Partial Agonist Activity of Bucindolol Is Dependent on the Activation State of the Human β1-Adrenergic Receptor

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Background—In contrast to other β-blockers, bucindolol has failed to reduce mortality in patients with chronic heart failure. It is currently debated whether this is due to partial agonist activity of this agent. We investigated whether conflicting results previously reported concerning the intrinsic activity of bucindolol can be explained by species differences or by different activation states of β-adrenergic receptors (β-ARs) in the respective tissues.

Methods and Results—On isolated right atria from transgenic mice with cardiac overexpression of human β1-ARs, bucindolol led to a greater increase in beating frequency (P<0.05) compared with wild-type mice. The increase amounted to 47% of the effect of xamoterol and was blocked by propranolol. On isolated, electrically stimulated, left ventricular muscle-strip preparations from failing human myocardium, bucindolol did not change the force of contraction under control conditions. In myocardial preparations pretreated with metoprolol (30 μmol/L, 90 minutes, subsequent washout), bucindolol significantly increased the force of contraction (P<0.001 vs control). In nonfailing atrial myocardium, isoproterenol pretreatment (1 μmol/L, 60 minutes) abolished the positive inotropic effect of xamoterol that was present under control conditions (P<0.05 vs control). The inotropic effects of bucindolol or xamoterol were inversely correlated to the inotropic response to forskolin in the respective specimens (r = −0.75 and −0.74, respectively; P<0.005).

Conclusions—We conclude that bucindolol is a partial agonist at the human β1-AR. In human failing myocardium, its partial agonist activity is masked by increased activation states of β-ARs and is unmasked after in vitro pretreatment with metoprolol. Thus, the partial agonist activity of bucindolol is dependent on the activation state of the human β1-AR.

Key Words: receptors, adrenergic, beta ■ inotropic agents ■ heart failure ■ genetics

Beta-adrenergic receptor (β-AR)–blocking agents are currently regarded as standard therapy for chronic heart failure.1 Clinical studies, however, have demonstrated differences among β-blockers. Although carvedilol, metoprolol, and bisoprolol have significantly reduced mortality (reviewed in Bristow1), xamoterol2 and, most recently, bucindolol3 have shown adverse or neutral effects on mortality. The reason why β-blockers differ in their efficacy to reduce mortality in heart failure patients might be related to different intrinsic activities of β-blockers. β-Blockers can be categorized as inverse agonists, neutral antagonists, or partial agonists.4 Whereas inverse agonists reduce the basal activation state of β-ARs, neutral antagonists bind to the receptor without changing its activation state. In contrast, partial agonists increase the activation state of β-ARs, leading to coupling of the receptor to the stimulatory G protein (Gs) with subsequent stimulation of adenyl cyclase. Recent data from both in vitro and in vivo experiments5–7 suggest that continuous stimulation of cardiac β1-ARs is detrimental to the heart. Xamoterol causes β1-adrenergic stimulation in animal8,9 and human10 tissue, and this partial agonist activity is believed to be the culprit for the disappointing performance of xamoterol in heart failure.2

Whereas it has been clearly demonstrated that xamoterol exerts partial agonist activity at the human β1-AR,9,10 the situation for bucindolol is less clear. Although partial agonist activity has been described in several animal species,8,11 studies investigating the effect of bucindolol on human tissue led to conflicting results. Some groups did not observe partial agonist activity,12,13 whereas we and others observed partial agonist activity of bucindolol in human myocardium.14,15 Partial agonist activity of bucindolol, however, was not constant in human failing myocardium and varied, depending on the respective tissue investigated.14

To determine whether the putative partial agonist activity of bucindolol is species dependent, we compared the effect of bucindolol on atria from wild-type mice (murine β1-AR) and transgenic mice with cardiac-specific overexpression of the...
human β₁-AR. Furthermore, we sought to investigate reasons underlying the great variability of results reported for the intrinsic activity of bucindolol in human myocardium. Determination of partial agonist activity in human myocardium is complicated because of the different degrees of β-adrenergic desensitization in chronic heart failure. Desensitization of β-ARs has been shown to convert partial agonist activity of β-AR ligands to inverse agonist activity in Sf9 insect cells. Thus, we hypothesized that in human failing myocardium, partial agonist activity of bucindolol in vitro might be masked by desensitization of the β-adrenergic signaling cascade in vivo. To elucidate this issue, we pretreated human failing myocardium with either the agonist isoproterenol or the inverse agonist metoprolol to achieve further desensitization or resensitization of β-ARs, respectively.

Methods

Tissue
The generation of transgenic mice overexpressing the human β₁-AR under the control of the α-myosin heavy-chain promoter has been described previously. Male wild-type and transgenic littermates derived from crosses of a heterozygous, transgenic (line β₁TG4) and wild-type mice were studied at an age of 2 to 4 months. Human ventricular myocardium was obtained from 4 male patients with heart failure due to ischemic or dilated cardiomyopathy (n=2/2) during heart transplantation (mean age, 53±5 years). We used 17 male, 2 female myocardium was obtained from 19 patients undergoing cardiac arterial bypass grafting or valve replacement (n=15/4; 17 male, 2 female; mean±SEM age, 65±4 years; mean±SEM left ventricular ejection fraction, 64±4%).

Functional Studies
Mouse heart experiments were performed as described previously. In brief, mouse atria were placed in a carbonated, 35°C tissue bath with modified Tyrode’s solution. Before addition of the pharmacological substances (1 µmol/L, unless indicated otherwise), the bath solution was changed 5 times with 5-minute intervals between the individual washing steps. The atria were allowed to contract spontaneously. The basal frequencies were comparable and not significantly different in the groups studied in Figure 1. The respective values were 402±14 beats per minute (bpm) in the xamoterol group (n=6), 397±15 bpm in the bucindolol group (n=9), and 363±24 bpm in the isoproterenol group (n=4). Propranolol (1 µmol/L) was added 10 minutes before the addition of bucindolol. The median effective concentrations (EC₅₀) for bucindolol, xamoterol, and isoproterenol were 59±30, 84±4, and 5.4±1.2 nmol/L, respectively.

Experiments on human left ventricular and right atrial myocardium were performed as described previously. The bath solution was maintained at 37°C, pH 7.4, and aerated with 95% O₂ and 5% CO₂. Muscles were stretched to the length at which the force of contraction was maximal.

Protocol 1
Failing left ventricular myocardium (baseline) was preincubated with isoproterenol (1 µmol/L), metoprolol (30 µmol/L), or vehicle for 90 minutes. After a 4-fold washout of pretreatment agents (pretreatment), muscles were incubated with forskolin (0.3 µmol/L). Bucindolol was then applied in cumulative doses (0.001 to 1 µmol/L). Each concentration was allowed to equilibrate for 30 minutes.

Protocol 2
Human atrial myocardium was exposed to isoproterenol (1 µmol/L), metoprolol (30 µmol/L), or vehicle for 60 minutes. After a 4-fold washout of pretreatment agents, forskolin (0.1 µmol/L) was added. Hereafter, xamoterol was added in cumulative concentrations (0.001 to 1 µmol/L). After removal of xamoterol and forskolin, isoproterenol was added in cumulative concentrations (0.001 to 1 µmol/L).

Protocol 3
Human atrial myocardium was pretreated as in protocol 2. After removal of pretreatment agents, forskolin was added in cumulative concentrations (0.01 to 10 µmol/L). After maximum force was maintained at 37°C, pH 7.4, and aerated with 95% O₂ and 5% CO₂. Muscles were stretched to the length at which the force of contraction was maximal.

Figure 1. Effect of bucindolol on spontaneous right atrial beating frequency in β₁-AR–transgenic mice and β₁-AR phosphorylation in HEK293 cells. A, Representative original recording of a single experiment: bucindolol (1 µmol/L) caused rapid and pronounced increase of spontaneous beating frequency of isolated right atria from transgenic mice with cardiac-specific overexpression of human β₁-AR. B, Cumulative data: chronotropic effects of bucindolol (Buc, n=9), xamoterol (Xam, n=6), and isoproterenol (Iso, n=4) in β₁-AR–transgenic (TG) and wild-type (WT) mice. Coincubation with propranolol (Prop, n=7) abolishes bucindolol-induced increase of beating frequency. **P<0.001 vs TG unstimulated (WT/TG, respectively); †P<0.05 vs WT stimulated, ‡P<0.01 vs TG stimulated. C, Original autoradiograph (insert) and cumulative data (n=4 to 8) of β₁-AR phosphorylation in HEK293 cells. Hemagglutinin–tagged human β₁-AR was immunoprecipitated with anti–hemagglutinin antibody, and radioactivity incorporated was determined with PhosphorImager analysis after polyacrylamide gel electrophoresis. *P<0.05 vs control (C).
reached, forskolin was removed by replacing the bath solutions at least 4 times. Finally, isoproterenol was added in cumulative concentrations (0.001 to 10 μmol/L).

**Intact-Cell Phosphorylation**

To analyze agonist-mediated phosphorylation of the β₁-AR, a hemagglutinin tag was added to the human β₁-AR at its N-terminus, and the construct was transfected into HEK293 cells with the calcium phosphate method. Forty hours after transfection, the cells were loaded with 140 μCi/mL [³²P]orthophosphate in phosphate-free Dulbecco’s modified Eagle’s medium for 2 hours. Labeled cells were stimulated with isoproterenol or bucindolol (10 μmol/L) for 5 minutes and solubilized in lysis buffer containing (in mM, unless indicated otherwise) Tris-HCl 50 (pH 7.4), NaCl 300, EDTA 5, NaN₃ 0.01%, NaF 50, Na₃PO₄ 5, Na₂VO₃ 1, Triton X-100 1%, phenylmethylsulfonyl fluoride 1, and iodoacetamide 10; β₁-ARs were immunoprecipitated with 12CA5 antibodies (Roche Bio-Sciences) directed against the hemagglutinin tag. Immunoprecipitated receptors were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and phosphoimaging (PhosphorImager) analysis.

**Statistical Analysis**

Average data are presented as mean±SEM. Statistical analyses (t tests for pairwise comparisons or ANOVA) were used with the InStat software package (GraphPad). Differences were considered significant when \( P<0.05 \). Linear and nonlinear regression analyses and calculation of EC₅₀ values were performed with GraphPadPrism (GraphPad).

**Results**

To determine whether the intrinsic activity of bucindolol on β₁-ARs was species dependent, experiments were performed in murine and human myocardium. First, we compared the effect of bucindolol and 2 well-characterized β₂-adrenergic agonists on the spontaneous beating frequency of isolated right atria from wild-type and transgenic mice overexpressing the human β₁-AR. Addition of bucindolol at a concentration of 1 μmol/L resulted in a marked and rapid increase of spontaneous right atrial beating frequency, which was significantly greater in transgenic compared with wild-type mice (Figure 1A and 1B). Bucindolol induced a mean rise of right atrial beating frequency by 78 bpm in transgenic animals (Figure 1A and 1B). Bucindolol induced a mean rise of right atrial beating frequency by 78 bpm in transgenic animals, whereas isoproterenol increased force by 11 bpm in transgenic animals (Figure 1B). The inotropic effect of bucindolol and 2 well-characterized β₂-adrenergic agonists on the spontaneous beating frequency of isolated right atria from wild-type and transgenic mice overexpressing the human β₁-AR. Together with recent data demonstrating that after isoproterenol pretreatment, forskolin concentration-response curves were shifted to the left (increased sensitivity, Figure 3A and 3B), whereas isoproterenol variability in the inotropic effects of bucindolol in previous studies on human myocardium was due to different sensitization states of β₁-ARs, failing myocardium was pretreated with isoproterenol, metoprolol, or vehicle for 90 minutes. The baseline force of contraction was not different among the 3 groups (Table 1). Isoproterenol increased force by 122±26% (\( P<0.05 \)), and metoprolol reduced force by 68±3% (\( P<0.001 \)). After washout of the pretreatment drugs, forskolin (0.3 μmol/L) was added to the organ bath to facilitate the coupling of Gs to adenylyl cyclase. Under these conditions, bucindolol changed the force of contraction in neither untreated nor isoproterenol-pretreated muscles. In contrast, in muscles pretreated with metoprolol, bucindolol concentration-dependently increased the force of contraction by a maximal 43±9% at 1 μmol/L (\( P<0.005 \)), indicating partial agonist activity (Figure 2A).

To test whether these findings were specific for bucindolol or might also apply to other partial agonists, we studied the effect of different pretreatments on the inotropic effects of xamoterol in human myocardium from patients without heart failure. As indicated in Table 2, the baseline force of contraction was equal in the 3 groups. As expected from the strong partial agonist xamoterol, this agent exerted a positive inotropic effect in control as well as metoprolol-pretreated myocardium (Figure 2B). In contrast, in isoproterenol-pretreated myocardium, the positive inotropic effect of xamoterol was completely abolished (Figure 2B). Interestingly, the inotropic responses to xamoterol and bucindolol showed a close inverse correlation with the inotropic effects induced by forskolin in the respective tissue specimens (xamoterol, \( r=-0.74, P<0.0001 \), Figure 2C; bucindolol, \( r=-0.75, P<0.005 \), not shown). Because the sensitivity of adenylyl cyclase to forskolin stimulation is dependent on the amount of precoupled Gₛ₁₈,₁₉ this might indicate that the response to a partial agonist is dependent on the activation state of the receptor.

To further elucidate this issue, we sought to determine the activation and sensitization states of β₂-ARs after the respective pretreatment protocols in human myocardium. Figure 3 illustrates that after isoproterenol pretreatment, forskolin concentration-response curves were shifted to the left (increased sensitivity, Figure 3A and 3B), whereas isoproterenol

<table>
<thead>
<tr>
<th>TABLE 1. Force of Contraction in Human Ventricular Myocardium From Patients With Heart Failure (Protocol 1)</th>
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<tbody>
<tr>
<td><strong>Force, mN</strong></td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Baseline</td>
</tr>
<tr>
<td>After pretreatment</td>
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<tr>
<td>After forskolin</td>
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<tr>
<td>After bucindolol</td>
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</table>

Failing left ventricular myocardium was preincubated with isoproterenol (1 μmol/L), metoprolol (30 μmol/L), or vehicle for 90 minutes. After 4-fold washout of pretreatment agents, muscles were incubated with forskolin (0.3 μmol/L). Bucindolol was then applied in cumulative doses (0.001–1 μmol/L), \( n=xy \) samples from \( n=y \) hearts. \( *P<0.05 \) vs baseline (paired \( t \) test).
concentration-response curves were slightly shifted to the right (decreased sensitivity, Figure 3C and 3D). In contrast, after metoprolol pretreatment, forskolin sensitivity was significantly decreased and isoproterenol sensitivity increased (Figure 3A through 3D). This indicates that agonist-pretreated β-ARs are slightly desensitized but also more activated, whereas metoprolol-pretreated β-ARs are sensitized but less activated compared with control conditions.

### Discussion

The main results of the present study are that bucindolol displays partial agonist activity at the human β1-AR and that in human failing myocardium, this effect is masked by an increased activation state and desensitization of β-ARs and can be unmasked by in vitro resensitization of β-ARs by inverse agonist pretreatment.

Although stimulation of cardiac β-ARs represents the strongest mechanism to increase contractility of the heart, it is heart failure, chronic stimulation of the β-adrenergic signal-
dolol increased β-AR phosphorylation. Because only β-ARs in the activated conformation are a substrate for receptor phosphorylation by β-AR kinase,21,22 this clearly indicates that the partial agonist activity of bucindolol translates into postreceptor events involving the activation of adenyl cyclase and cAMP-dependent activation of kinases, ie, β-AR kinase and protein kinase A.22 Phosphorylation of the β-AR is a prerequisite for receptor desensitization by uncoupling from G, and (in the long term) downregulation by sequestration and internalization.22 This is in concert with results from Asano et al,23 who observed downregulation of β-ARs in chick heart myocytes after bucindolol treatment for 24 hours.

The studies by Chidiac et al16 suggest that partial agonist activity is best discovered in cells with sensitized β-ARs. Accordingly, when human myocardium was pretreated with the inverse agonist metoprolol followed by washout, an unambiguous partial agonist activity of bucindolol was observed. In contrast, in isoproterenol-treated or untreated myocardium, this partial agonist effect was absent. Similarly, agonistic effects of the (stronger) partial agonist xamoterol were observed in metoprolol-treated or untreated human atrial myocardium but not in isoproterenol-pretreated tissue.

The effects exerted by pretreatment are most likely induced by slowly reversible alterations in the activation and desensitization states of the β-adrenergic signal transduction system. The desensitization state of β-ARs—which is thought to be due to phosphorylation and β-arrestin binding—is best assessed by measuring agonist concentration-response curves. These experiments revealed that pretreatment with metoprolol sensitized and pretreatment with isoproterenol desensitized the β-AR-Gs–adenylyl cyclase system. The fact that control human myocardium had an intermediate position in these experiments indicates that the β-adrenergic system is partially desensitized in these samples.

The pretreatment also affected the responsiveness to forskolin. Forskolin directly activates adenyl cyclase; however, it has a higher efficacy and potency when Gs is precoupled to the adenyl cyclase complex.18,19 Accordingly, high-affinity [3H]forskolin binding can be applied to assay Gs–adenylyl cyclase complexes.19 In cell systems overexpressing the β-AR, increased basal [3H]forskolin binding indicates that Gs–adenylyl cyclase complexes reflect constitutive (nonliganded) receptor activity.24 In our functional experiments, the potency of forskolin (EC50 values) was dependent on the type of pretreatment. This indicates that pretreatment affects the amount of Gs coupled to adenyl cyclase and thus, the activation state of the system.

Pretreatment affected the responsiveness to isoproterenol and to forskolin in opposite directions. Isoproterenol pretreatment desensitized β-ARs (decreased potency of isoproterenol) but activated the Gs–adenylyl cyclase system (increased potency of forskolin). In contrast, metoprolol pretreatment

### TABLE 3. Force of Contraction in Human Atrial Myocardium (Protocol 3)

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Control Pretreatment (n=9)</th>
<th>Metoprolol Pretreatment (n=9)</th>
<th>Isoproterenol Pretreatment (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Force, mN</td>
<td>5.5±1.5</td>
<td>6.1±2.1</td>
<td>5.3±1.4</td>
</tr>
<tr>
<td>After pretreatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.9±1.3*</td>
<td>1.1±0.5†</td>
<td>3.7±1.0†</td>
</tr>
<tr>
<td>Fmax (forskolin)</td>
<td>6.9±1.4*‡</td>
<td>7.7±1.9‡</td>
<td>8.0±0.9‡</td>
</tr>
<tr>
<td>Fmax (iso)</td>
<td>6.9±1.5‡</td>
<td>7.8±1.9</td>
<td>7.7±0.9‡</td>
</tr>
</tbody>
</table>

Human myocardium was pretreated as in protocol 2. After removal of pretreatment agents, forskolin was added in cumulative concentrations (0.01–10 µmol/L). After maximum force was reached, forskolin was removed by exchanging bathing solutions at least 4 times. Finally, isoproterenol was added in cumulative concentrations (0.001–10 µmol/L). Fmax indicates maximum force after forskolin and isoproterenol, respectively.

*P<0.005, †P<0.05 vs baseline; ‡P<0.001, §P<0.01 vs after pretreatment (paired t test).
increased the potency of isoproterenol but decreased the potency of forskolin. These effects explain the inverse correlation between partial agonist effects of both bucindolol and xamoterol and the potency of forskolin in individual human myocardial samples (Figure 2C). It suggests that partial agonist effects are more readily detected in samples with a low level of β-AR pretreatment (as indicated by low sensitivity to forskolin).

These observations might explain the highly variable results that were obtained in earlier studies with partial β-AR agonists in general and with bucindolol in particular on human myocardium. Especially in failing myocardium, detection of partial agonist activity might be critically hampered by pronounced activation and desensitization of β-ARs due to chronic stimulation with endogenous catecholamines. This is supported by the fact that in failing myocardium, maximal stimulation of β-ARs with isoproterenol did not change the inotropic effect of bucindolol compared with control conditions. In stark contrast, under desensitized conditions (ie, metoprolol pretreatment), the partial agonist activity of bucindolol could be unmasked in this tissue. Bucindolol is structurally similar to carvedilol. However, despite these similarities, carvedilol never displayed partial agonist activity, even in systems extremely sensitive to test this property, including studies from our own laboratory and experiments in the pithed rat. This is in agreement with studies on human myocardium, where carvedilol is equivalently classified as an inverse agonist.

Regarding the ongoing debate on why bucindolol is less effective than other β-blockers in reducing mortality in heart failure patients, it should be noted that in human failing myocardium, the partial agonist activity of bucindolol was observed only after metoprolol pretreatment. However, the extensive sympathetic activation in heart failure might be mimicked best by isoproterenol pretreatment—a condition wherein partial agonism was observed for neither bucindolol nor xamoterol. In contrast, the therapeutically beneficial compounds metoprolol and bisoprolol behave as inverse agonists in a broad range of experimental tissues and conditions.

The present data add further evidence to the hypothesis that the initial activation state of human β-ARs rather than the species investigated determines the intrinsic activity of a β-AR ligand. Furthermore, these observations provide an explanation for different results obtained in different studies and experimental systems concerning the intrinsic activity of bucindolol. We conclude that bucindolol is a partial agonist at the human β1-AR and that detection of partial agonist activity in human failing myocardium is dependent on the activation state of the human β1-AR.

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