Cardioprotective and Survival Benefits of Long-Term Combined Therapy with \( \beta_2 \) Adrenoreceptor (AR) Agonist and \( \beta_1 \) AR Blocker in Dilated Cardiomyopathy Postmyocardial Infarction

Ismayil Ahmet, Melissa Krawczyk, Weizhong Zhu, Anthony Yiu-Ho Woo, Christopher Morrell, Suresh Poosala, Riu-ping Xiao, Edward G. Lakatta, and Mark I. Talan

Laboratory of Cardiovascular Sciences, National Institute on Aging, Intramural Research Program, National Institutes of Health, Baltimore, Maryland

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ABSTRACT

We have reported therapeutic effectiveness of pharmacological stimulation of \( \beta_2 \) adrenoreceptors (ARs) to attenuate the cardiac remodeling and myocardial infarction (MI) expansion in a rat model of dilated cardiomyopathy (DCM) post-MI. Furthermore, the combination of \( \beta_2 \) AR stimulation with \( \beta_1 \) AR blockade exceeded the therapeutic effectiveness of \( \beta_1 \) AR blockade. However, these studies were relatively short (6 weeks). In this study, in the same experimental model, we compared different effects, including survival benefit, of combined therapy with the \( \beta_1 \) AR blocker, metoprolol, plus the \( \beta_2 \) AR agonist, fenoterol (\( \beta_1 \), \( \beta_2 \)), and either therapy alone (\( \beta_1 \) or \( \beta_2 \)) during the 1-year study. Therapy was started 2 weeks after permanent ligation of the left coronary artery. Cardiac remodeling, MI expansion, and left ventricular function were assessed by serial echocardiography and compared with untreated animals (nT). Sixty-seven percent mortality in nT was reduced to 33\% in the \( \beta_1^-\beta_2^- \) (\( p < 0.01 \)). Progressive cardiac remodeling observed in nT and \( \beta_1^- \) was significantly attenuated in \( \beta_1^-\beta_2^+ \) during the first 6 months of treatment. In \( \beta_1^-\beta_2^+ \), MI expansion was completely prevented, and functional decline was significantly attenuated during the entire year. Myocardial apoptosis was significantly reduced in both \( \beta_1^-\beta_2^+ \) and \( \beta_1^- \). A reduction of cardiac \( \beta_2 \) AR density and decreases in chronotropic and contractile responses to \( \beta_2 \) AR-specific stimulation in the absence of a reduction of \( \beta_2 \) AR density in nT were precluded in rats receiving combined therapy. The results demonstrate the cardioprotective and survival benefit of long-term combination therapy of \( \beta_2 \) AR agonists and \( \beta_1 \) AR blockers in a model of DCM.

During the last decade, our understanding of \( \beta \)-adrenergic receptor (AR) subtype signaling and its role in development of chronic heart failure (CHF) have significantly increased, and \( \beta_1 \) AR blockade has become the therapy of choice in the treatment of CHF (Bristow, 1997; Sabbah, 1999; Hjalmarson et al., 2000; Pönicke et al., 2003). Stimulation of \( \beta_1 \) ARs, which activates the \( G_s \)-protein pathway, can promote apoptosis of cardiomyocytes, whereas \( \beta_2 \) ARs, in contrast, couple not only with \( G_s \), but also with \( G_t \)-protein, and, on the basis of the later, their stimulation is antiapoptotic and cardioprotective (Communal et al., 1999; Chesley et al., 2000; Zaugg et al., 2000; Zhu et al., 2001; Shizukuda and Buttrick, 2002; Xiao et al., 2004). However, despite growing experimental evidence of beneficial effects of \( \beta_2 \) AR agonists in CHF, this evidence has not been translated into clinical trials based on three main arguments. First, \( \beta_2 \) AR stimulation has a potent chronotropic effect and is known to elicit arrhythmias (Pearce et al., 1989; Lindmark and Ottoossen, 1998; Martin et al., 1998). Second, animal studies in the 1990s demonstrated that chronic \( \beta \) AR stimulation leads to down-regulation and desensitization of \( \beta_2 \) AR (for review, see Brodie et al., 1995); thus, their selective activation cannot be sustained. Finally, the attempts in the early 1980s to treat CHF patients with \( \beta_2 \) AR agonist monotherapy were not successful (Irmer et al., 1981).

ABBREVIATIONS: AR, adrenoreceptor; CHF, chronic heart failure; MI, myocardial infarction; DCM, dilated cardiomyopathy; LV, left ventricular; Echo, echocardiographic; SH, sham operation; EF, ejection fraction; nT, untreated animals group; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; ANOVA, analysis of variance; EDV, end-diastolic volume; ESV, end-systolic volume.
Several years ago, we (Ahmet et al., 2004), for the first time, tested the effect of selective pharmacological stimulation of β2 AR in an in vivo rat model of post-myocardial infarction (MI) dilated cardiomyopathy (DCM). Starting 2 weeks after coronary ligation, we compared the effects of 6-week treatment with the β2 AR agonist, fenoterol and zintelor, with the traditional β1 AR blocker therapy, metoprolol. The progression of left ventricular (LV) remodeling and MI expansion was monitored by serial echocardiography. At study termination, cardiac function was analyzed by pressure-volume loop measurements, and hearts were evaluated histologically. The effectiveness of the β2 AR agonists in attenuating LV dilatation and functional decline significantly exceeded that of the β1 AR blocker. Only β2 AR agonists prevented infarct expansion and actually reversed the decline of systolic function. Moreover, β2 AR agonists, but not the β1 AR blocker, attenuated diastolic dysfunction and prevented the hypertrophy of cardiomyocytes. Antian apoptotic effects of β2 AR stimulation also exceeded that of the β1 AR blocker.

More recently, in the same experimental model, we compared 6 weeks of combined therapy of the β2 AR agonist, fenoterol, plus the β1 AR blocker, metoprolol, with 6 weeks of metoprolol monotherapy (Ahmet et al., 2005). The combined therapy was superior to metoprolol monotherapy with respect to LV remodeling, functional decline, MI expansion, and apoptosis, as was previously demonstrated with β2 AR stimulation alone. However, the effects of β1 AR blockade and β2 AR stimulation were not additive. Lack of synergy with metoprolol but positive effects on cardiac remodeling have also been reported recently for another β2 AR agonist, clenbuterol (Xydas et al., 2006).

Thus, in the rodent experimental model of DCM after permanent coronary ligation and myocardial infarction, β2 AR activation therapy alone or combined β1 AR blockade and β2 AR AR activation therapy are superior to β1 AR blockade alone for the treatment of CHF. However, these prior studies were relatively short term, i.e., only 6 weeks of treatment. Given potentials for adverse effects of β2 AR agonists, the ultimate experimental, preclinical evidence of therapeutic benefit of β2 AR agonists or combination of β2 AR agonists and β1 AR blockers for CHF requires a long-term assessment, and survival benefit must be included in measurable outcomes. In the present study, we followed mortality, echocardiographic MI expansion, and progression of LV dysfunction during a full year after induction of MI by permanent coronary artery ligation in five groups of rats. Additional objectives of the present study were to measure the effect of treatment on β AR subtype density and responsiveness to specific stimulation to understand the mechanism of superior therapeutic effectiveness of combined β1 AR blockade and β2 AR activation in comparison with β1 AR blockade as a monotherapy. The effectiveness of long therapeutic antian apoptotic and vasodilator properties of β2 AR activation was also revisited. Starting 2 weeks after coronary ligation rats, matched for MI size, LV dilatation, and LV function, were assigned to treatment groups: treated with the β1 AR blocker, metoprolol; β2 AR agonist, fenoterol; the combination of β1 AR blocker and β2 AR agonist; as well as nontreatment and sham-operated groups.

Materials and Methods

Experimental Design. Male Wistar rats (Charles River Laboratories, Inc., Wilmington, MA), weighing 350 to 380 g, were housed and studied in conformance with the National Institutes of Health guide (1996; manual 3040-2) and institutional animal care and use committee approval. After baseline echocardiographic (Echo) assessment, the left descending coronary artery was ligated in 150 rats. An additional 10 rats underwent a sham operation (SH) without actual coronary ligation. Two weeks after surgery, LV dimensions, ejection fraction (EF), and MI size were measured by Echo. Animals with an MI size more than 20% and less than 50% of LV were divided into four groups of similar average MI size and variability. In three groups of rats, treatment was initiated either with metoprolol, a selective β1 AR blocker (β1), or with fenoterol, a selective β2 AR agonist (β2), or the combination of metoprolol and fenoterol (β1 β2; Sigma-Aldrich, St. Louis, MO). Fenoterol and metoprolol were dissolved in the drinking water; the daily dose was adjusted to 250 μg/kg for fenoterol and 100 mg/kg for metoprolol, as we described previously (Ahmet et al., 2004). The fourth group remained untreated (nT), and the fifth group was sham operated. Treatment was started 2 weeks after coronary ligation and continued for 12 months (4 weeks were counted as 1 month). Echo was repeated every month after the initiation of treatment. After the final Echo, some rats representing average Echo indices within each group were selected for experiments involving measurements of single cardiomyocytes responses to β AR stimulation. The remaining rats underwent an invasive hemodynamic study, and their hearts were harvested for histological and β AR density evaluation.

Coronary Artery Ligation. Rats were anesthetized with isoflurane (2% in oxygen). The surgical procedure was performed as described previously (Hochman and Bulkley, 1982).

Echocardiography. Echocardiography (Sonos 5500, a 12-MHz transducer) was conducted under light anesthesia by sodium pentobarbital (30 mg/kg i.p.) as described previously (Ahmet et al., 2005; see supplemental data). The ECG recorded during 10 min of each Echo was evaluated to detect and record rhythm disturbances. Arrhythmic events that were widespread throughout the measurement period were classified according to the Lambeth convention criteria (Walker et al., 1988).

Hemodynamic Measurements. Invasive LV pressure-volume loop analyses were conducted as described previously (Ahmet et al., 2004; see also supplemental data).

In Vivo β AR Stimulation. After conclusion of pressure-volume loop analyses, a “stress test” was conducted by measuring the heart rate responses to β1 AR stimulation (20 μg/kg/min dobutamine) or to β2 AR stimulation (200 μg/kg/min zintelor). Drugs were delivered through femoral vein on alternative sides in the volume of 400 μl (80 μl/min) for 5 min. Drug deliveries were separated by a 5-min drug-free period.

Histological Acquisition. Histological staining and analyses were performed as described previously (Pearce et al., 1989). In brief, the hearts were isolated and weighed. Myocardial sections from the midpapillary muscle level were subjected to Masson’s trichrome, hematoxylin and eosin, and TUNEL staining. MI size was expressed as an average percentage of the LV endocardial and epicardial circumferences that were identified as infarct in the Masson’s trichrome staining sections. The number of TUNEL-positive myocytes was counted throughout the myocardium, excluding the scar. The total number of cardiomyocytes per slide was calculated on the basis of average cardiomyocyte density per field of vision from hematoxylin and eosin-stained sections. The number of apoptotic myocytes was expressed as a number per 105 total myocytes. Care was taken to verify every putative apoptotic myocyte under high amplification (×100).

Membrane Preparation and Radioligand Binding Assay of β AR. The procedure for membrane preparation and radioligand
binding assay of β AR has been described elsewhere (K-Laflamme et al., 1997; see supplemental data).

**Measurement of Cardiac Myocyte Contraction.** The procedure for measurement of cardiac myocytes contraction has been described elsewhere (Spurgeon et al., 1990; see supplemental data).

**Statistical Analyses.** All data are expressed as mean ± S.E.M. Mortality was described using Kaplan-Meier survival curves. Comparison among untreated and three treatment groups was followed by pair-wise comparisons of untreated group with each of the three treated groups and evaluated using the Wilcoxon test. A preliminary power analysis was conducted to determine the number of experimental animals per group based on the assumption that statistically significant differences in mortality between groups should be not less than 30%. Reported Echo indices were compared using a mixed effects model for repeated measurements. If the group-time interactions were statistically significantly different, a one-way ANOVA and Bonferroni’s post hoc test were used to test for statistical differences at each time point.

Group differences in hemodynamic or histological data among groups were assessed by Student’s t test or by one-way ANOVA with Bonferroni’s or Dunnett’s post hoc test as appropriate. Statistical significance was assumed at \( p < 0.05 \).

**Results**

**Early Mortality and Cardiac Remodeling after Coronary Ligation before Treatment and Treatment Group Assignment.** Thirty-five percent of 150 rats subjected to a coronary ligation died during the first 24 h after surgery. Ninety-eight coronary-ligated rats and all 10 sham-operated rats survived and 2 weeks after surgery were subjected to the second echocardiography, at which time the baseline (pretreatment) infarct size was estimated. Twenty-eight coronary-ligated rats with an MI size of less than 20% or more than 50% of LV were removed from the experiment before assignment to treatment groups. For the remaining 70 rats, the average Echo-estimated MI size at 2 weeks after coronary ligation was 32.3 ± 0.86% of LV. Compared with presurgical values, the average Echo-measured end-diastolic LV volume (EDV) during the 2 weeks after coronary ligation was increased by 137%, the end-systolic LV volume (ESV) increased by 366%, and the EF was reduced by 59.1% (\( p < 0.05 \) for all). The degree of early (pretreatment) remodeling correlated with the MI size \( (r = 0.45, 0.55, \) and \(-0.44\) for EDV, ESV, and EF, respectively). In comparison, as a result of a rapid growth period, the EDV in SH animals \( (n = 10) \) during the same time increased by only 25%, the ESV increased by 31%, and the EF was reduced by 2.8%. MI rats were assigned to experimental treatment groups at this time \( (nT, 18; \beta_2^+, 18; \beta_1^-, 17; \) and \( \beta_2^+\beta_1^- \) ) in such a way that pretreatment Echo-derived LV morphometric parameters (EDV and ESV), EF, and MI size did not differ among the groups (Figs. 1 and 2; one-way ANOVA with post hoc comparison at month 0, \( p > 0.05 \)).

**Effect of 12 Months of Different Treatments on Mortality in DCM Rats.** Figure 3 illustrates the Kaplan-Meier survival curves for four groups of experimental animals and one sham-operated group during 1 year after initiation of treatment. No SH animals died during observation. Among MI animals, no mortality occurred during the first 3 months after the initiation of treatment. Rats in the nT group began to die earlier than in other groups, and at 7 months after initiation of therapy \( (7.5 \text{ months after coronary ligation}) \), mortality in nT exceeded 50%. Survival analyses at 6 months revealed significant differences among groups \( (p < 0.03, \) Wilcoxon test); however, the pair-wise analyses showed statistically significant differences only between nT and combined \( \beta_2^+\beta_1^- \) therapy groups \( (p = 0.01) \). The mortality in \( \beta_2^- \) and \( \beta_1^- \) monotherapy groups reached 50% 3 and 3.5 months later, respectively, than in the nT group. Mortality in the combined \( \beta_2^+\beta_1^- \) therapy group at that time was only 25%, and 70% of animals in the combined therapy group were still alive at the end of 1 year. At the end of the observation period \( (12 \text{ months}) \), the Wilcoxon test was marginally significant among all groups \( (p = 0.074) \) and significant \( (p = 0.018) \) for the pair-wise comparison between the nT and \( \beta_1^- \) and \( \beta_2^+ \) groups only. At that time, the difference in mortality between \( \beta_2^+\beta_1^- \) and nT was 34%. The trend for enhanced survival in monotherapy groups \( (288 \text{ days in } \beta_2^+ \text{ alone and } 313 \text{ days } \beta_1^- \text{ alone}) \) compared with nT \( (205 \text{ days}) \) did not reach statistical significance \( (p = 0.1) \).

**MI Size Estimated by Echo**

![Fig. 1. The average MI size of untreated and three treatment groups of rats estimated by monthly Echo and presented as percentage of LV. Left, all animals; middle, animals that survived 12 months after the MI induction; right, scatter diagram comparing MI size in the same animals measured during the final, 12-month echocardiography and subsequently histologically. * \( p < 0.05 \), \( \beta_2^+\beta_1^- \) versus nT or \( \beta_1^- \); † \( p < 0.05 \), \( \beta_2^+\beta_1^- \) versus \( \beta_2^+ \) (one-way ANOVA and post hoc Bonferroni comparison).](image-url)
Infarct Expansion and Late Cardiac Remodeling. Figure 1 (left) documents monthly results of Echo-derived measurements of MI size expressed as the percentage of LV perimeter, i.e., MI expansion, in different experimental groups. The original MI size in nT group (31% of LV) increased by more than 40% and reached the size of 43% of LV during the first 3 months of observation (starting 2 weeks after surgery) and by another 30% during the next 9 months, reaching a size of 48% of LV at 12 months. The progression of MI expansion in the $\beta_1^-$ group did not differ from that in the nT, and at the end of treatment, the average MI size in this group was only slightly less than in nT (43.4% of LV, $p > 0.05$). The MI size in the $\beta_2^+$ group did not change during the first 2 months but then began to expand. However, the rate of increase slowed down from month 2 to month 4, and its magnitude was less than that of either nT or $\beta_1^-$. During the first 3 months of treatment, the MI size in the $\beta_2^+$ group remained statistically smaller than in nT, but this beneficial effect was subsequently lost. In contrast, the MI size in the SH group. 

Figure 2. Body mass-adjusted LV end-diastolic volume (A), end-systolic volume (B), and ejection fraction (C) in untreated and three treatment groups estimated by monthly Echo during 12 months after induction of MI. Top panels, all animals subjected to measurement. Bottom panels, only animals that survived 12 months after induction of MI. Left, entire 12 months of observation and treatment. Panels in the second to fourth columns, experiment divided into three time intervals: beginning of treatment (first 3 months), mid-term (from 3rd to 7th months), and end term (from 7th to 12th months), respectively. LV volumes are adjusted for individual body mass and normalized for the corresponding average body mass of the animals in the SH group. $p < 0.05$, $\beta_2^+$ versus nT; $p < 0.05$, $\beta_2^+$ versus $\beta_1^-$. $\beta_2^+$ versus $\beta_1^-$. $\beta_2^+$ versus nT (one-way ANOVA and post hoc Bonferroni comparison).
MI expansion only in animals that survived to the end of observation and thus excludes animals that died during the prior 12 months. The general pattern of the MI expansion among survived animals was essentially similar to the pattern characteristic for all animals presented in Fig. 1, left. The MI size measured histologically after 12 months of treatment (Supplemental Table 1) was highly correlated (r² = 0.64) with Echo-estimated MI size just before sacrifice (Fig. 1, right). The histological MI size (Supplemental Table 1) was similar among nT, β₁⁻, and β₂⁺ groups but was 22% lower (p < 0.05) in combined β₁⁻β₂⁺.

Figure 2 illustrates the progression of LV remodeling (EDV and ESV expansion, Fig. 2, A and B, respectively) and functional decline (EF reduction; Fig. 2C) during the 1 year of treatment. The LV volumes were normalized for body mass of individual animals at the time of the measurement and further indexed to the average body mass of the SH group at the same time. Top panels of Fig. 2, A to C, represent data for all animals subjected to Echo, and bottom panels include only data of animals that survived at 12 months. The left panels present data for the entire year. The progression of EDV and ESV expansion and EF fall varied among MI groups (ANOVA, group × time interaction, p < 0.05). For clarity of presentation, these data are divided into three time periods (right panels): early treatment period (first 3 months of treatment), midterm treatment (from the 3rd to 7th months), and late-term treatment (from the 7th to 12th months).

**Early Treatment Period.** The results during the first 3 months of treatment essentially replicated the results reported in our previous short-term studies of 2-month therapy (Ahmet et al., 2004, 2005). In the nT group, the EDV (Fig. 2A) expanded by 30% and was similar with the rate of EDV expansion in the β₁⁻ group. The EDV in β₂⁺β₁⁻ and β₂⁺ groups were similar to each other and expanded at a much slower rate. EDV was significantly lower in the β₂⁺β₁⁻ than in the nT group during the first 2 months of treatment.

The changes in ESV (Fig. 2B) during the first 3 months of treatment essentially paralleled those in EDV, but differences among groups were much more pronounced. The ESV in nT group increased by 40%, and the pattern of ESV increase was identical to the β₁⁻ group. The combined β₂⁺β₁⁻ treatment reduced the expansion of ESV (p < 0.05 versus nT for each of the first 3 months of treatment), and this effect was similar to the effect of β₂⁺ monotherapy.

The greater differences in the effects of treatment occurred in EF (Fig. 2C). Without treatment (nT group), EF (which fell to the level of 25% during the first 2 weeks after coronary ligation) continued to fall and at the end of the 3rd month reached 21%. The EF in the β₁⁻ group showed no indication of the effect of treatment and did not differ from nT. In contrast, the EF in the β₂⁺β₁⁻ group increased during the 1st month of treatment, from 24 to 31%, remained above 30% during the first 3 months of treatment, and was statistically different from both nT and β₁⁻. The 1st month after initiation of treatment, the EF in the β₂⁺β₁⁻ group was even greater than in β₂⁺β₁⁻ but then subsequently started to decline.

**Midterm Treatment.** During the midterm, the pattern of LV volume expansion observed during the early stage of treatment continued; both the EDV and ESV were similar between the nT and β₁⁻ groups, whereas the β₂⁺β₁⁻ group showed the attenuation of LV volume expansion. However, the effect of β₂⁺β₁⁻ treatment on the LV remodeling began to wane during the middle term, and at the 6th month, the LV volumes in the β₂⁺β₁⁻ group were similar to that in nT and β₁⁻. Nevertheless, the beneficial effect of combined β₂⁺β₁⁻ treatment on EF persisted; the EF in β₂⁺β₁⁻ group was significantly higher than in nT or β₁⁻ during the entire middle term.

A remarkable change in the effects of β₂ AR stimulation on LV remodeling and function occurred between the early and midterm treatment. Although during the first 3 months of treatment, the attenuation of LV expansion and functional decline in β₂⁺ group was similar to β₂⁺β₁⁻ group, during the midterm, the LV expansion and functional decline in the β₂⁺ group accelerated and reached that in nT; at months 5 and 6, the ESV and EF in β₂⁺ were significantly higher and lower, respectively, than those in β₂⁺β₁⁻.

**End-Term Treatment.** During the end term (final 5 months) of treatment, the differences in LV volumes between the β₂⁺β₁⁻ and nT groups significantly reduced and were statistically nonsignificant. However, the EF in the β₂⁺β₁⁻ group remained significantly (p < 0.05), 6%, higher than that in nT even at the very end of the experiment.

The general pattern of the LV remodeling and functional decline among animals that survived to the end of observation, i.e., excluding animals that died during the prior 12 months (bottom panels of Fig. 2, A–C), was similar to that of all animals (Fig. 2, A–C, top panels).

In SH animals, the LV remodeling reflects the normal growth and aging, and data are not shown in the figure. In SH, the EDV increased by 62% and the ESV by 91%, the EF reduced by 13%, and, at the end of the year, the average EDV in SH was 0.505 ± 0.03 ml, the ESV was 0.239 ± 0.02 ml, and the EF was 52 ± 1.5%.

To summarize, the nT group showed progressive expansion of LV volume and decline of LV function, the LV remodeling and functional decline in the β₁⁻ group was similar with that in nT, and the β₂⁺β₁⁻ treatment attenuated the LV EDV and ESV expansion up to 6 months after beginning of treatment and the fall of EF during the entire 12-month of treatment. The β₂⁺ monotherapy was effective only during the first 2 months.

**Rhythm Disturbances.** The presence or absence of rhythm disturbances was noted during repeated monthly
Echo (see Supplemental Results). Figure 4 illustrates the records of total incidences of premature ventricular contractions in different experimental groups expressed as per-centage of the current number of animals in that group at every time point. The occurrence and increase of the number of animals having an arrhythmia over the time of experiment was lower in the combined treatment group comparing with nT and other treatment groups (p < 0.05).

Response to Selective in Vivo β AR Stimulation in Stress Test. Figure 5 illustrates the HR response to in vivo stimulation of β1 AR with dobutamine or β2 AR with zinterol in different treatment groups of rats that survived 12 months after MI induction. The HR increase in response to dobutamine stimulation was similar in SH, nT, and different treatment groups and ranged from 33 to 44% (p > 0.05), suggesting that acute β1 AR responsiveness was not affected by post-MI remodeling or by different treatments. In contrast, the 10% HR increase observed in response to stimulation by zinterol (a β2 AR agonist) in SH rats was diminished in both nT and β2+ groups (p < 0.05) but was completely unaffected in both β1− and β2+ groups, suggesting that β2 AR responsiveness becomes reduced with development of CHF, cannot be rescued by β2 stimulation, but can be rescued by chronic β1 AR blockade.

The Density of Myocardial β AR Subtypes. Figure 6 shows the average LV myocardium β AR density (Fig. 5A) and their β1 and β2 subtypes (Fig. 6, B and C, respectively) of rats 12 months after MI induction. Total β AR density was significantly reduced in rats in untreated CHF (nT). β1 AR blockade, alone (β1−) or in combination with β2 AR stimulation (β1−β2−), restored β AR density, but in the β2− group, β AR density remained low. These bindings largely reflected β1 AR density, which was significantly reduced in nT and β2− groups but was normal in β1− and β2+ groups. The β2 AR density, in the contrast, was not affected in the nT or in groups treated with the β1 AR blocker (β1− and β2−) but was significantly reduced by treatment involving β2 AR stimulation alone (β2−). Thus β1 AR density is reduced in CHF, and this reduction can be rescued by β1 AR blockade. β2 AR density is not affected by CHF but is reduced when CHF is accompanied by chronic β2 AR stimulation. This later effect is also reversed by β1 AR blockade.

Terminal Hemodynamics. For more information regarding terminal hemodynamics, see Supplemental Results, Supplemental Table 2, and Supplemental Fig. 1.

Myocardial Apoptosis. Figure 7 presents the number of TUNEL-positive cardiomyocytes in rats that survived 12 months after induction of MI. The average number of TUNEL-positive cardiomyocytes was 3 to 6 times lower in the myocardium of the hearts in treated groups than in the hearts of untreated animals. However, this reduction was statistically significant only in β1− and β2−β1− groups.

Responses of AR Stimulation in Isolated Single Cardiomyocytes. Figure 8 illustrates the contractile response of single cardiac myocytes isolated from the hearts of rats harvested at 12 months after induction of MI. Responses to different concentrations of norepinephrine stimulation (left panel) did not differ among SH, nT, and either single-therapy groups, but the β1 AR response was augmented in β2−β1− (p < 0.05) in comparison with SH. Responses to zinterol stimulation (right panel) were markedly depressed in nT (p < 0.05) and to a lesser degree in the β2− group (p < 0.05) compared with SH. In contrast, in the β1− and β2−β1− groups, the response to zinterol stimulation was preserved and was similar to SH. These results are in concert with results of in vivo AR stimulation and suggest that the β2 AR contractile response of cardiomyocytes is reduced with development of CHF and cannot be rescued by β2 stimulation but can be rescued by chronic β1 AR blockade.

Discussion

The present study extended the previous, short-term studies (Ahmet et al., 2004, 2005) and demonstrated a definite superiority of long-term combined therapy with the β2 AR agonist and β1 AR blocker over any monotherapy alone. In particular, DCM rats treated with combined therapy showed a 34% increase in survival compared with untreated rats, whereas the survival benefit of single therapy with β1 AR blocker (the β1− group) or β2 AR agonist (the β2− group) was not statistically significant. The arrest of MI expansion shown previously in 6-week-long combined therapy with the β1 AR blocker and β2 AR agonist persisted during year-long therapy; the original, pretreatment, MI size did not expand at all. Moreover, the reduction of LV remodeling shown pre-
During 6 weeks of combined therapy (β₁ AR blocker and β₂ AR agonist) persisted in the present study for more than 6 months. However, after 6 months, there was no difference between combined therapy and single therapy with the β₁ AR blocker or β₂ AR agonist with respect to LV remodeling. However, with respect to functional decline, the combined therapy was significantly more beneficial than either single therapy during the entire year, i.e., the EF was significantly higher in the β₂⁺β₁⁻ group than in the nT group at all time points. It is noteworthy that beneficial effects of combined therapy on LV remodeling and function were accompanied with a significant reduction of arrhythmic events, thus alleviating a major concern associated with consideration of use of β₂ AR agonists in treatment of CHF.

One important finding is that beneficial effects of a monotherapy with a β₂ AR agonist alone, previously shown in our present study, waned when observation was extended in our present study, and, after 3 months of a single β₂ AR agonist therapy, the sonographic indices of LV dilatation and function as well as the rate of MI expansion rapidly approached that of untreated animals. In vitro studies conducted at the end of 1-year observation shed some light on the mechanisms of effectiveness of combined (β₁ AR blocker and β₂ AR agonist) therapy and helped to explain the loss of effectiveness of β₂ AR agonist monotherapy.

Measurements of the number of apoptotic cardiomyocytes generally agreed with results obtained from Echo. Twelve months after coronary ligation, all treated animals showed improvements in comparison with untreated animals. It is interesting to note that the reduction in the number of apoptotic nuclei was less pronounced in the myocardium of β₂⁺ than in β₂⁺β₁⁻ and β₁⁻ rats, paralleling the effects of Echo-derived EF.

With respect to βAR density, the present study confirmed previously reported observations of reduction of the cardiac β₁ AR density in DCM with preservation of β₂ AR density (Bristow et al., 1986). β₁ AR blockade alone or in combination with the β₂ AR agonist rescued the reduction of β₁ AR number. Furthermore, in the present study, we observed that β₂ AR density in DCM rats becomes reduced after a year-long single therapy with a β₂ AR agonist. This finding is in concert with well recognized effects of β₂ AR agonist tachyphylaxis associated with its long-term dosing (Lipworth et al., 1989) and thought to be related to a reduction of β₂ AR subtype density in the heart (Qing et al., 1997). Our findings showed that addition of β₁ AR blocker (i.e., combination of β₂⁻β₁⁻) prevented the reduction in β₂ AR density and, as was shown in Echo measurements, prevented the tachyphylaxis. Results of Echo measurements showed that beneficial effect of a single therapy with β₂ AR agonist on LV remodeling and function observed at the beginning of the therapy disappeared with it continuation beyond 2 months, but the benefit of combined therapy on EF persisted for a full year. The mechanism of prevention of β₂ AR density reduction and, therefore, the prevention of tachyphylaxis by addition of β₁ AR antagonist to a treatment, as shown in our present study, is unclear and remains to be elucidated.

Although we did not observe any reduction of the density of cardiac β₂ AR 12 months after coronary ligation in the untreated MI group, the chronotropic response in vivo experiments and contractile responses in experiment with single cardiomyocytes (supplemental data) to β₂ AR-specific stimulation (zinterol) were both reduced. This reduction in responsiveness without reduction in density is compatible with currently generally accepted point of view that in failing heart the β₂ AR are uncoupled from the effector systems (for review, see Port and Bristow, 2001; Lohe et al., 2003; Brodde et al., 2007). It is interesting to note that the β₁ AR blockade in combined therapy prevented (rescued) this reduced responsiveness. The mechanism of this “rescue” effect of β₁ AR blockade on reduced β₂ AR responsiveness is not clear; however, the role of heterodimerization of β AR shown previously (Zhu et al., 2005) might be considered.

It is difficult to speculate about exact mechanisms of therapeutic effectiveness of combined β₂ AR stimulation and β₁
AR blockade on the basis of a long-term preclinical animal trial with mortality as an endpoint, such as the present study. However, it is possible to make some inferences, considering the results of the present study in the context of our previous findings and of those previously reported in the literature. The antiapoptotic effect of $\beta_1$ AR blockade is well known (Bristow, 1997; Communal et al., 1999; Hjalmarson et al., 2000; Zaugg et al., 2000; Shizukuda et al., 2002; Pönicke et al., 2003). It had been reported previously by us and others that $\beta_2$ AR stimulation protects single cardiomyocytes from apoptosis induced by adverse stimuli (Chesley et al., 2000; Zaugg et al., 2000; Shizukuda et al., 2002; Xiao et al., 2004).

We have also reported that in the post-MI DCM rat model therapeutic effect of 6-week-long treatment with $\beta_2$ agonist alone or in combination with $\beta_1$ blocker was accompanied by substantial reduction of apoptosis in the MI border zone, as well as in remote areas of the myocardium (Ahmet et al., 2004, 2005). A reduction of the number of cardiomyocytes stained for apoptosis in the MI hearts of rats subjected to 12 months of treatment with the combination of the $\beta_2$ AR agonist and $\beta_1$ AR antagonist, the $\beta_1$ AR antagonist alone, and, to the lesser degree, with the $\beta_2$ AR agonist alone was also shown in the present study. Thus, it is plausible to conclude that a reduction of apoptosis was one of the important mechanisms responsible for success of combined therapy in the present study. The direct antiapoptotic effect of $\beta_2$ AR on cardiomyocytes was, at least in part, a contributing factor. The antiapoptotic effect of “unloading” of the heart associated with vasodilatory properties of fenoterol and reported previously by us and others (Ahmet et al., 2004, 2005; Schena et al., 2004) was also undoubtedly a contributing factor. On another front, it has been reported that $\beta_2$ AR stimulation inhibits the production of some cytokines, i.e., interleukin-3 and interleukin-12, in vitro (Panania-Bordignon et al., 1997; Borger et al., 1998). In the recent report (Nishii et al., 2006), $\beta_2$ AR treatment (formoterol and salbutamol) reduced IFN-y myocardial expression in the model of autoimmune myocarditis in rats. Thus, anti-inflammatory potential of $\beta_2$ AR stimulation might also be a contributing factor.

In summary, the present study proves that in the rat experimental model of post-MI DCM, combined therapy with the $\beta_1$ AR blocker and the $\beta_2$ AR agonist is effective and exceeds either single therapy alone, including the clinically proven treatment with $\beta_1$ AR blockers. Compared with untreated MI animals, the combined therapy increased survival by more than 30%, prevented the MI expansion, improved the LV function, and attenuated the LV remodeling for a longer period than either monotherapy. Moreover, combined with the $\beta_1$ AR blocker, the $\beta_2$ AR agonist did not increase the number of arrhythmic events.

**Study Limitations.** The study was powered for limited pair-wise comparison of the untreated group with three treatment groups, and statistically significant differences between groups were assumed to be in excess of 30%. As a result, the 34% improvement in survival in rats treated with combined therapy ($\beta_1^{-}\beta_2^{+}$) compared with untreated MI rats was statistically significant, whereas the obvious trend in survival benefit of the $\beta_1$ AR blocker monotherapy (below 30%) was not statistically significant. However, the study was designed to prove the survival benefit of combined therapy ($\beta_1^{-}\beta_2^{+}$). The effect of $\beta_1^{-}$ AR blockade on survival is well known.

Two weeks after induction of MI, animals were divided into experimental groups in such a way as to provide for similar average MI size and its variability. Rats, which had small (<20% of LV) or very large (>50% of LV) MI, were excluded from the study. The MI size was measured at the 2nd week (pretreatment baseline) echocardiography. Although this method is clearly superior to a random group assignment, its accuracy is limited by the accuracy of sonographic measurements, which in our hands correlated with histology at $r^2 = 0.64$.

**References**


Address correspondence to: Dr. Mark I. Talan, National Institute on Aging, Intramural Research Program, Gerontology Research Center, 5600 Nathan Shock Drive, Baltimore, MD 21224-6825. E-mail: talanm@grc.nia.nih.gov