Effectiveness of β-Blockade in Experimental Chronic Aortic Regurgitation

Eric Plante, MSc; Dominic Lachance, BSc; Martin Gaudreau, MSc; Marie-Claude Drolet, MSc; Élise Roussel, MSc; Marie Arsenault, MD; Jacques Couet, PhD

Background—Past studies have suggested that the adrenergic system becomes abnormally activated in chronic volume overload, such as in severe aortic valve regurgitation (AR). However, the effectiveness of agents directed against this adrenergic activation has never been adequately tested in chronic AR. We therefore tested the effects of metoprolol treatment on the left ventricular (LV) function and remodeling in severe chronic AR in rats.

Methods and Results—Severe AR was created in adult male Wistar rats by retrograde puncture of the aortic leaflets under echocardiographic guidance. Two weeks later, some animals received metoprolol treatment (25 mg/kg) orally for 24 weeks, and some were left untreated. LV dimensions, ejection fraction, and filling parameters were evaluated by echocardiography. Hearts were harvested at 1, 2, 14, and 180 days for the evaluation of hypertrophy, β-adrenergic receptor status, and extracellular matrix remodeling. We found that metoprolol treatment prevented LV dilatation and preserved the ejection fraction and filling parameters compared with untreated animals. Metoprolol increased the expression of β1-adrenoreceptor mRNA and reduced G protein receptor kinase 2 levels. Collagen I and III mRNA levels were reduced. Cardiac myocyte hypertrophy was also prevented.

Conclusions—In our experimental model of severe AR, metoprolol treatment had a significant beneficial global effect on LV remodeling and function. These results suggest that the adrenergic system is important in the development of volume-overload cardiomyopathy in AR and that adrenergic-blocking agents may play a role in the treatment of this disease. (Circulation. 2004;110:1477-1483.)

Key Words: valve, aortic, insufficiency ■ echocardiography ■ regurgitation ■ receptors, adrenergic, beta

Aortic valve regurgitation (AR) is a chronic volume-overload disease that induces progressive left ventricular (LV) dilatation and eccentric hypertrophy. Patients suffering from significant levels of AR will often remain asymptomatic for decades before heart failure develops.1,2 Current treatment guidelines suggest that they should be treated primarily with vasodilators.1 Significant alterations of the adrenergic system and adrenergic receptors have been reported in animal models of chronic volume overload.3–12 Despite these interesting findings, the hypothesis that β-adrenergic blocking agents might be effective to protect the volume-overloaded LV has not been adequately tested. A few studies have suggested that a short-term treatment with β-blockers might be beneficial in volume overload.3,11,13,14 However, the long-term effectiveness of this type of drugs in chronic AR has never been adequately evaluated. In the present study, we assessed the effects of a 6-month treatment with the β-blocker metoprolol on the LV function and remodeling of rats with severe chronic AR.

Methods

Animals

Acute Study

Forty male Wistar rats (400 to 450 g; Charles River, St-Constant, QC, Canada) had severe AR induced for 1, 2, or 14 days until they were euthanized to evaluate the acute adaptations of the LV. A sham-operated group was used as control (n=10).15

Chronic Study

Thirty-eight male Wistar rats (body weight, 400 to 450 g) were divided into 3 groups as follows: group 1, normal controls (sham-operated; n=10); group 2, untreated AR (n=18); and group 3: AR receiving 25 mg · kg⁻¹ · d⁻¹ of metoprolol tartrate (n=10) (Sigma) in drinking water. Drug treatment was started 2 weeks after the surgical procedure described below and continued for 24 weeks thereafter.16 This protocol was approved by the Université Laval’s Animal Protection Committee and was consistent with the recommendations of the Canadian Council on animal care. Severe AR was induced in the animals as previously described by retrograde puncture of the aortic valve leaflets.15–17 Echocardiographic studies were performed as described elsewhere15–17 at each time point of the protocol (at 0, 1, 2, or 14 days for the acute study and at 0 and 180 days for the chronic study). Cardiac output was...
calculated by Doppler echocardiography and indexed to the animal’s body weight. AR was considered severe by echocardiography by the presence of all of the following criteria: color-Doppler ratio of regurgitant jet width to LV outflow tract diameter >50%, retrograde holodiastolic flow in proximal descending aorta with end-diastolic velocity >18 cm/s, ratio of time-velocity integral of reversed diastolic flow to forward systolic flow in descending thoracic aorta >60%, and acute increase in LV dimensions. Echocardiographic criteria of AR severity had to be accompanied by an acute drop of aortic diastolic pressure >30% to qualify. Ejection fraction, diastolic filling, relative wall thickness (RWT), and LV mass were evaluated as described elsewhere.15–18 Normal echocardiographic diastolic filling parameters were assessed previously in a cohort of normal age-matched Wistar rats.15

At the end of the protocol, the animals were euthanized, their hearts were quickly dissected, and LVs as well as the other cardiac chambers were weighed and then snap-frozen in liquid nitrogen and kept at ~80°C.

Tissue Catecholamine Determination
LV tissue total catecholamines were measured by radioimmunoassay (Amersham).

β-Adrenergic Receptors
LV tissues were homogenized in the following buffer (in mmol/L): 25 Tris–HCl, 2 MgCl2, 250 sucrose, 5 HEPES (pH 7.4). Homogenates were centrifuged for 30 minutes (1000g, 4°C) and the supernatant for 30 minutes at 35 000g. Membranes were suspended in a homogenization buffer, and protein concentration was determined. Binding activity was evaluated by use of [125I]iodocyanopindolol (specific activity: 200 Ci/mmol; Amersham). Nonspecific binding was determined in the presence of propanolol (1 μmol/L) (Sigma) and subtracted from the total count. The total density of β-adrenergic receptors was determined by use of increasing concentrations of propanolol (1 μmol/L to 0.1 mmol/L). The β1/β2-receptor ratio was evaluated with metoprolol. Competition curves and analysis were performed using GraphPad Prism version 3.02 (GraphPad Software).

Immunoblotting
Crude LV homogenates were separated by SDS-PAGE. Volumes of samples loaded on gel were corrected for the amount of protein. Immunoblotting was performed as described elsewhere.20 Membranes were hybridized with primary antibodies directed against G protein receptor kinase (GRK) 2 and GRK5, sarcomeric α-actin, and smooth muscle α-actin. Bands were visualized and quantified with a Chemilumager system (Alpha Innotech Corporation).

Messenger RNA Accumulation
cDNA synthesis and reverse transcription–polymerase chain reaction (RT-PCR) analyses were performed as described elsewhere19,20 using the following oligonucleotide pairs: glyceraldehyde phosphate dehydrogenase (GAPDH), 5'-ATCCCATCACCACATCTCCAG-3' and 5'-CCATCACCGCACAGTTTCC-3'; β-adrenoreceptor (β1, AR): 5'-GCACACTGGCAAATGTAATG-3' and 5'-GGTGAAACGAAGAAGTATT-3'; β2-AR: 5'-GCCGTGCAAGTGATT-3' and 5'-TATACAGTGCTTTGCTT-3'; collagen type 1 (Col1): 5'-CGAGGTAACAGAGGTGAAAGA-3' and 5'-CGAGACTCTGATGCTTCTG-3'; and proc-matrix metalloprotease 2 (MMP2): 5'-CTATTTGCTGACAGCTTTGG-3' and 5'-GCAGACTTCTTCTCCT-3'. Denaturation, annealing, and amplification temperatures were 94°C, 60°C (50°C and 55°C for Col3 and MMP2, respectively), and 68°C, respectively.

Cardiomyocyte Cross-Sectional Area
LV sections fixed in paraffin were stained with Masson’s trichrome. Myocyte cross-sectional area was measured.16 Sections from at least 10 animals per group were studied. Results are presented as mean±SEM in arbitrary units.

<table>
<thead>
<tr>
<th>TABLE 1. Hemodynamics (Day 180)</th>
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<tbody>
<tr>
<td>Parameters</td>
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<tr>
<td>Pulse pressure, mm Hg</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
</tr>
<tr>
<td>Stroke volume, mL</td>
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<tr>
<td>Cardiac output, mL/min</td>
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<tr>
<td>Cardiac index, mL·min⁻¹·g⁻¹</td>
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</table>

Values are mean±SEM. Stroke volume is from pulsed Doppler measurement in the LV outflow tract. *P<0.05 and †P<0.01 vs AR control rats.

Immunohistochemistry
The number of fibronectin-positive cells per field was evaluated in LV sections as previously described.16 The mean of sham controls was arbitrarily fixed at 100, and the results of the other groups are expressed relative to the sham controls.

Statistical Analysis
Results are presented as mean±SEM unless specified otherwise. One-way ANOVA was performed to compare serial data. Statistical significance was set at a probability value of P<0.05 using a post hoc Tukey test. Data and statistical analysis were performed using GraphPad Prism.

Results

Clinical Data
Drug treatment was well tolerated. All animals were alive at the end of the protocol. No animals developed clinical signs of heart failure in either group. Lung weight at death tended to be slightly increased in untreated AR rats, but this did not reach statistical significance. Metoprolol treatment had no significant effect on lung weight (not shown).

Hemodynamics
All rats remained normotensive (Table 1). Cardiac index was significantly increased in all AR compared with sham animals (+36%, P<0.05). We noted a trend toward lower heart rates in the metoprolol group that did not reach statistical significance. The stroke volumes and cardiac indexes of all metoprolol AR animals were comparable and remained significantly higher than those of normal controls.

Echocardiographic Evaluation of LV Function and Remodeling
Eccentric hypertrophy was present before drug treatment was started 14 days after AR, and ejection fraction was still in the normal range at that time (Table 2). After 180 days, all nontreated animals had developed significant LV hypertrophy compared with normal controls (sham) (LV mass index: AR, 2.8±0.1 mg/g versus sham, 1.8±0.1 mg/g, P<0.01). Metoprolol decreased the eccentric remodeling, eccentricity being defined as a decrease in RWT (RWT AR-metoprolol, 0.36±0.02 versus RWT in untreated AR, 0.30±0.01, P<0.05). There was a trend toward a decrease in calculated and measured LV mass in metoprolol-treated animals. AR resulted in a significant dilatation of the LV cavity, as shown in Figure 1. End-diastolic and end-systolic diameters increased in nontreated animals compared with controls. Meto-
prolol decreased the end-systolic dimensions but had a milder effect on diastolic dimensions. LV ejection fraction decreased significantly in untreated animals (Figure 1) but remained normal and similar to controls in rats treated with metoprolol. Diastolic filling parameters (Doppler E/A wave ratio of the mitral valve outflow) remained normal in 80% of the animals treated with metoprolol, compared with 60% in the non-treated group ($P<0.05$).

### Table 2. Echo Parameters Baseline at Week 2 Before the Initiation of Metoprolol vs Vehicle Alone ($n=28$)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Week 2</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>End-diastolic diameter, mm</td>
<td>7.8±0.10</td>
<td>9.6±0.10</td>
<td>&lt;0.0001</td>
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<tr>
<td>End-systolic diameter, mm</td>
<td>4.0±0.08</td>
<td>5.5±0.10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>72.3±1.49</td>
<td>66.9±1.11</td>
<td>0.01</td>
</tr>
<tr>
<td>Relative wall thickness</td>
<td>0.39±0.010</td>
<td>0.34±0.006</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LV mass index, mg/g</td>
<td>1.9±0.11</td>
<td>2.8±0.16</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

### Adrenergic System and Regulatory Pathways

Myocardial total catecholamines were elevated in all AR rats compared with normal controls (Figure 2A). Metoprolol treatment did not affect tissue catecholamines levels.

A decrease in total βAR density was recorded 2 days after AR induction, but this parameter returned to normal at day 14 and remained normal after 6 months (Figure 2B). Metoprolol treatment had no effect on β-receptor density, and $\beta_1/\beta_2$ ratio in the LV remained relatively stable (not shown). $\beta_1$-Adrenoreceptors ($\beta_1$AR) mRNA expression by RT-PCR was similar in non-treated AR animals compared with normal controls, whereas metoprolol treatment increased $\beta_1$AR expression. $\beta_2$AR mRNA levels remained unchanged (Figure 3).

In acute AR, GRKs 2 and 5 were upregulated in the LV (Figure 4). GRK2 protein content remained elevated after 6 months, whereas GRK5 levels were not statistically different from controls. Metoprolol treatment decreased the LV protein content of GRK 2 (Figure 4) as well as the GRK2 mRNA levels (not shown). A similar trend was observed for the expression of the Gs protein. Gs protein mRNA levels were significantly elevated in AR compared with sham (+45%,
and metoprolol treatment tended to attenuate this increase (17% versus AR, *P*<0.05). Myocyte Hypertrophy and Extracellular Matrix Remodeling (at 180 Days)

Myocyte cross-sectional area tended to be larger in AR animals compared with controls (+9%, *P*=NS). Metoprolol treatment decreased myocyte hypertrophy (−49% versus untreated AR, *P*=0.001).

Sarcomeric α-actin protein content was only moderately increased in AR LVs (at 180 days) (Figure 5). Conversely, smooth muscle α-actin protein levels were greatly increased in AR LVs. Metoprolol treatment did not reverse the effect of AR on either type of α-actin. The density of fibronectin-positive cells in the LV doubled in all AR animals. Metoprolol treatment had no effect on this parameter. AR rats had higher LV mRNA levels of fibronectin, collagens I and III, and MMP2 than sham-operated controls (Figure 5). Metoprolol treatment significantly decreased collagen I and III mRNA levels compared with untreated AR animals. β-Blockade normalized fibronectin mRNA levels but did not alter MMP2 expression in AR rats.

**Discussion**

In this study, we demonstrate for the first time in an animal model of chronic AR that a long-term treatment with a β-blocker can help prevent LV remodeling, maintain normal LV ejection fraction and filling parameters, prevent myocyte hypertrophy, and inhibit some aspects of extracellular matrix remodeling.

In heart failure, β-blockers protect the failing myocardium against catecholamine toxicity and counteract the neurohormones by improving βAR density and affinity and decreasing fibrosis and extracellular matrix remodeling. Significant abnormalities of the adrenergic system have also been identified in humans and animals submitted to chronic volume overload. Others have reported significant decreases...
in β-adrenoreceptor density in the LV of patients with chronic volume overload compared with pressure-overload and normal controls.23 In subjects with clinical heart failure from severe AR, intense adrenergic activity is present, as in other causes of heart failure.24 β-Blockers have been proved effective in the treatment of residual LV dysfunction in patients who underwent aortic valve replacement for symptomatic AR.25 However, the adrenergic system status and the efficacy of preventive β-blocker treatment in subjects with compensated AR are unknown. Small anecdotal studies in a few subjects with Takayasu arteritis and AR suggest that β-blockers are well tolerated and may decrease LV hypertrophy.26,27

β-Blockers have never been adequately tested in a chronic animal model of AR. In rabbits with severe AR, significant changes in catecholamine levels were reported.3–6 βARs are also significantly affected, as well as the activity of adenylate cyclase. It has been shown in a similar model that sympathectomy can protect against volume overload, thereby suggesting an important role of the sympathetic system in this hemodynamic state.7 In acute AR in rabbits, short-term β-blocker treatment (7 days) was well tolerated and decreased cardiac remodeling.8 β-Blockers have been studied in dogs with chronic mitral regurgitation, another form of volume-overload cardiomyopathy. In this model, β-blockers alone or in combination with an ACE inhibitor significantly improved LV function.13,14

β-Blockers are traditionally avoided in AR because it occurs in diastole, and it is feared that bradycardia might increase the regurgitant volume. β-Blockers are usually not given in patients with AR for fear of this bradycardia, but this relies on little scientific proof. Although regurgitant volume per beat may increase with severe bradycardia, total regurgitant volume per minute usually remains unchanged because the number of cardiac cycles per minute also decreases. There is a potential risk of increased afterload because of an increased stroke volume. In one study, however, bradycardial pacing in a model of severe AR unexpectedly improved cardiac work and myocardial capillary growth.12

Our animals tolerated very well the long-term β-blocker treatment at a relatively small dose for rats. Metoprolol treatment was effective against LV remodeling and helped preserve a normal ejection fraction and better filling parameters. It is not known whether higher doses of metoprolol would have the same beneficial effects.

These beneficial effects of metoprolol may be attributable to several factors. We observed only minor changes in the LV β-adrenergic density in AR rats, although metoprolol treatment increased β-receptor mRNA expression. This suggests an increased turnover of the receptor in this situation. However, most of the changes we observed took place downstream in the signaling pathway. The GRK system is considered to play a pivotal role in the desensitization and downregulation of G protein–coupled receptors. We thus were interested in investigating GRK 2 and 5 protein contents in the LV of our AR rats. Changes in the expression of the GRK2, GRK5, and G1 levels suggest an activation of the adrenergic system not only during the acute but also in the chronic phase because high levels of GRK2 persist even after 6 months. The presence of elevated LV tissue catecholamine concentration is also an indicator of neurohormonal activation. Metoprolol normalized the GRK2 expression. High levels of GRK2 have been associated with cardiac hypertrophy and heart failure.28 Inhibition of GRK2 activity in these models was shown to improve cardiac function and to elicit changes similar to β-blockade.29 Because β-adrenergic receptor internalization is necessary for activation of pathways that elicit cellular growth, the effect of metoprolol on GRK2 levels is of significant importance.28–31 The minimal effects of β-blockade on β-adrenergic receptor density in our model seem to come in contrast with previous observations made in animals and humans with congestive heart failure, in which β-blockade usually results in a significant increase in receptor density. Our animals were not in overt heart failure, however, and were still relatively early in the evolution of their disease. For these reasons, we did not expect dramatic variations
in receptor density, and, in fact, there were few variations of the overall density of β-adrenergic receptors throughout the protocol, even in untreated animals (Figure 2B). Our data suggest that β-blockade favorably affects the receptor’s turnover and its regulatory pathways. However, the exact mechanisms by which this occurs remain to be further explored. In our study, metoprolol treatment also reduced the cross-sectional area of cardiomyocytes. Although such effects of prolol treatment also reduced the cross-sectional area of cardiomyocytes, they have been shown in other models of cardiac diseases, this is the first time they have been shown in a model of chronic AR. Metoprolol also had a beneficial effect on extracellular matrix remodeling by inhibiting collagen and fibronectin expression. These parameters were increased in AR rats. An increase in the expression of MMP2 is also an indicator of an active remodeling process. The increase in the expression of collagens and fibronectin, as well as smooth muscle α-actin, suggests an increased number of fibroblasts in the AR rats. Metoprolol had no effect on the number of fibroblasts present in AR rats. We previously observed that the number of fibroblasts in the LV of AR rats rises abruptly in the first 2 weeks after AR, thus before the beginning of treatment in our study. Nevertheless, our results suggest that β-blockade alone can inhibit LV remodeling by inhibiting the expression of several extracellular matrix components despite the presence of an increased number of fibroblasts in the LV.

Study Limitations

Great care must be taken before results of animal studies are transposed to humans. Our model of AR was not designed to evaluate long-term morbidity and mortality and has significant differences compared with the disease in humans, as most animal models do. Drug dosages in rats and humans are radically different and cannot be interchanged. The doses of metoprolol we used induced only marginal reductions of heart rate in our animals. The effects of higher doses potentially resulting in significant bradycardia need to be assessed. Because most patients with severe AR who are not yet candidates for valve surgery use oral vasodilators, the combination of these drugs with β-blockers needs to be evaluated. Further studies will be performed to answer those questions.

Conclusions

In our animal model of chronic AR, a 6-month treatment with the β-blocker metoprolol prevented LV dilatation, preserved LV ejection fraction, helped maintain normal filling parameters, prevented myocyte hypertrophy, and inhibited some aspects of extracellular matrix remodeling. These results suggest an important role of the adrenergic system in the development of volume-overload cardiomyopathy associated with severe chronic AR.

Acknowledgments

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