Specific $\beta_2$AR Blocker ICI 118,551 Actively Decreases Contraction Through a $G_i$-Coupled Form of the $\beta_2$AR in Myocytes From Failing Human Heart

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**Background**—We have observed direct (noncatecholamine-blocking) negative inotropic effects of the selective $\beta_2$-adrenoceptor (AR) antagonist ICI 118,551 in myocytes from failing human ventricle. In this study we characterize the effect in parallel in human myocytes and in myocytes from animal models where $\beta_2$ARs or $G_i$ proteins are overexpressed.

**Methods and Results**—Enzymatically isolated, superfused ventricular myocytes were exposed to $\beta_2$AR agonists and antagonists/inverse agonists, and contraction amplitude was measured. ICI 118,551 decreased contraction in ventricular myocytes from failing human hearts by 45.3±4.1% (n=20 hearts/31 myocytes, P<0.001) but had little effect in nonfailing hearts (4.9±4.4%, n=5 myocytes/3 hearts). Effects were significantly larger in patients classified as end-stage. Transgenic mice with high $\beta_2$AR number and increased $G_i$ levels had normal basal contractility but showed a similar negative inotropic response to ICI 118,551. Overexpression of human $\beta_2$AR in rabbit myocytes using adenovirus potentiated the negative inotropic effect of ICI 118,551. In human, rabbit, and mouse myocytes, the negative inotropic effects were blocked after treatment of cells with pertussis toxin to inactivate $G_i$, and overexpression of $G_ia_2$ induced the effect de novo in normal rat myocytes.

**Conclusions**—We hypothesize that ICI 118,551 binding directs the $\beta_2$AR to a $G_i$-coupled form and away from the $G_i$-coupled form (ligand-directed trafficking). ICI 118,551 effectively acts as an agonist at the $G_i$-coupled $\beta_2$AR, producing a direct negative inotropic effect. Conditions where $\beta_2$ARs are present and $G_i$ is raised (failing human heart, TG$\beta_2$ mouse heart) predispose to the appearance of the negative inotropic effect. *(Circulation. 2002;105:2497-2503.)*

**Key Words:** myocytes ■ receptors, adrenergic, beta ■ contractility ■ heart failure
those of nontransgenic littermate controls. Inhibitory G-protein (G_i) was found to be increased, and pertussis toxin treatment, which inactivates G_i, restored both β2AR responses and raised basal myocyte shortening. Contrary to predictions, we continued to observe inverse agonism in myocytes from these adapted mice, even though basal activity was not increased.

Even more surprisingly, we have seen a similar effect of ICI 118,551 in myocytes from failing human heart. β2ARs are present in myocytes from failing human heart and can contribute to increases in contraction after exposure to isoproterenol, but they are far from the excess in the TGβ2-NHLI mice. Inverse agonism attributable to reduction of tonically activating R*, secondary to excess β2ARs, is therefore unlikely. However, failing human heart does have in common with TGβ2-NHLI mice an increased level of G_i.

In the present study, we have characterized the negative inotropic effect of β2AR antagonists in myocytes from failing human heart, TGβ2-NHLI mice, and other models of β2AR or G_i overexpression. The results lead us to hypothesize that β2AR blockers can act as agonists at a G_i-coupled form of the β2AR, producing a direct negative inotropic effect without influencing cAMP levels. This form of stimulus trafficking or ligand-directed trafficking of receptors between different G-proteins has been described for β2AR and other G-protein coupled receptors (GPCRs) in several tissues.

**Methods**

**Contraction Measurements in Myocytes**

Tissue was obtained from failing and nonfailing human ventricular myocardium from TGβ2-NHLI mice and littermate controls, with transgenic status confirmed as previously described, and from normal rats (Sprague-Dawley; Charles River, Margate, UK) and rabbits (New Zealand white; Charles River, Margate, UK). Ethical procedures followed were in accordance with institutional guidelines in all cases. Isolation of adult myocytes was performed as described for human, mouse, rat, and rabbit myocardium. Myocytes in a bath volume of 200 μL were superfused with Krebs-Henseleit (KH) solution at 2 mL/min, 37°C. Composition of the KH solution was (in mmol/L) NaCl 119, KCl 4.7, MgSO4 0.94, KH2PO4 1.2, NaHCO3 25, and glucose 11.5, gassed with 95% O2/5% CO2 to bring the pH to 7.4. Calcium concentrations were adjusted to give contraction amplitudes 50% to 75% of maximum (8 mmol/L human, 4 mmol/L mouse, rat, and rabbit) to increase accuracy of measurements of negative inotropic effects. Cells were paced at 0.2 Hz (human), 0.5 Hz (rat, rabbit), or 1 Hz (mouse) using field stimulation. Contraction was measured using a video-motion detector as previously described. ICI 118,551 and alprenolol were superfused over stably contracting myocytes and remained in contact until the decrease in contraction had reached a steady state, usually after 5 minutes. Only myocytes showing full reversal of the negative inotropic effect on washout of ICI 118,551 were used in the analysis.

**cAMP Measurement**

cAMP was measured using an ELISA kit (Amersham). Freshly isolated myocytes were incubated for 5 minutes at 37°C in KH, in the presence and absence of 1 μmol/L ICI 118,551. Cells were pelleted by centrifugation and quickly disrupted using the lysis buffer.
supplied with the ELISA kit. Protein was measured using the Bradford reagent.

**Pertussis Toxin Treatment**
Freshly isolated myocytes were incubated with pertussis toxin (PTX) (1.5 μg/mL) at 35°C for 2 to 3 hours for mouse and up to 6 hours for human. For treatment of rabbit myocytes cultured with Adv.β1R, pertussis toxin was added with the virus, and cells were cultured for an additional 24 hours. After PTX treatment, both PTX-treated and nontreated cells were kept at room temperature until the time of experiments.

**Infection of Myocytes With Adenovirus**
E1-deficient-type adenovirus was constructed to express the human β2AR (Adv.β2AR) or Gαi3 plus green fluorescent protein (Adv.Gαi3-GFP). The control virus, Adv.GFP, was a gift from Drs. Hajjar and del Monte at the Cardiovascular Research Center (Massachusetts General Hospital, Charlestown, Mass). Rat or rabbit myocytes were cultured as previously described. Adenovirus expressing the required protein was initially added to each well, containing 2×10^4 myocytes in 2 mL medium at 3.5×10^5 PFU for Adv.Gαi3-GFP or 10^7 to 10^8 for Adv.β2AR and Adv.GFP. Gαi3 overexpression was confirmed by immunoblotting techniques as previously described.

**Materials**
Pertussis toxin, (-)-isoproterenol, (-)-alprenolol, and CGP 20712A were obtained from Sigma, and BRL 37344, ICI 118,551, and ICI 215,001 were from Tocris.

**Statistical Analysis**
Numbers are quoted for myocytes and hearts, but for statistical purposes, results for several cells from one heart were pooled. Differences between means were determined using a paired or unpaired Student’s t test with a level of P<0.05 taken to be statistically significant. For groups of 3 or more, one-way ANOVA was used with the Fisher test for pair-wise comparison of means.

**Results**

**Negative Inotropic Effects of β2AR Antagonists in Human and Mouse Ventricular Myocytes**
Contracting myocytes were exposed to ICI 118,551, a highly specific antagonist/inverse agonist at β2ARs, at a concentration of 1 to 3 μmol/L. Significant decreases in amplitude were observed in myocytes from failing human heart and from TGβ2−NHLI mice (Figure 1). A small decrease was also observed in myocytes from littermate mice, although this was significantly less than that in TGβ2−NHLI (P<0.001), whereas no significant effect was seen in myocytes from nonfailing human heart (Figure 1). In failing human myocytes, effects started to become apparent at concentrations as low as 3 nmol/L ICI 118,551 (Figure 1). A representative trace in Figure 1 shows that the negative inotropic effect was rapidly and completely reversible. Alprenolol had a similar negative inotropic effect (data not shown).

In failing human heart, ICI 118,551 had significant effects on beat duration, with time-to-peak contraction and time-to-90% relaxation reduced compared with basal contraction (Figure 2). An expanded trace shows the marked effect on the second phase of relaxation (Figure 2).

**Relation of Inverse Agonist Effect to Patient Characteristics**
Effects were significantly larger in patients classified as end-stage, NYHA IV (48.8±4.9%, n=7) than patients in NYHA classes II and III (29.9±6.4%, n=6, P<0.05). Dividing by disease etiology did not reveal systematic differences, with reductions of 42.6±6.1% (n=8) for idiopathic dilated cardiomyopathy, 43.8±9.9% (n=5) for ischemic heart disease, and 40.3±8.6% (n=3) for congenital heart disease. Negative inotropic effects were also observed for patients with primary pulmonary hypertension, hypertrophic obstructive cardiomyopathy, and doxorubicin-related cardiomyopathy. Fewer left ventricular samples were studied, but the average reduction was also significant (33.5±3.9%, n=7 patients, P<0.001). Little difference was observed between patients who had been treated with β-blockers (40.6±6.2% decrease, n=6) and those untreated (45.8±5.9, n=10), although decreases were slightly greater in 2 patients receiving inotropes1 μmol/L (60% in each case).

**Subtype Selectivity for Negative Inotropic Effect of ICI 118,551**
Myocytes were challenged with ICI 118,551 in the presence of the highly selective β1AR antagonist CGP 20712A at a concentration (0.3 μmol/L) that would be expected to produce a 3- to 4-log unit shift in the effect of an agent acting at the β1AR (Figure 3). There was no effect of CGP 20712A itself on basal contraction, and the decrease in amplitude with ICI 118,551 was unaffected. This indicates that the negative inotropic effect was not mediated by the β1AR subtype. Isoproterenol, in the continued presence of the β1AR antagonist, was able to surmount the effects of ICI 118,551. The effects of ICI 118,551 were not mimicked by β2AR agonists in either mouse or human myocytes (data not shown).
Negative Inotropic Effect of ICI 118,551 Is Not cAMP-Related

cAMP concentrations were measured in myocytes from TGβ2-NHLI mice (Figure 4). Consistent with our previous observations in these animals, cAMP levels were not raised compared with control. Nor did ICI 118,551 significantly decrease cAMP in either control or TGβ2-NHLI myocytes (Figure 4). Measurement of cAMP is less reliable in human myocytes because of the variable number of viable cells. We therefore tested the effects on contraction of RpcAMPS, which competes with cAMP for binding to protein kinase A. RpcAMPS was used at a concentration (100 μmol/L) that could inhibit completely the response of the myocyte to isoproterenol.6,15 Basal contraction of myocytes from failing human heart could inhibit completely the response of the myocyte to isoproterenol.6,15 Basal contraction of myocytes from failing human heart or TGβ2-NHLI mice. Isoproterenol (Iso, 10 μmol/L) in the presence of β1AR blockade reversed negative effects of ICI 118,551. Human myocytes, n=7 for ICI/CGP, n=5 for Iso; mouse myocytes, n=7. Significantly different from control (Con), *P<0.05, **P<0.01; significantly different from CGP+ICI, ###P<0.001.

Involvement of Gι in the Negative Inotropic Effect of ICI 118,551

For both mouse and human myocytes, PTX treatment to inactivate Gι abolished the negative inotropic effect of ICI 118,551 (Figure 5). Conversely, overexpression of Gια2 induced the effect de novo in rat myocytes, which have a minor population of β1-ARs. In untreated rat myocytes, ICI 118,551 at inverse agonist concentrations had little effect on contraction. After culture for 48 hours with an adenoviral vector (Adv.Gια2,GFP), ICI 118,551 induced a significant negative response (Figure 6). Myocytes cultured for the same period without adenovirus or with adenovirus expressing GFP alone were unaffected.

Overexpression of β2AR in Rabbit Myocytes Enhances Negative Inotropic Effects of ICI 118,551

Rabbit myocytes were transfected with Adv.β2AR, and the total β2AR number was increased from 44.5±8.6 fmol/mg protein (mean±SEM, n=7) to 284.8±55.6 fmol/mg protein (P<0.002). In control cells, the percentage of β2ARs was 85.5±3.4%, and in β2AR-overexpressing cells, 16.3±5.6% (P<0.001). Unlike rat, normal untransfected rabbit myocytes showed a small depression of contraction in response to ICI 118,551 (Figure 7), which was similar in freshly isolated or cultured cells. β2AR-overexpressing myocytes had enhanced negative inotropic responses to ICI 118,551 (48.5±4.7% [n=19] decrease in contraction versus 18.5±2.2% [n=23] in cultured myocytes not exposed to Adv.β2AR [P<0.001]). Pertussis toxin treatment abolished the increase in response to ICI 118,551 brought about by β2AR overexpression (Figure 7).

Discussion

We have observed a substantial direct negative inotropic effect of the classical β2AR-selective antagonist/inverse agonist, ICI 118,551, on myocytes from failing human heart. In many aspects, this effect resembled that observed in myocytes from transgenic mice overexpressing the human β2AR, which we have characterized in parallel. Various trivial explanations for the negative inotropic effect can be excluded; first, block of endogenous catecholamines is unlikely, because these will not be present in superfused single
myocytes. Second, nonspecific or membrane-stabilizing effects, where the lipophilic nature of some \( \beta \)-blockers is thought to depress contractility by an effect on sarcolemmal ion channels, would be expected in normal mouse and rat or in nonfailing human myocytes also. Third, general fatigue or rundown of myocyte contraction is excluded, because only reversible negative inotropic effects were analyzed.

Several lines of evidence suggest that the effect is mainly associated with the \( \beta_2 \)AR. It occurred in TG\( \beta_2 \) and failing human heart, which have in common the strong contribution of \( \beta_2 \)ARs in ventricular myocardium. Negative inotropic effects of ICI 118,551 were not prevented by \( \beta_1 \)AR blockade and could be reversed by isoproterenol in the presence of the \( \beta_1 \)AR blocker. Overexpression of the \( \beta_2 \)AR in rabbit myocytes markedly enhanced the negative response in this species. Importantly, stimulation of the \( \beta_2 \)AR, which has previously been reported to depress myocardial contraction, did not mimic the response to ICI 118,551.

However, it is clear that the negative inotropic effect is not related to a reduction of the constitutively active form of the \( \beta_2 \)AR, \( R^* \). In neither the TG\( \beta_2 \)-NHLI myocytes nor those from failing human heart was basal contraction raised above control values. cAMP did not tonically support basal contraction in mouse myocytes or myocytes from failing or nonfailing human heart. The original hypothesis for inverse agonism, that preferential binding of ICI 118,551 to \( R \) (the inactive form of the \( \beta_2 \)AR) shifts the equilibrium away from \( R^* \) and so reduces adenylate cyclase activation, cannot provide an explanation for these results. Evidence from this study implicates \( G_i \) in the negative inotropic effect of ICI 118,551. Overexpression of \( G_\alpha_3 \) induced the negative inotropic of ICI 118,551 de novo in rat myocyte, whereas

Figure 5. Pertussis toxin treatment abolished the negative inotropic effect of ICI 118,551. Paired experiments are shown, with responses to ICI 118,551 in myocytes from the same hearts before and after pertussis toxin treatment (human, \( n=3 \) hearts with 4 myocytes before and 5 myocytes after PTX; mouse, \( n=5 \) hearts/myocytes). Human myocytes were studied in 8 mmol/L Ca\(^{2+} \) and mouse in 4 mmol/L Ca\(^{2+} \). Significantly different from control. *\( P<0.05 \), **\( P<0.01 \). Pertussis toxin treatment had no significant effect on basal contractility in either human (\( P=0.17 \)) or mouse (\( P=0.28 \)) myocytes.

Figure 6. Overexpression of \( G_\alpha_3 \) using an adenoviral vector induced the negative inotropic effect of 1 \( \mu \)mol/L ICI 118,551 in normal rat myocytes. Bar graph, contraction amplitude (% shortening, 4 mmol/L Ca\(^{2+} \)) of myocytes from 5 animals cultured for 48 hours either with (13 myocytes) or without (9 myocytes) Adv\( .G_\alpha_3 \).GFP. Infected cells were identified by green fluorescence. ***\( P<0.001 \) vs control. Right, Western blot of infected myocyte preparation. Con indicates cultured 48 hours without virus; Adv\( .G_\alpha \), cultured 48 hours with Adv\( .G_\alpha_2 \).GFP; and \( G_\alpha_1, G_\alpha_2, G_\alpha_3 \), standards.
pertussis toxin treatment to inactivate $G_i$ prevented the effect in mouse, rabbit, or human myocytes.

To reconcile these conflicting results, we propose a modification to the original hypothesis for inverse agonism. In the new scheme, ICI 118,551 binds to and stabilizes an additional active isoform (R#) of the $\beta_2$AR that couples through $G_i$ to a pathway with a direct negative inotropic effect in the ventricular myocyte. ICI 118,551 is, in effect, an agonist at R#. This occurs in parallel to the normal R*-Gs coupling that activates adenylate cyclase. R* and R# are in equilibrium, possibly with $G_i$, and the increased amount of R# and Gs is now significantly different from respective basal, **$P<0.01$, ***$P<0.001$; significantly different from control or $\beta_2+PTX$, ##$P<0.01$.

**Figure 7.** Enhancement of the negative inotropic effect of 1 $\mu$mol/L ICI 118,551 after overexpression of the human $\beta_2$AR using an adenoviral vector in rabbit myocytes and its reversal by pertussis toxin. Contraction amplitude (% shortening) of rabbit myocytes cultured for 24 hours without additions (control, 23 myocytes/11 hearts), with Adv.$\beta_2$AR ($\beta_2$, 19 myocytes/10 hearts), or with Adv.$\beta_2$AR plus pertussis toxin ($\beta_2+PTX$, 10 myocytes, 3 hearts). Basal, 4 mmol/L Ca$^{2+}$; ICI 118,551, 1 $\mu$mol/L. Significantly different from respective basal, **$P<0.01$, ***$P<0.001$; significantly different from control or $\beta_2+PTX$, ##$P<0.01$.

In TG$\beta_2$ mice (original phenotype), total $\beta_2$AR levels are massively increased, although the equilibrium between isoforms is not necessarily altered. The excess R* is sufficient to activate the adenyl cyclase pathway to produce levels of cAMP that will stimulate contraction above basal. In the original hypothesis, inverse agonists have negative inotropic effects mainly by decreasing levels of R* via a shift in equilibrium toward R, reducing Gi activation of adenyl cyclase and the raised basal contraction. Inverse agonists bind to R but have little effect on basal contraction when Gi is in the normal range.

In TG$\beta_2$-NHLI mice, Gi has upregulated. If the $\beta_2$AR (R) binds directly to Gi, the excess Gi will shift the equilibrium toward R and away from R*. This accounts for the decrease in basal contraction relative to the original phenotype. In our TG$\beta_2$-NHLI myocytes, the level of R* has dropped below that which can activate basal contraction. Inverse agonists bind to R, and the increased amount of R* and Gi, is now sufficient to mediate a negative inotropic response. A similar situation occurs in myocytes from failing human ventricle; i.e., $\beta_2$ARs are active, Gi is increased, and there is no basal activation of adenyl cyclase through Gi. These special circumstances reveal a direct negative inotropic effect of $\beta$-blockers mediated through the $\beta_2$ARs.

Evidence for ligand-specific receptor active states, or stimulus-trafficking of receptors, has been obtained previously for several G-protein-coupled receptors, including the $\beta_2$AR.7 When receptors can activate distinct subcellular pathways through 2 different G-proteins, the rank order of potency of agonists often differs for the 2 effects. Furthermore, mutations of the receptor can preferentially affect responses through one pathway but not the other.8 Evidence for a $\beta_2$AR/Gi link has been accumulating for some time, with Gi tonically inhibiting the positive inotropic and apoptotic effects of $\beta_2$ARs in normal rat myocytes.16-18 PKA-dependent phosphorylation of the $\beta_2$AR was shown to switch the $\beta_2$AR from Gi to Gs coupling in HEK29 cells.19 In human atria, immunoprecipitation studies have shown stimulation of Gi through the $\beta_2$AR, with inhibition by pertussis toxin increasing activation though Gi/adenylate cyclase.20 The present study is the first demonstration of such a link in human ventricle, although it differs from previous reports in that an inverse, rather than conventional, agonist produces the effect.

The mechanism of the negative inotropic effect is not yet known, and we can only speculate at this point. The abbreviation of the second phase of relaxation is consistent with a decrease in APD and resembles our previous findings with the $I_{KATP}$ channel opener Lemakalim in human myocytes.21 The rapidity of the effect is also more consistent with a direct action on a membrane channel rather than a second messenger-mediated mechanism.

Are these pharmacological effects likely to be relevant to the clinical use of $\beta$-blockers? Clearly, the unopposed $\beta_2$AR responses and high Gi levels in heart failure predispose to the demonstration of negative inotropic effects of $\beta$-blockers. It is known that $\beta$-blockers must be titrated carefully in patients with heart failure, and it has been assumed that the initial decrease in cardiac output22 is a consequence of withdrawal of tonic sympathetic support. The present study suggests that direct negative inotropic effects of $\beta$-blockers might also contribute to this initial decline in contractility. ICI 118,551 is an experimental compound, not used in patients with heart failure. However, previous studies23 have shown negative inotropic effects of carvedilol and metoprolol in muscle strips from failing human heart at clinically relevant concentrations.

The $\beta_2$AR/Gi-mediated negative inotropic effect of $\beta$-blockers may also be the tip of the iceberg. There are many potential Gi-activated pathways that would not have immediate contractile effects but could modify the response of ventricular muscle to apoptotic stimuli17,24 or hypertrophic agents (MAP kinase19). The fact that a $\beta$-blocker can act directly through $\beta_2$ARs and Gi allows the possibility that these pathways are active during $\beta$-blockade and could contribute to the recovery of ventricular function produced by these agents in failing human heart.

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