonos 4500 and a 15-6 MHz transducer (Philips). Mice were lightly anesthetized with 2.5% Avertin (0.06 mL/10 g) and fixed. When they had recovered consciousness (~10 minutes), good 2D short-axis views of the left ventricle (LV) were obtained for guided M-mode measurements of the LV posterior wall thickness, LV end-diastolic diameter, and LV end-systolic diameter.

NO Measurement

The plasma levels of nitrite and nitrate (NOx) were measured as previously described.17 Cardiac myocardial protein level of eNOS, inducible NOS (iNOS), neuronal NOS (nNOS), and eNOS phosphorylated activity were checked by use of Western blot analy-
Expression of these proteins was detected by use of the diaminobenzidine method and quantified by densitometry.

**RNA Analysis**

Assessment of the cardiac gene expression for natriuretic peptide precursor type B (BNP), protein inhibitor of NOS (PIN), and sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2) was performed by real-time polymerase chain reaction (RT-PCR). The Quantitect SYBR Green RT-PCR kit (Qiagen) was used to perform amplifications with the One-step protocol as described by the manufacturer.

**Statistical Analysis**

For all statistical tests, multiple comparisons were performed by 1-way ANOVA with the Tukey-Kramer exact probability test. The least-squares method was used for linear correlation between selected variables. Data were reported as the mean ± SEM. A value of P < 0.05 was considered statistically significant.

**Results**

**Celiprolol Inhibits Myocyte Protein Synthesis Induced by Either Isoproterenol or PE**

Isoproterenol, a nonselective β-AR agonist, increased myocyte protein synthesis. The selective β1-AR antagonist celiprolol suppressed the enhancement by 70% (Figure 1A), suggesting that it is predominantly β1-ARs that mediate the isoproterenol-induced increase in protein content. As shown in Figure 1B, celiprolol also reduced the increase in protein synthesis stimulated by PE, indicative of an antihypertrophic effect independent of β1-AR blockade. PE increased cell size, whereas celiprolol significantly suppressed the increase (Figure 1, C and D). The inhibition of eNOS by L-NAME partially abolished the antihypertrophic effect of celiprolol on the PE-induced increase in protein synthesis (Figure 1, B–D).

**Celiprolol Attenuates Pathological Cardiac Hypertrophy**

Consistent with the results in vitro, low-dose celiprolol markedly attenuated cardiac hypertrophy 4 weeks after TAC (Figure 2, A and B). Histological examination also revealed that the extent of myocyte hypertrophy (Figure 3 A–C) was reduced and both myocardial and perivascular fibrosis were ameliorated in celiprolol-treated mice (Figure 2, C and D and Figure 3, D and E). The same results were achieved at a higher dose of celiprolol.

The results of hemodynamics and echocardiography measured just before the animals were killed are shown in the Table. Celiprolol reduced heart rate significantly but did not affect tail-cuff systolic blood pressure. The LV wall became thinner in celiprolol-treated mice than in TAC mice. Between the 2 celiprolol groups, no significant differences were noted in hemodynamic and echocardiographic findings, although there was a tendency for the LV cavity to be smaller, the LV wall to be thicker, and LV fractional shortening to be higher in mice treated with the higher dose (data not shown).

We recorded echocardiographs and hemodynamics for all mice before surgery and found no differences among the 6 groups (data not shown). The transstenosis pressure gradient measured in 2 to 3 mice in each group showed no significant difference (Table).
L-NAME Partially Abolishes the Antihypertrophic Effect of Celiprolol

Treating TAC mice with celiprolol plus L-NAME decreased but did not completely abolish the antihypertrophic effect of celiprolol, whereas L-NAME alone did not further increase cardiac hypertrophy in the TAC mice (Figure 2, A–D).

Celiprolol Prevents Transition to Heart Failure

TAC induced congestive heart failure, manifested by increases in lung weight and reductions in fractional shortening. Compared with values in sham-operated mice, the ratio of lung weight to body weight (LW/BW) increased by ∼84% in TAC mice but only by 27% in low-dose celiprolol–treated mice (Figure 4, A and B), respectively. LV fractional shortening was also significantly higher in celiprolol–treated mice than TAC mice (Figure 4C). The same results on heart function were achieved at the higher dose of celiprolol. Furthermore, L-NAME markedly counteracted these beneficial effects produced by celiprolol.

NO Production Is Associated With Cardiac Hypertrophy and Heart Failure

To further clarify the role of NO in cardiac hypertrophy and heart failure, we evaluated the time course of cardiac hypertrophy, heart failure, and the plasma levels of NOx. Heart weight-to-body weight ratio (HW/BW) and LW/BW were increased time-dependently from 1 to 4 weeks (Figure 5, A and B), whereas plasma levels of NOx were decreased time-dependently (Figure 5C). Significant negative linear correlations were found between HW/BW and plasma NOx level (D) and between LW/BW and plasma NOx level (E). Numbers of mice in Sham, TAC 1 week (1w), TAC 2w, TAC 4w, and TAC + Celi groups are 6, 5, 5, 7, and 6, respectively. *P<0.01, †P<0.05 vs sham; ‡P<0.01 vs TAC 4w.

Celiprolol Increases eNOS Protein and Its Activity

Myocardial protein levels of eNOS and its phosphorylation were decreased in TAC mice; celiprolol treatment increased eNOS and its phosphorylation significantly (Figure 6, A and B). We also tried to check the protein levels of iNOS and nNOS in myocardium with Western blot analysis and found that they were hardly detectable (data not shown), which is similar to previous reports.16,20

Celiprolol Induces Altered Transcriptional Expression

As shown in Figure 7, quantitative real-time PCR demonstrated that celiprolol treatment decreased levels of the hypertrophic molecular marker BNP, downregulated expression of PIN, and upregulated expression of SERCA. These changes further support our findings in vitro and in vivo that celiprolol attenuates cardiac hypertrophy and improves heart function partially by increasing the release of NO.

Discussion

Our study demonstrated that celiprolol attenuates cardiac myocyte hypertrophy both in vitro and in vivo and halts the
process leading from hypertrophy to heart failure. These effects are mediated by a selective $\beta_1$-AR blockade and NO-dependent pathway. In this study, we found a negative correlation between plasma NOx and cardiac remodeling, indicating that NO production diminished time-dependently in the process of cardiac remodeling, which is in agreement with previous reports.21-24 Celiprolol inhibits cardiac remodeling via attenuating myocardial hypertrophy, decreasing cardiac fibrosis, and ameliorating pulmonary edema; this process is mediated, at least in part, by augmented eNOS signaling, because L-NAME partially abrogated this effect. Previous studies also demonstrated that celiprolol inhibited cardiac fibrosis,25 and overexpression of eNOS improved both cardiac and pulmonary function.26 Although no significant difference was noted in tail-cuff pressure between the TAC mice treated with L-NAME + celiprolol and celiprolol alone, a vascular disparity really existed. Our echocardiographic data demonstrated an increase of LV chamber and myocardial hypertrophy and a decrease of LV ejection fraction in L-NAME + celiprolol-treated mice, indicating an increase of LV resistance.

The main finding in this study is that celiprolol inhibits the myocyte hypertrophy via activation of the NO signaling pathway. Experimental27 and clinical28 studies have also shown that activation of the NO-dependent pathway attenuates the effect of submaximal concentrations of catecholamines in the heart. These findings serve to support the results of the present study that PE-induced myocyte hypertrophy can be inhibited by celiprolol via the NO signaling pathway. Because stimulation of the adrenergic system is also involved in the pathogenesis of LV hypertrophy induced by pressure overload,29 activation of the NO-dependent pathway by celiprolol should make some contribution to the attenuation of hypertrophy shown in our in vivo study.

Furthermore, ours is the first evidence that celiprolol can downregulate PIN, one of the potential mechanisms for the antihypertrophic effect of celiprolol. PIN has attracted significant attention for its potential importance as a regulator of nNOS,30 and it also inhibits another 2 types of NOS isoenzymes (eNOS and iNOS).31 The mechanisms for the linkage of $\beta_1$-blockade to eNOS upregulation have been clarified by other investigators. Kalinowski et al10 demonstrated that some $\beta$-blockers augment NO production via activation of ATP efflux, with consequent stimulation of P2Y purinoceptor. Celiprolol was also reported to activate eNOS through the PI3K-Akt signaling pathway.18 Conversely, the impact on PIN may be a specific effect of $\beta_1$-blockers. It was reported that an association exists between nNOS decrease in the hypothalamus and enhanced sympathetic tone in rats with heart failure.32 Because $\beta_1$-blockers are known to reduce sympathetic tone, it seems plausible that upregulation of nNOS via inhibiting PIN may make some contribution to the sympathetic inhibition of $\beta_1$-blockers.

We showed in this study that celiprolol completely inhibited the decrease in myocardial eNOS protein levels, whereas its inhibition was partial in plasma NOx levels. This discrepancy might be likely. In the chronic heart failure state, in addition to the reduced myocardial eNOS expression, platelet-derived NO production33 and nNOS expression in neural tissue was also reported to be decreased.34,35 However, there seems to be no evidence to show that celiprolol can completely inhibit the decrease in platelet-derived NO production or nNOS expression in neural tissue, which may be one reason that celiprolol only partially prevented the plasma NOx decrease in TAC mice.

Considering its excellent level of safety,36 the favorable effect on serum lipid metabolism and insulin sensitivity in chronic heart failure,37 the benefit from the blockade of $\beta_1$-ARs confirmed in large-scale clinical trials,38 and its ability to activate the eNOS signaling pathway, a further
large-scale and long-term clinical trial is needed to provide conclusive evidence for the effect of celiprolol in patients with chronic heart failure, especially those with hypertension, cardiac hypertrophy, diabetes mellitus, or hyperlipidemia.

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