Long-Term Endothelin Receptor Antagonist Administration Improves Alterations in Expression of Various Cardiac Genes in Failing Myocardium of Rats With Heart Failure

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Background—We reported that long-term (3-month) treatment with the endothelin (ET) type A (ET\textsubscript{A}) receptor antagonist BQ-123 markedly improved survival in rats with chronic heart failure (CHF). However, it is not known whether long-term treatment with an ET receptor antagonist improves alterations in the expression of cardiac genes in failing hearts.

Methods and Results—CHF rats and control sham-operated rats were treated with BQ-123, SB209670 (ET\textsubscript{A/B} dual receptor antagonist), or saline (vehicle) for 3 months. The survival of CHF rats was markedly higher in the BQ-123 or SB209670 treatment group than in the saline treatment group. The changes in the gene expression of classic molecular markers for failing hearts (mRNA levels of atrial natriuretic peptide and \(\beta\)-myosin heavy chain) were greatly inhibited by BQ-123 or SB209670 treatment in CHF rats. Long-term BQ-123 treatment also normalized the alterations in the expression of functional molecular markers in failing hearts (e.g., mRNA levels of ryanodine receptor, sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase, angiotensin-converting enzyme, angiotensin II type 1 receptor, and prepro-ET-1).

Conclusions—We demonstrated for the first time that long-term (3-month) treatment with an ET receptor antagonist improves the alterations in the expression of various cardiac genes of classic molecular markers (e.g., mRNA in atrial natriuretic peptide and \(\beta\)-myosin heavy chain) and of functional molecular markers (e.g., mRNA levels of ryanodine receptor, sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase, angiotensin-converting enzyme, angiotensin II type 1 receptor, and prepro-ET-1) in the failing hearts of CHF rats, suggesting that the great improvement of survival in CHF rats by an ET blocker is partly attributed to the prevention of molecular changes in failing hearts. (Circulation. 2000;101:2849-2853.)

Key Words: endothelin • heart failure • genes

Endothelin (ET)-1, a potent vasoconstrictive peptide produced by endothelial cells,\textsuperscript{1} is also produced by cardiac myocytes.\textsuperscript{2} ET-1 induces cardiac hypertrophy\textsuperscript{3} and cellular injury in cardiac myocytes\textsuperscript{4} in addition to its potent positive inotropic and chronotropic actions.\textsuperscript{2} We and other groups have reported that plasma ET-1 concentrations are elevated in heart failure in humans\textsuperscript{5,6} and in experimental animal models.\textsuperscript{7}

We have reported that the production of ET-1 is markedly increased in the failing hearts of rats with chronic heart failure (CHF) due to chronic myocardial infarction and that the enhancement of myocardial ET-1 contributes to the modulation of cardiac function in the failing heart.\textsuperscript{8} Moreover, we have reported that chronic (3-month) treatment with the ET type A (ET\textsubscript{A}) receptor antagonist BQ-123 greatly ameliorates long-term survival and hemodynamic parameters in rats with CHF.\textsuperscript{9} Mulder et al\textsuperscript{10} have also reported that the ET\textsubscript{A/B} dual receptor antagonist bosentan improves long-term survival in rats with CHF. Regarding the effect of an ET receptor antagonist on altered gene expression in failing myocardium, only 1 study exists,\textsuperscript{11} and it demonstrates that medium-term (2-week) treatment with an ET receptor antagonist (bosentan) does not alter gene expression in failing myocardium even though this same treatment has been shown to ameliorate the hemodynamics and ventricular remodeling in rats with heart failure.\textsuperscript{11} Although there are several reports showing the effectiveness of the blockade of ET receptors in ameliorating survival,\textsuperscript{9,10,12,13} hemodynamic features,\textsuperscript{9,10,12–14} and histological features by use of Masson’s trichrome staining in heart preparations from animals with heart failure,\textsuperscript{9} to date, there is no report indicating how long-term blockade of ET receptors affects cardiac gene expression in the failing myocardium. The present study was designed to answer this question.

In the failing myocardium resulting from CHF, it is reported that qualitative changes occur, such as the switching of some sarcomere proteins associated with cardiac hypertrophy,\textsuperscript{15} abnormalities of intracellular Ca\textsuperscript{2+} handling,\textsuperscript{16} and the activation of several neurohumoral factors\textsuperscript{17} produced in the

Received August 17, 1999; revision received January 10, 2000; accepted January 31, 2000.
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heart. Furthermore, it has been reported that the above qualitative changes are attributed to alterations in the expression of various cardiac genes in the failing heart. Al-though these qualitative changes are regarded as a compensatory mechanism for cardiac failure, they have an aspect of promoting the deterioration of the failing heart.

In the present study, the effects of long-term (3-month) ET-1 blockade on the changes in the gene expression of classic molecular markers in the failing heart (mRNA levels of atrial natriuretic peptide [ANP] and β-myosin heavy chain [β-MHC]) were studied with the use of the ET\(_A\) receptor antagonist BQ-123 or the ET\(_{AXB}\) dual receptor antagonist SB209670, and the changes in the gene expression of functional molecular markers in the failing heart (mRNA levels of ryanodine receptor, sarcoplasmic reticulum Ca\(^{2+}\)-ATPase [SERCA], ACE, angiotensin II type 1 [AT\(_1\)] receptor, and prepro-ET-1) were studied with the use of BQ-123 in CHF rats.

**Methods**

**Experimental Heart Failure and Study Protocol**

Left ventricular (LV) free-wall myocardial infarction was induced in 6-week-old male rats (180 to 210 g) by the method of Pfeffer et al., the details of which can be found in our previous reports. To investigate the effects of long-term administration of the ET\(_A\) receptor antagonist BQ-123, which was a gift from Dr Masaru Nishikibe (Tsukuba Research Institute, Banyu Pharmaceutical Co, Tsukuba, Japan), and the ET\(_{AXB}\) dual receptor antagonist SB209670, which was a gift from Dr Eliot Ohlstein (SmithKline Beecham Pharmaceuticals, King of Prussia, Pa), on the survival of rats with CHF, an osmotic minipump was subcutaneously implanted. These rats were called CHF rats. Both compounds were dissolved in saline. Because there was a possibility that the infarct size of rats with coronary artery ligation would be affected by blocking of the ET-1 pathway in the acute phase of myocardial infarction, we started BQ-123 or SB209670 administration 10 days after surgery to induce myocardial infarction. The rats were divided into the following 4 groups: (1) sham+saline group, consisting of sham-operated rats receiving saline infusion for 12 weeks; (2) CHF+saline group, consisting of CHF rats receiving saline infusion for 12 weeks; (3) CHF+BQ-123 group, consisting of CHF rats receiving BQ-123 infusion (7.5 mg/d per rat) for 12 weeks; and (4) CHF+SB209670 group, consisting of CHF rats receiving SB209670 infusion (1.0 mg/d per rat) for 12 weeks.

**Hemodynamic Measurement and Tissue Sampling**

Twelve weeks after the administration of BQ-123, SB209670, and saline was begun, the hemodynamic parameters of all surviving rats were measured according to the methods described in our previous reports. After hemodynamic measurements, the heart was excised and divided into the right ventricle and LV, including the septum, and a part of this sample was frozen in liquid nitrogen for determination of the mRNA expression of several cardiac genes. These LV samples were stored at −80°C until use. Moreover, part of the LV samples obtained just after the animals were euthanized were used for the evaluation of the production of inositol phosphates in the heart.

**Quantification of mRNA Levels by Reverse Transcription and PCR**

The mRNA levels of ANP, α-MHC, β-MHC, ryanodine receptor, SERCA, ACE, AT\(_1\) receptor, and prepro-ET-1 were analyzed by reverse transcription–polymerase chain reaction (PCR). The expression of GAPDH was also determined as an internal control.
Total RNA from LV was isolated, and total RNA (10 μg) was primed according to methods described in our previous studies.12,21–23 cDNA was diluted 1:10, and 1 μL was used for PCR. Each PCR reaction contained 0.5 μmol/L of each specific primer and 0.025 U/μL Taq polymerase (Takara Ltd), as described in our previous studies.24–26 The sequences of the specific primers were as follows: ANP (sense), 5'-ATGGGCTCTTTCCACACC-3'; ANP (antisense), 5'-TCCGGCTGGCCTCAATCCGT-3'; ACE (sense), 5'-GCTCAGAACAAGGCGACACT-3'; and ACE (antisense), 5'-ACACCTTTTGGAAGACCTTT-3'. PCR was carried out by use of a TaKaRa Thermal Cycler MP (TP-3000, TaKaRa Ltd), as described in our previous studies.24–26

Distinction between α-MHC and β-MHC was determined by a previously described method.24 The identical sequences of the oligonucleotides for α-MHC and β-MHC were as follows: MHC (sense), 5'-GAGCTTGGCGATCTTG-3'; GAPDH (antisense), 5'-CTGGCAATGTAATGC-3'; and GAPDH (sense), 5'-GAGCTTGGCGATCTTG-3'. PCR was carried out by use of TaKaRa PCR Thermal Cycler MP (TP-3000, TaKaRa Ltd), as described in our previous studies.24–26

Physiological Responses to AT1 Receptor Stimulation in Hearts of CHF Rats With and Without Treatment by ET Receptor Antagonist

Angiotensin II (Ang II)–induced changes in phosphoinositol turnover were studied in CHF rats with and without BQ-123 treatment. Ang II–stimulated phosphoinositol turnover was measured by the method of Meggs et al.25

Data Statistics

Data are expressed as mean±SEM. One-way ANOVA followed by a post hoc test was used for statistical comparisons among the various treatment groups (Figures 3 and 4). The survival data are presented as Kaplan-Meier curves and compared by the log-rank test. Differences were considered significant at P<0.05.

Results

Effect of Long-Term BQ-123 or SB209670

Treatment on Long-Term Survival and Hemodynamic Parameters

The 12-week survival rates of CHF rats treated with BQ-123 (85%; 11 of 13 CHF rats survived) and of CHF rats treated with SB209670 (80%; 16 of 20 CHF rats survived) were significantly higher than those of CHF rats treated with saline (43%; 6 of 14 CHF rats survived), as seen in Figure 1. The mean myocardial infarct size did not differ among the 3 groups. The survival curve for BQ-123–treated rats is identical to that shown in Figure 1 of Reference 9. The survival curve for SB209670-treated CHF rats is shown for the first time in Figure 1 of the present study.

In sham-operated rats (n=8), the values of LV +dP/dt max and LV −dP/dt max were 8485±461 and −7928±659 mm Hg/s, respectively. In CHF rats treated with BQ-123, the magnitudes of LV +dP/dt max and LV −dP/dt max (6912±244 mm Hg/s [n=8] and −5229±336 mm Hg/s [n=8], respectively) were significantly higher than the respective magnitudes in CHF rats treated with saline (5692±196 mm Hg/s [n=6], P<0.01; −3884±231 mm Hg/s [n=6], P<0.05). In CHF rats treated with SB209670, LV +dP/dt max was significantly higher (6577±256 mm Hg/s [n=16], P<0.05) than LV +dP/dt max in CHF rats treated with saline. Thus, long-term administration of BQ-123 or SB209670 greatly and comparably improved the survival and hemodynamic parameters in CHF rats.

Effect of Long-Term BQ-123 or SB209670

Treatment on Expression of Various Cardiac Genes in Hearts of Rats With CHF

The expression of ANP mRNA in the LV of CHF rats was markedly higher than that in the LV of sham-operated rats, and long-term BQ-123 or SB209670 treatment inhibited the increase in the expression of ANP mRNA in CHF rats (Figures 2 and 3A). The ratio of the expression of β-MHC mRNA to α-MHC mRNA in the LV of CHF rats was markedly higher than that in the LV of sham-operated rats, and BQ-123 or SB209670 treatment prevented the alteration of this ratio in CHF rats (Figures 2 and 3B).

The expression of ryanodine receptor mRNA in the LV of CHF rats was significantly lower than that in the LV of sham-operated rats, and chronic BQ-123 treatment normal-
ized the expression in CHF rats (Figures 2 and 4A). The expression of SERCA mRNA in the LV of CHF rats was also significantly lower than that in the LV of sham-operated rats, and BQ-123 also reversed the alteration of the expression in CHF rats (Figures 2 and 4B).

The expression of ACE and AT₁ receptor mRNA in the LV of CHF rats was significantly higher than that in the LV of sham-operated rats, and chronic BQ-123 treatment normalized these expressions in CHF rats (Figures 2, 4C, and 4D).

The expression of prepro-ET-1 mRNA in the LV of CHF rats was markedly higher than that in the LV of sham-operated rats, and chronic BQ-123 treatment normalized the expression of prepro-ET-1 mRNA in CHF rats (Figures 2 and 4E).

Physiological Responses to AT₁ Receptor Stimulation in Hearts of CHF Rats With and Without Treatment by ET Receptor Antagonists

Ang II was found to stimulate phosphoinositol turnover by 17% in LV myocytes of sham-operated rats (n=8), by 51% in LV myocytes of CHF rats treated with saline (n=6), and by 19% in LV myocytes of CHF rats treated with BQ-123 (n=8). These findings indicate that the alteration of Ang II–stimulated phosphoinositol turnover corresponds to the change at the mRNA level of AT₁ receptors.

Discussion

In the present study, we have demonstrated for the first time that long-term (3-month) treatment with an ET receptor antagonist improves the alteration in the expression of various cardiac genes (e.g., mRNA levels of ANP, β-MHC, ryanodine receptor, SERCA, ACE, AT₁ receptor, and prepro-ET-1) in the hearts of CHF rats; this alteration is a phenomenon that occurs in the failing heart. These findings are the major novelty of the present study. For these experiments, the ETA receptor antagonist BQ-123 and the ET A/B dual receptor antagonist SB209670 were used, and we investigated the expression of various cardiac genes (classic molecular markers and functional molecular markers in the failing heart) in the LVs of rats that survived. The changes in the gene expression of classic molecular markers in the failing heart (mRNA levels of ANP and β-MHC) were greatly inhibited by BQ-123 or SB209670 treatment in CHF rats. We also investigated whether an ET receptor antagonist improves the alteration of functional molecular markers in the failing heart by use of BQ-123; in the failing hearts of CHF rats, long-term BQ-123 treatment normalized the alteration of the expression of mRNA levels of the ryanodine receptor, SERCA, ACE, AT₁ receptor, and prepro-ET-1. At present, because only a few drugs (e.g., ACE inhibitors) are reported to be effective in the amelioration of cardiac gene expression in the failing heart, the present finding indicating that long-term treatment with an ET receptor antagonist improves the alterations in the expression of various cardiac genes in the failing heart is considered to be very important in the treatment of heart failure.

Another important new finding of the present study is that long-term (3-month) administration of BQ-123 or SB209670 greatly and comparably improves the long-term survival and hemodynamic parameters in rats with CHF. Therefore, the present data suggest that the use of an ET₁-selective receptor antagonist or an ET A/B dual receptor antagonist could become a beneficial strategy in the treatment of CHF. Indeed, in the present study, the changes in the gene expression of classic molecular markers in the failing heart (mRNA levels of ANP and β-MHC) were greatly inhibited by BQ-123 or SB209670 treatment in CHF rats.

It has been reported that the ET A/B dual receptor antagonist bosentan was effective in ameliorating the survival of rats with heart failure due to hypertension caused by salt loading despite the finding that the α₁-adrenergic receptor antagonist doxazosin, a potent vasodilator, did not affect the survival of these rats. Therefore, it is considered that the beneficial
effects of bosentan in these rats are mainly attributable to the direct humoral effects on the cardiomyocytes and that the contribution of the vasodilatory effect is minimal.

In the present study, the expression of ryanodine receptor mRNA and of SERCA mRNA was significantly lower in the hearts of CHF rats than in the hearts of sham-operated rats, and the expression of these genes was significantly higher in CHF rats treated with BQ-123 than in CHF rats treated with saline. Systolic and diastolic functions were improved in CHF rats treated with BQ-123 compared with CHF rats treated with saline. Thus, it is suggested that improvement in the impaired intracellular Ca²⁺ mobilization by chronic BQ-123 administration occurs and that this improvement results in the amelioration of hemodynamics in CHF rats; i.e., there is improvement in systolic and diastolic function that is brought about by BQ-123 treatment.

The expression of ACE mRNA and AT₁ receptor mRNA is significantly increased in the failing hearts of CHF rats, and BQ-123 administration prevents these increases, suggesting that the enhancement of the cardiac renin-angiotensin system in these CHF rats is inhibited by BQ-123 administration.

Furthermore, we revealed for the first time that long-term BQ-123 treatment normalized the increase in the expression of cardiac prepro-ET-1 mRNA in the failing heart. Because ET-1 itself induces prepro-ET-1 mRNA expression and BQ-123 inhibits ET-1–induced prepro-ET-1 mRNA expression, we propose that BQ-123 interferes with the cascade of the autoenhancement by ET-1 in the failing heart.

In conclusion, we demonstrate for the first time that long-term (3-month) treatment with an ET receptor antagonist improves the alterations in the expression of various cardiac genes of classic molecular markers (eg, mRNA levels of ANP and β-MHC) and of functional molecular markers (eg, mRNA levels of the ryanodine receptor, SERCA, ACE, AT₁ receptor, and prepro-ET-1) in the failing hearts of rats with CHF. Therefore, it is proposed that the great improvement of survival and hemodynamic parameters in CHF rats after long-term treatment with an ET receptor antagonist is partly attributed to the prevention of molecular changes in the failing heart.

Acknowledgments

This study was supported by a grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan (9770473, 10670629, and 11557047), by a research grant from Japan Heart Foundation, by a grant from the Uehara Memorial Foundation, and by a grant from the Miyachi Project from Tsukuba Center for Advanced Research at University of Tsukuba.

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_Circulation_. 2000;101:2849-2853
doi: 10.1161/01.CIR.101.24.2849

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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