Cardioprotection by long-term ET<sub>A</sub> receptor blockade and ACE inhibition in rats with congestive heart failure: mono- versus combination therapy

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Abstract

Objectives: We investigated the effects of long-term endothelin A (ET<sub>A</sub>) receptor blockade and ACE inhibition, either alone or in combination, on the hemodynamics, neurohormonal activation and cardiac remodeling in rats with congestive heart failure (CHF) after extensive myocardial infarction (MI).

Methods: Rats were treated with placebo, the ET<sub>A</sub> antagonist LU135252 (30 mg/kg/d), the ACE inhibitor trandolapril (0.3 mg/kg/d), or a combination of both for 11 weeks, starting 7 days after MI.

Results: Despite comparable effects on left ventricular (LV) systolic pressure among all drug treatments, only combined ET<sub>A</sub> and ACE inhibition significantly reduced LV end-diastolic pressure (P<0.01), improved LV dP/dt<sub>max</sub> (P<0.01) and normalized sympathetic activation (P<0.05) in rats with CHF. The combination therapy was more effective in reducing type I and III collagen mRNA levels, MMP-2 zymographic activity and collagen accumulation in the surviving LV myocardium. Moreover, the increases in cardiac β-myosin heavy chain and skeletal α-actin mRNAs, markers of hypertrophy or failure, were attenuated to a greater degree by the combination therapy than monotherapy, whereas right ventricular hypertrophy and ANF mRNA upregulation were significantly (P<0.01) prevented only by combined ET<sub>A</sub> and ACE inhibition.

Conclusion: Long-term combined ET<sub>A</sub> receptor and ACE inhibition improved cardiac failure after extensive MI more effectively than monotherapy. We show additive effects on LV fibrosis and fetal gene expression. ET<sub>A</sub> receptor antagonists could be a therapeutic option in CHF in addition to an ACE inhibitor.

Keywords: Infarction; Heart failure; Endothelins; Angiotensin; Fibrosis

1. Introduction

Left ventricular (LV) remodeling after myocardial infarction (MI) involves myocyte hypertrophy and chamber dilation. In addition, increased collagen accumulation remote from the infarct site increases myocardial stiffness and contractile dysfunction, and contributes to the progression of ventricular enlargement and heart failure [1–4].

The beneficial effects of ACE inhibitors after MI are related to peripheral vasodilatation, ventricular unloading, and reduction of myocyte hypertrophy and interstitial fibrosis [5–8]. Although ACE inhibitors improve symptoms and reduce overall mortality, heart failure remains a progressive disease process and is the leading cause of death in humans. Thus, the development of novel therapeutic strategies for heart failure represents an important priority in cardiovascular medicine.

Plasma endothelin-1 (ET-1) levels are elevated in patients with heart failure and correlated with the severity of the disease [9]. In addition, the local ET system is activated in the failing heart [10,11] and endothelin is involved in postinfarct remodeling [10–14]. Whether ET<sub>A</sub> receptor antagonists provide a benefit additional to ACE inhibitor treatment in congestive heart failure (CHF) remains unknown.

The aim of this study was to analyze the long-term effects of an ET<sub>A</sub> receptor blocker (LU135252), an ACE

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inhibitor (trandolapril), and their combination, on hemodynamics and LV remodeling (fibrosis, hypertrophy, fetal gene expression) in rats with CHF after extensive MI. In addition, we studied the effects of pharmacologic interventions on neurohormonal activation and right ventricular hypertrophy.

2. Methods

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

2.1. Myocardial infarction, study protocols, hemodynamic measurements

Left coronary artery ligations [11] were performed in adult male Wistar rats (200–250 g). Briefly, under ether anaesthesia, the thorax was opened, the heart exteriorised, and a ligature placed around the proximal left coronary artery. The heart was returned to its normal position and the thorax closed. Mortality was 40% within the first 24 h. Sham-operated controls (n=10) underwent the same surgical procedure except that the suture around the coronary artery was not tied. Of the 140 rats that had undergone left coronary artery ligation, 66 died before the seventh postoperative day. Starting on the seventh postoperative day, sham-operated animals received placebo treatment, and surviving MI rats (74) were allocated randomly to one of the following four treatment groups: placebo (n=20), the ET\textsubscript{A} antagonist LU135252 (30 mg/kg/d, n=18), the ACE inhibitor trandolapril (0.3 mg/kg/d, n=18), or a combination of LU135252+trandolapril (n=18) given in drinking water according to the manufacturer’s instructions (Knoll AG). Twenty-three rats died after randomization during the 11 weeks of treatment: placebo (n=7), LU135252 (n=6), trandolapril (n=5), and the LU135252+trandolapril combination (n=5).

2.2. Sample collection, infarct size, ventricular dilation

The heart was divided into right ventricle and LV, including septum, in ice-cold saline. The LV was cut into three transverse sections: apex, middle ring (~3 mm), and base. From the middle ring, 5-μm sections were cut at 100-μm intervals and stained with picrosirius red. The boundary lengths of the infarcted and non-infarcted endocardial and epicardial surfaces were traced with a planimeter digital image analyser (Sony). Infarct size was calculated as the average of all slices and only rats with extensive infarcts (>45%) were included in the study (placebo, n=8; LU135252, n=7; trandolapril, n=7; and LU135252+trandolapril, combination, n=9). LV cavity area (area enclosed by LV endocardial circumference) normalized by body weight was taken as an index of LV dilation [15].

2.3. Quantification of cardiac gene expression

Total RNA was isolated from surviving LV (septum) and right ventricular (RV) myocardium using TRIzol reagent (Life Technologies). Transforming growth factor-β\textsubscript{1} (TGF-β\textsubscript{1}), collagen α1(I) and α1(III), atrial natriuretic factor (ANF) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) gene expression was determined by competitive polymerase chain reaction (PCR) (Table 1). Heterologous internal standards were constructed with a Competitive DNA PCR Kit (Takara Shuzo) and purified using a PCR Purification Kit (QIAGEN). Products of PCR amplification were separated on 2% agarose gel. A given mRNA level was expressed as a ratio with respect to the level of mRNA for GAPDH. α- and β-myosin heavy chain (MHC) mRNA and skeletal and cardiac α-actin mRNA were amplified by PCR as previously described [16] using DIG-labeled forward primers. After digestion with the restriction en-

Table 1

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequences (5’–3’)</th>
<th>Genebank Acc. No.</th>
<th>PCR product size (bp)</th>
<th>T\textsubscript{m} (°C) cycles</th>
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<tr>
<td>Collagen α1(I)</td>
<td>TGCCGTGACCTCAAGATGTG-se CACAACGTGCTGTAGGTGA-as</td>
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zyme Tru9I for MHC and SacI for α-actin, fragments of the PCR amplification product were separated, respectively, on 8% and 6% polyacrylamide gel. The ratio of β- to α-MHC mRNA and skeletal to cardiac α-actin mRNA was quantified using chemiluminescence.

2.4. Myocardial hydroxyproline, collagen phenotypes

For hydroxyproline determination, LV myocardial samples (septum) were freeze-dried, weighed and hydrolyzed in 6 N HCl at 110 °C for 24 h. Hydroxyproline concentration was measured spectrophotometrically [17]. Collagen content was expressed in μg/mg dry tissue weight assuming that collagen contains an average of 13.4% hydroxyproline.

Collagen extraction and phenotyping was performed as described [18]. Briefly, after removal of the bulk of noncollagen protein with SDS (2%), LV (septum) collagen was digested with cyanogen bromide in formic acid (70% vol/vol) for 18 h at 25 °C. The supernatant was dried, directly dissolved in loading buffer and electrophoresed on a 12% SDS–polyacrylamide gel. The amount loaded was determined by hydroxyproline determination. After electrophoresis, the gel was stained with Coomassie blue R250 (0.125%). The relative amounts of type I/III collagen were determined from the relationship between the relative area under the densitometry curve corresponding to band G [αI(1)-CB-8] for collagen type I and band M [αI(111)-CB-5 plus αI(III)-CB-9] for collagen type III.

2.5. Tissue homogenization, zymography, reverse zymography

LV myocardial samples (septum) were homogenized in ice-cold extraction buffer [19], incubated with continuous agitation for 20 h and centrifuged at 8000×g for 30 min at 4 °C. Proteins were determined by Bradford assay. Observed collagen fragments were separated, respectively, on 8% and 6% polyacrylamide gel containing gelatin (type A from porcine skin) under nonreducing conditions [20]. Reverse zymography was performed in a similar manner except that purified MMP-2 (30 μg/ml, Oncogene) was incorporated into a 15% SDS–polyacrylamide gel along with 1 mg/ml gelatin as described [21]. Human recombinant purified enzymes (MMP-2, TIMP-2, Oncogene) were used as a positive control and standardization among gels. Molecular weights were determined using prestained SDS–PAGE standard and precision protein standards (Bio-Rad).

2.6. Neurohormonal assay

After hemodynamic measurement, a blood sample was collected from the right carotid artery. Plasma renin activity (PRA) and aldosterone levels were measured by radioimmunoassay (Sorin Biomedica). Plasma norepinephrine levels were measured with high-performance liquid chromatography and plasma ET-1 levels by radioimmunoassay, as reported [11].

2.7. Statistical analysis

Statistical analysis was performed by two-factor ANOVA followed by the least-squares mean test. Results are reported as mean±S.E.M. P<0.05 was considered statistically significant.

3. Results

3.1. Global parameters

Infarct size and body weight were similar among the experimental groups (Table 2). Trandolapril monotherapy and combination therapy attenuated the rise in LV weight observed in CHF placebo rats. RV weight was markedly higher in placebo-treated CHF rats compared with sham-operated controls and was significantly reduced only by combined ET₄ receptor and ACE inhibition. CHF resulted...

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<td>Global parameters in rats with congestive heart failure 12 weeks after myocardial infarction compared with control</td>
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<td>---------------------------------</td>
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<tr>
<td>Sham (n=10)</td>
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<tr>
<td>Infarct size (%)</td>
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<td>BW (g)</td>
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<td>RV/BW (mg/g)</td>
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<td>LV/BW (mg/g)</td>
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<td>LVCA/BW (mm²/g)</td>
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<td>LVSP (mmHg)</td>
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<td>MAP (mmHg)</td>
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<td>RAP (mmHg)</td>
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<td>Heart rate (bpm)</td>
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LVSP, left ventricular systolic pressure; MAP, mean arterial pressure; RAP, right atrial pressure; LVCA, left ventricular cavity area. Values are mean±S.E.M. *P<0.05, **P<0.001 vs. sham; †P<0.05, ‡P<0.01, ††P<0.001 vs. placebo CHF.
in an increase in LVCA, which was significantly attenuated by both monotherapies and further reduced by combined ET<sub>a</sub> and ACE inhibition (Table 2).

3.2. Hemodynamics

LV systolic pressure (LVSP) and mean arterial pressure (MAP) were reduced in CHF rats on placebo compared with sham-operated animals and were slightly lowered further to a comparable degree in all active treatment groups. Right atrial pressure (RAP) was elevated in untreated CHF rats and reduced more significantly by combination therapy than monotherapies (Table 2). CHF rats developed elevated LV end-diastolic pressure (LVEDP), which was significantly reduced by combination therapy with LU135252 and trandolapril (Fig. 1). LV dP/dt<sub>max</sub> was reduced in CHF placebo rats, tended to be higher in LU135252- and trandolapril-treated animals, and was significantly improved only in the combined treatment group (Fig. 1).

3.3. Left ventricular collagen and TGF-β<sub>1</sub> gene expression

LV collagen type I and type III mRNA levels (Fig. 2) were increased in CHF rats and significantly reduced in all treatments groups. However, combined ET<sub>a</sub> and ACE inhibition suppressed the upregulation of LV collagens more than the individual agents. LV dP/dt<sub>max</sub> significantly correlated with the reduction in collagen type I (P<0.001) and type III (P<0.05) mRNA levels. TGF-β<sub>1</sub> mRNA levels markedly increased in the LV of placebo CHF rats compared with controls and were lowered by all drug treatments. TGF-β<sub>1</sub> gene expression closely correlated with collagen type I (r=0.81, P=0.0001) and type III (r=0.90, P=0.0001) mRNA levels.

3.4. Left ventricular collagen concentration and phenotypic ratio

LV collagen concentrations were markedly increased in CHF placebo rats. Treatment with LU135252 or trandolapril alone was associated with a significant reduction in collagen concentration. Combination therapy further suppressed collagen accumulation in the failing LV myocardium (Fig. 2). Enhanced ratios of collagen type I/III were observed in CHF rats compared with controls (2.48±0.08 versus 1.96±0.04, P<0.005). All drug treatments significantly reduced the collagen phenotype ratio compared with CHF placebo (LU CHF, 2.11±0.12; T CHF, 2.21±0.05; LU+T CHF, 2.19±0.06; P<0.05).

3.5. Left ventricular MMP-2 activity and TIMP-2 inhibitory activity

LV MMP-2 (66- and 58-kDa gelatinases) activity was increased in placebo CHF rats compared with controls (382±17 versus 248±7 densitometric units/μg protein, P<0.001). This increase was blunted by ET<sub>a</sub> receptor blockade (330±24, P=0.07), significantly reduced by ACE inhibition (313±21, P<0.05) and further reduced by combination therapy (300±22, P<0.01). TIMP-2 (21 kDa) inhibitory activity was higher in CHF rats than in sham-operated rats (29.2±3 versus 14.7±2 densitometric units/μg protein, P<0.05) and was similarly enhanced in all treatment groups (LU CHF, 29.9±5; T CHF, 32.1±4; LU+T CHF, 27.2±3).

3.6. Left and right ventricular fetal gene expression

mRNA expressions of α-actins, MHCs and ANF were examined as molecular markers of the hypertrophic response to CHF. The ratios of LV skeletal to cardiac α-actin
mRNA and LV β-MHC to α-MHC mRNA were substantially increased in placebo CHF rats, significantly attenuated by trandolapril monotherapy and further reduced by combined ET\textsubscript{A} and ACE inhibition (Fig. 3). RV ANF mRNA expression was markedly higher in CHF rats than in controls and was significantly reduced only by combined ET\textsubscript{A} receptor and ACE inhibition. Moreover, only combined ET\textsubscript{A} and ACE inhibition prevented the CHF-associated rise in the RV ratio of skeletal to cardiac α-actin mRNA (Fig. 4). The ratio of β-MHC to α-MHC mRNA was increased in RV and significantly reduced by ACE inhibition alone. However, combination therapy led to a
substantial further decrease in the RV $\beta$- to $\alpha$-MHC ratio compared with trandolapril monotherapy (Fig. 4).

4. Discussion

The major novel findings of this study are the following. (1) The combination of an ET$_A$ receptor antagonist and an ACE inhibitor reduced LV end-diastolic pressure, improved cardiac function and normalized sympathetic activation in rats with CHF after extensive MI. The combination was more effective in reducing cardiac fibrosis and fetal gene expression than the individual drugs. (2) The rise in RV weight and ANF mRNA associated with CHF was prevented only by the combination. (3) ET$_A$ receptor blockade alone attenuated gene expression of fibrillar type I/III collagens and of TGF-$\beta_1$ in the surviving LV myocardium of CHF rats.

4.1. Left ventricular hemodynamics

Coronary artery ligation in the rat produces a broad spectrum of cardiac dysfunction, ranging from minor impairment to overt heart failure [7,22], depending on MI size. Several studies in rats have demonstrated that monotherapy with either ACE inhibitors [7,8] or ET receptor antagonists after MI provided beneficial effects on LV hemodynamics and remodeling [10–13]. In rats with extensive MI, which mimics severe CHF, the effect of ACE inhibition was minor [7]. We show that the combina-
receptor blockade [12,13]. We show for the first time that ET\textsubscript{A} receptor blockade effectively decreased collagen type I and III mRNA expression in the LV myocardium, leading to reduced collagen deposition. The close correlation of the reduction in collagen I/III expression by ET\textsubscript{A} receptor blockade with TGF-β\textsubscript{1} gene expression, a cytokine known to play a crucial role in mediating collagen synthesis [1,4], suggests a mechanistic interdependence. ET\textsubscript{A} receptor inhibition also reversed the rise in the ratio between collagen type I and collagen type III at the protein level. It has previously been shown that ET-1 increased cardiac fibroblast collagen synthesis [28], and activated the procollagen I promoter [29]. Moreover, involvement of ET-1 in cardiac collagen deposition in mineralocorticoid hypertension has been suggested by reduction of procollagen synthesis after ET\textsubscript{A} receptor blockade [30]. Our observations provide evidence that the upregulated cardiac endothelin system plays an important role in the pathogenesis of postinfarction reactive LV fibrosis, by promoting collagen gene expression.

The combination therapy most effectively normalized LV collagen expression and accumulation. Alterations in the collagen matrix raise cardiac muscle stiffness and impair LV performance, leading to progressive dysfunction and heart failure [1]. It has been proposed that fibrosis in the remote noninfarcted myocardium is a major determinant of ventricular remodeling in ischemic cardiomyopathy [2]. Therefore, the more marked improvement of LV remodeling and failure by combined ACE and ET\textsubscript{A} inhibition may be mediated, in part, by the reduction of LV fibrosis.

Upregulation of MMPs also plays an important role in LV collagen expression and accumulation. Alterations in the collagen matrix raise cardiac muscle stiffness and impair LV performance, leading to progressive dysfunction and heart failure [1]. It has been proposed that fibrosis in the remote noninfarcted myocardium is a major determinant of ventricular remodeling in ischemic cardiomyopathy [2]. Therefore, the more marked improvement of LV remodeling and failure by combined ACE and ET\textsubscript{A} inhibition may be mediated, in part, by the reduction of LV fibrosis.

4.2. Left ventricular fibrosis

Remodeling post-MI is characterized by increased TGF-β\textsubscript{1} and deposition of collagen in residual LV myocardium [1], which is reduced by ACE inhibition [6,8] or ET receptor blockade [12,13]. We show for the first time that ET\textsubscript{A} receptor blockade effectively decreased collagen type I and III mRNA expression in the LV myocardium, leading to reduced collagen deposition. The close correlation of the reduction in collagen I/III expression by ET\textsubscript{A} receptor blockade with TGF-β\textsubscript{1} gene expression, a cytokine known to play a crucial role in mediating collagen synthesis [1,4], suggests a mechanistic interdependence. ET\textsubscript{A} receptor inhibition also reversed the rise in the ratio between collagen type I and collagen type III at the protein level. It has previously been shown that ET-1 increased cardiac fibroblast collagen synthesis [28], and activated the procollagen I promoter [29]. Moreover, involvement of ET-1 in cardiac collagen deposition in mineralocorticoid hypertension has been suggested by reduction of procollagen synthesis after ET\textsubscript{A} receptor blockade [30]. Our observations provide evidence that the upregulated cardiac endothelin system plays an important role in the pathogenesis of postinfarction reactive LV fibrosis, by promoting collagen gene expression.

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Upregulation of MMPs also plays an important role in LV dilation during heart failure [31,32]. Both ET-1 and angiotensin II increase MMP activity in isolated myocytes [33]. Accordingly, in the present study the addition of the ET\textsubscript{A} antagonist to ACE inhibition further reduced MMP-2 activity in the surviving LV myocardium, thus contributing to improved LV remodeling.

4.3. Fetal gene expression

Loading conditions [34], angiotensin II [6,35] and ET-1 [16,36] are involved in fetal gene re-expression of hypertrophic myocytes. Downregulation of α-MHC coupled with upregulation of β-MHC [37,38] has been demonstrated in failing human myocardium. Enhanced expression of fetal isoforms of contractile proteins may play a critical role in the impairment of cardiac performance and pathophysiology of heart failure [6,37]. Ruf et al. provided evidence that alterations of cross-bridge kinetics may reflect the mechanical and energetic consequences of α-MHC downregulation in the failing human myocardium [39]. In the present study, combined ET\textsubscript{A} receptor and ACE inhibition prevented alterations in the gene expression of MHCs and α-actins in the surviving LV and RV myocardium more than either monotherapy. Thus, normali-
zation of the molecular phenotype and the improvement of myocardial failure are likely to be causally related.

The effectiveness of combination therapy in preventing RV hypertrophy may be mediated through improvement of LV failure. In fact, the hypertrophic growth of the right ventricle post-MI is related to LV dilation and pump failure, resulting in increased pressure load on the ventricle [7,22]. However, a more effective direct interference with the RV renin–angiotensin system and/or endothelin system by combination therapy may have contributed to the pronounced improvement of RV remodeling.

Several studies have reported a link between the renin–angiotensin and endothelin systems. ET-1 is involved in angiotensin II-induced hypertrophy in cultured cardiomyocytes [40]. In a transgenic angiotensin II-dependent rat model of hypertension, upregulation of the endothelin system plays an important role in the transition from LV hypertrophy to LV dysfunction via decreased sarcoplasmic reticulum Ca$^{2+}$ uptake [41]. Moreover, in transgenic rats for both the human renin and angiotensinogen genes, LU135252 given in combination with a low dose of losartan reduced blood pressure, cardiac hypertrophy and renal collagen type III gene expression, suggesting that angiotensin II-induced end-organ damage might arise via an endothelin-related mechanism [42]. This is compatible with our present findings in rats with CHF.

4.4. Neurohumoral activation

In the present study, consistent with past reports [43,44], ET$_A$ receptor inhibition lowered plasma renin activity in rats with CHF. The underlying mechanisms may include indirect effects on renin release secondary to improvement of renal perfusion [45] and/or decreased sympathetic activation. A direct effect of ET$_A$ receptor blockade appears unlikely as, in other models with high renin activity, ET$_A$ antagonism did not affect plasma renin activity [42].

In the present study, plasma norepinephrine levels tended to be reduced by ET$_A$ receptor blockade and ACE inhibition alone, but were significantly suppressed only by the combination therapy. Hemodynamic improvement likely accounted for reflex inhibition of sympathetic activation. New et al. [44] also reported that combined AT$_1$ and ET receptor blockade reduced plasma norepinephrine and improved LV pump function to a greater extent than monotherapy in developing heart failure. A reduction in plasma norepinephrine may contribute to increased benefit of combined ET$_A$ and ACE inhibition by preventing the adverse cardiovascular effects of excessive sympathetic stimulation. In fact, circulating norepinephrine concentrations are elevated in CHF and contribute to further decompensation and poor prognosis [3].

The beneficial effect of combined ET$_A$ receptor and ACE inhibition on hemodynamics and sympathetic activation could also have contributed to the reduction of plasma ET-1 levels. Supporting this notion, circulating endothelin concentrations are increased and correlate with the severity of heart failure [9]. In addition, changes in circulating ET-1 levels reflect the magnitude and the direction of the hemodynamic and neurohormonal response to ACE inhibitors as well as β-blocker therapy [46,47].

Whether selective ET$_A$ or mixed ET$_A$ and ET$_B$ receptor blockade should be favored in the treatment of CHF is still unclear. Selective ET$_A$ antagonists may offer advantages over mixed ET$_A$ and ET$_B$ antagonists. ET$_B$ receptor inhibition reduces endothelin clearance and NO-mediated vasodilatation [14]. In addition, ET$_B$ receptor inhibition may abrogate the beneficial effects of ET$_A$ receptor blockade on vascular superoxide formation [43]. However, Mulder et al. [13] reported that the inhibition of both ET$_A$ and ET$_B$ receptors improved hemodynamics and LV remodeling to the same extent as ET$_A$ receptor blockade alone in rats with CHF. Clearly, randomized clinical trials are needed to compare the effects of ET$_A$ with mixed ET$_A$ and ET$_B$ receptor antagonists on top of ACE inhibition on clinical outcome of CHF patients.

4.5. Clinical implications

The beneficial effect of ET$_A$ receptor blockade on top of ACE inhibition may have important clinical implications for patients with CHF. Although to date there are no clinical endpoint studies using ET antagonists in CHF patients, several short-term studies demonstrate hemodynamic and symptomatic improvement [14,25,27,48,49]. ET$_A$ receptor blockade causes pulmonary vasodilation [50] and reduces forearm vascular resistance in CHF patients treated with ACE inhibitors [24,26]. However, long-term studies investigating the effects of combination therapy with ET antagonists and ACE inhibitors on cardiac remodeling are lacking.

Our work provides experimental evidence that long-term combined ET$_A$ receptor and ACE inhibition improves myocardial failure and prevents fibrosis and fetal gene expression more effectively than either monotherapy. Thus, ET$_A$ receptor blockers may be useful when added to ACE inhibitors in patients with CHF.

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References