reduce infarct size (Miura et al., 1997; Reffelmann and Kloner, 2003). Klein et al. (2000) reported that the reduction of infarct size was detectable only when cariporide was infused during the first 30 min after ischemia. In their study, cariporide infused after 45 min of ischemia until 10 min of reperfusion failed to reduce infarct size. In the ESCAMI trial, pretreatment with eniporide in patients undergoing reperfusion therapy for acute ST-elevation myocardial infarction did not reduce infarct size assessed by cardiac enzyme release (Zeymer et al., 2001). The present study revealed that pretreated cariporide significantly reduced interstitial myoglobin level from the very early phase after myocardial ischemia. This fact may be a reason why cariporide should be administered before ischemia to exert its cardioprotective effects. Thus, the reduction of interstitial myoglobin level during reperfusion observed in the present study may reflect amelioration of structural damage caused by ischemia and not necessarily the damage induced by reperfusion. Nevertheless, cariporide has a certain cardioprotective effect against structural damage during ischemia and reperfusion.

Methodological considerations

The responses of heart rate and arterial pressure to myocardial ischemia/reperfusion were small in the present study. Because Kashihara et al. (2004) reported that Bezold–Jarisch (B–J) reflex induced by phenylbiguanide blunted arterial baroreflex via the shift of the neural arc toward lower sympathetic nerve activity, small responses in heart rate and arterial pressure might be due to weak B–J reflex during acute myocardial ischemia in rabbits.

Conclusions

Intravenous cariporide significantly reduced myocardial interstitial myoglobin level during ischemia/reperfusion and decreased NE release during ischemia. When administered before ischemia, treatment with cariporide may be an effective cardioprotective strategy against structural damage during ischemia/reperfusion and excessive NE release during ischemia.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Adding the acetylcholinesterase inhibitor, donepezil, to losartan treatment markedly improves long-term survival in rats with chronic heart failure

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Aims Modulation of vagal tone using electrical vagal nerve stimulation or pharmacological acetylcholinesterase inhibition by donepezil exerts beneficial effects in an animal model of chronic heart failure (CHF). Considering different treatment mechanisms underlying renin–angiotensin system (RAS) suppression and parasympathetic activation, we hypothesized that parasympathetic activation together with RAS inhibition could attenuate CHF progression. To test this hypothesis, we investigated the therapeutic effects of a combination of donepezil and losartan in CHF rats with extensive myocardial infarction (MI).

Methods and results Rats ($n = 85$) that had survived extensive MI were implanted with a blood pressure transmitter and were randomly assigned to receive either a combination of donepezil and losartan (DLT group) or losartan alone (LT group). Compared with the LT group, the DLT group showed a significantly lower heart rate without hypotension. DLT therapy further improved 280-day overall survival relative to the LT group (31% vs. 8%, $P = 0.022$) by preventing cardiac dysfunction (LV dP/dt_{max}, 4064 ± 170 vs. 3430 ± 117 mmHg/s, $P < 0.01$; LV end-diastolic pressure, 17 ± 2 vs. 22 ± 2 mmHg, $P < 0.05$). DLT therapy was also associated with lower plasma BNP and catecholamine levels, lower cardiac angiotensin II concentrations, and higher capillary density in the peri-infarct region.

Conclusions Combined treatment with donepezil and losartan prevented the progression of cardiac dysfunction and improved the long-term survival of CHF rats with extensive MI, suggesting that this combination could be a novel pharmacotherapy for severe CHF.

Keywords Donepezil • Losartan • Heart failure • Myocardial infarction • Survival

Introduction

Chronic heart failure (CHF) is characterized by overactivity of the sympathetic nervous system and the renin–angiotensin system (RAS), and reduced activity of the parasympathetic nervous system.[1–4] Currently, treatment for CHF has been centred on reducing the sympathetic effects, leading to the widespread clinical use of pharmacotherapeutic agents, including beta-blockers,[5–7] ACE inhibitors, and ARBs,[8–10] which have demonstrated favourable long-term effects on mortality and morbidity. The combination of beta-blockers with an ACE inhibitor and/or an ARB was subsequently recommended by the American Heart Association, and this combination is presently considered a standard therapy for CHF.[11] Despite these potent drugs, CHF is one of the main reasons for hospitalization.[12] Patients with CHF have low quality of life, and their mortality rate remains high.

Because diminished parasympathetic activity is another important independent risk factor underlying CHF[13] and myocardial

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infarction (MI),[14] restoring parasympathetic activity could be an alternative treatment for CHF. As a first step towards developing a therapeutic strategy targeting the parasympathetic system, we demonstrated that electrical vagal nerve stimulation (VNS) markedly improved the long-term survival of CHF rats with extensive MI, by preventing the progression of pumping failure and cardiac remodelling.[15,16] This treatment strategy had been evaluated in clinical trials and was shown to be feasible and beneficial for CHF patients.[17,18] Nonetheless, because VNS is an invasive approach, further large-scale and long-term clinical studies are necessary to verify its safety and effectiveness. Furthermore, the cost of the stimulation device and implantation surgery may become limiting factors for its standard clinical use. Since the efferent vagal nerve activity is transmitted by acetylcholine, drugs that increase local acetylcholine concentration can yield a bradycardic effect similar to that of VNS.[19,20] Previously, we found that administration of donepezil, an acetylcholinesterase inhibitor used in the palliative treatment of Alzheimer's disease, significantly increased vagal tone[21] and improved long-term survival in rats with extensive MI.[22]

Any potential therapy to be considered for clinical translation should be examined for its compatibility with established treatments. Considering that different treatment mechanisms are presumably involved in RAS suppression and parasympathetic activation, we hypothesized that parasympathetic activation together with RAS inhibition could attenuate CHF progression. RAS inhibition by a specific ARB, such as losartan, has been shown to prevent ventricular remodelling and improve the prognostic outcome in CHF, and ARBs are prescribed as routine drugs for CHF patients. In the present study, we investigated whether donepezil is effective in further suppressing cardiac dysfunction and improving long-term survival when administered in combination with losartan in rats with extensive MI.

Methods

The care and use of the animals were in strict accordance with Directive 2010/63/EU of the European Parliament and the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, which have been approved by the Physiological Society of Japan. All protocols were reviewed and approved by the Animal Subject Committee in the National Cerebral and Cardiovascular Center.

Experimental heart failure

Extensive MI was induced by occluding the proximal left coronary artery in male Sprague–Dawley rats ($n = 180$; body weight: 250–280 g; SLC, Hamamatsu, Japan) under halothane inhalation anaesthesia, as described previously (3% at induction and 1.2% during surgery).[15,22] Approximately 50% ($n = 95$) of the animals with extensive MI survived beyond the first 24 h. One week later, we measured LVEF by echocardiography after inhalation of the anaesthetic halothane (1%), and those rats ($n = 85$) with EF <40% were enrolled in this study. We confirmed the infarct size by post-mortem examination.

Study design

At 2 weeks after extensive MI, the surviving rats were randomly assigned to receive treatment with a combination of donepezil and losartan (DLT group, $n = 41$) or treatment with losartan alone (LT group, $n = 40$) for remodelling and prognosis studies (*Figure 1A*). Donepezil (Eisai, Inc., Tokyo, Japan) was administered by dissolving the drug in drinking water to a concentration of 50 mg/L. The dosage of donepezil (5 mg/kg/day) was chosen to decrease the heart rate (HR) by 20–30 b.p.m. in MI rats. Losartan (LKT Laboratories, Inc., St. Paul, MN, USA) was administered by admixing the drug into food pellets, at a dose of 10 mg/kg/day during the first 2 weeks, then increased to 30 mg/kg/day throughout the study period (*Figure 1B*). The dosages of donepezil and losartan were determined by a preliminary study as the maximum levels that did not obviously affect daily food consumption or normal growth in MI rats.

Telemetric measurements of blood pressure and heart rate

One week after inducing extensive MI, we surgically implanted a blood pressure (BP) transmitter (TA11PA-C40, DSI, St. Paul, MN, USA) after inhalation of the anaesthetic halothane (3% at induction and 1.2% during surgery) in 32 CHF rats for the remodelling study, in accordance with previously described procedures.[15,22] The catheter was inserted into the abdominal aorta, and the transmitter was positioned in the abdomen. The BP and HR data were recorded continuously in freely moving animals. The recording was sampled at 500 Hz.

Haemodynamic measurements under anaesthesia

After the 6-week treatment, we conducted an acute haemodynamic study in the surviving CHF rats (LT, $n = 13$; DLT, $n = 14$) under anaesthesia (3% induction, 1.2% surgery, and 0.6% halothane during data recording). Left ventricular and arterial pressures were measured with a 2 Fr catheter-tip micromanometer (SPC-320, Millar Instruments, Inc., Houston, TX, USA), right atrial pressure was measured with a fluid-filled transducer, and aortic flow was measured by a transonic flow probe, as described previously.[15] All the signals were digitized at a rate of 500 Hz for 5 min. After completing the haemodynamic measurements, blood samples were collected from the right carotid artery, and all rats were sacrificed with an overdose of *i.v.* sodium pentobarbital (100 mg/kg). The heart was excised for weighing, and frozen heart samples and 4% phosphate-buffered paraformaldehyde solution-fixed paraffin sections were prepared for subsequent analysis.

Neurohumoral study

Plasma catecholamine concentrations were measured by high-performance liquid chromatography with electrochemical detection after alumina adsorption. Plasma levels of BNP, arginine vasopressin (AVP), and angiotensin II (Ang II), and cardiac Ang II concentration were determined using enzyme-linked immunosorbent assay (ELISA). For details, see the Supplementary material online, *Methods*.

Immunohistochemistry and microvessel density

Biventricular sections (4 μm thickness) were deparaffinized, placed in citrate buffer, and heated in an autoclave to enhance specific immune staining. The sections were incubated overnight with rabbit antihuman von Willebrand factor (vWF) polyclonal antibody at 4 °C and were then incubated with Alexa 633-conjugated goat antirabbit IgG

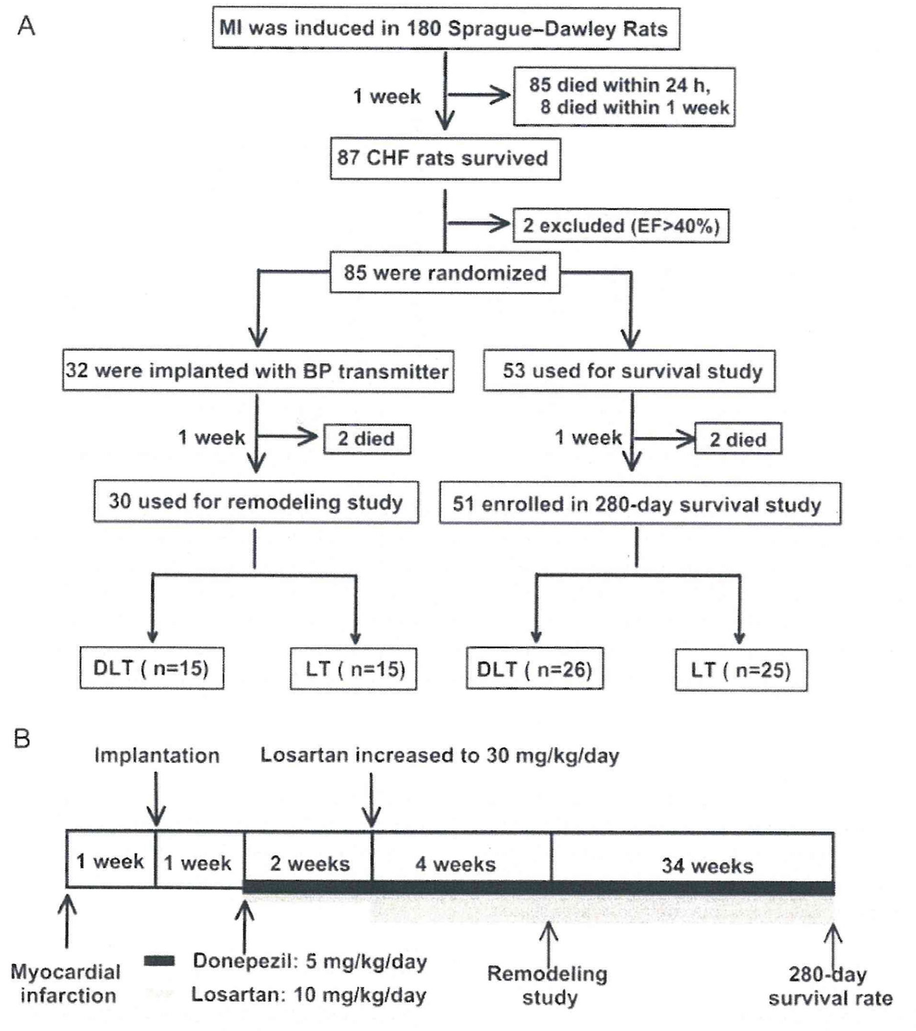

Figure 1 Schematic description of study design and timeline. (*A*) Study design of combined donepezil and losartan therapy in rats with chronic heart failure (CHF). The surviving CHF rats were randomly assigned to receive treatment with a combination of donepezil and losartan (DLT group) or to treatment with losartan alone (LT group) for remodelling and prognosis studies. (*B*) Study events and timeline. MI, myocardial infarction; EF, left ventricular ejection fraction; BP, blood pressure. At 2 weeks after extensive MI, donepezil (5 mg/kg/day) was administered by dissolving the drug in drinking water. Losartan was administered by admixing the drug into food pellets at a dose of 10 mg/kg/day during the first 2 weeks and increasing the dose to 30 mg/kg/day throughout the study period.

for microvessel analysis. Fluorescence of Alexa 633 was observed with a laser scanning microscope. Capillary vessels in the peri-infarct area, excluding the scar region, were counted using a laser scanning microscope system at $\times 20$ magnification. Data obtained from high-power fields were averaged and expressed as the number of capillary vessels. Immunohistochemical double staining of Ang II type 1 (AT_1) receptors and smooth muscle actin (SMA) was performed in the sections. For details, see Supplementary materials online, *Methods*.

Western blotting and detection of proteins

For the western blotting investigation, a normal control group (N) and a CHF group without treatment (UT, 8 weeks post-MI) were analysed in addition to the DLT and LT groups. Frozen samples from the non-infarct left ventricle were homogenized for measuring cardiac Ang II concentrations by ELISA, and using western blotting for detecting AT_1 and AT_2 receptors. For details, see Supplementary materials online, *Methods*.

Determination of infarct size and histological examination

The heart was rapidly excised, rinsed to remove blood, weighed, and sliced. Biventricular sections (4 μm thickness) from the basal, middle, and apical portions were stained using Masson's trichrome method. Histological images were analysed using a frame grabber. Infarct size

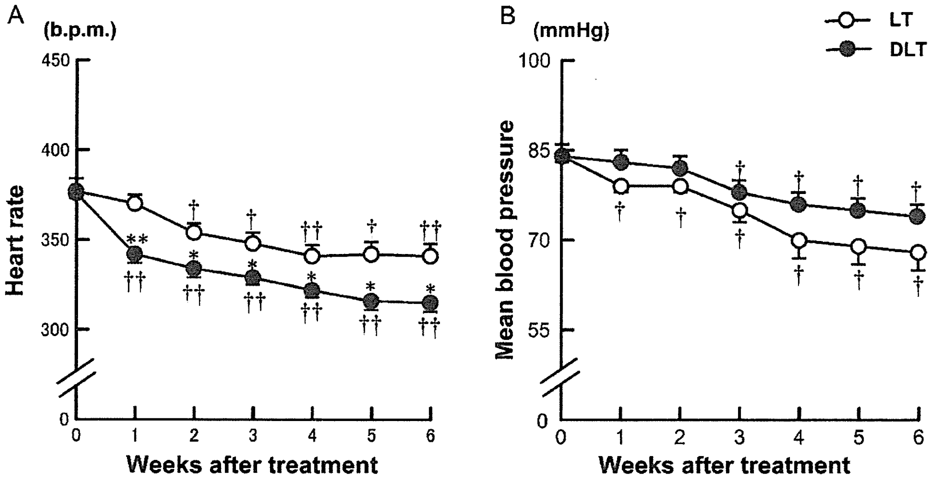

Figure 2 Telemetric haemodynamic changes in rats with chronic heart failure. (*A*) Mean heart rate (HR). (*B*) Mean blood pressure (MBP). Each point represents the average of 1 week's data from all animals in each group [losartan alone (LT), $n = 13$; donepezil + losartan (DLT), $n = 14$]. The average HR decreased significantly from the second week of treatment in both groups, but to a greater degree in the DLT group than in the LT group. The difference in HR between the DLT and LT groups reached ~30 b.p.m. at the sixth week of treatment (315 ± 6 b.p.m. vs. 341 ± 6 b.p.m., $P < 0.05$), while the MBP in the DLT group was not lower than that in the LT group (74 ± 6 mmHg vs. 68 ± 3 mmHg, *P*, non-significant). The values of the data points are given in the Supplementary material online, *Table S1*. Values are means ± SEM. *$P < 0.05$; **$P < 0.01$ vs. LT; †$P < 0.05$, ††$P < 0.01$ vs. pre-treatment values (week 0) of each group by one-way ANOVA with repeated measures and post-hoc Dunnett's test.

was calculated from the three slices by dividing the sum of the endocardial lengths of infarcted regions by the sum of endocardial circumferences. The extent of myocardial fibrosis was evaluated using a light microscope at × 20 magnification. The myocardial fibrosis index was calculated from eight high-power fields in the non-infarct septum area in each rat.

Long-term survival study

To examine the outcomes of DLT and LT therapies, we evaluated survival over 280 days. The rats were inspected daily, and gross post-mortem examinations were conducted on dead rats. At the end of the observation period, surviving animals were sacrificed with an overdose (200 mg/kg) of sodium pentobarbital, injected intraperitoneally. The heart was removed for the subsequent measurement of infarct size. The cause of death was classified as pump failure if oedema, extreme weight loss accompanied by panting over a 24 h period before death, or obvious pleural effusion were observed. Otherwise, the cause of death was classified as sudden cardiac death.

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). For data from the haemodynamic and remodelling study, differences between the DLT and LT groups were tested using Student's *t*-test. Differences in HR and BP before and during the treatment in each group were examined using one-way analysis if variance (ANOVA) with repeated measures and post-hoc Dunnett's test. For the neurohumoral data, the Mann–Whitney U-test was used to compare differences between the DLT and LT groups. For the western blot, the differences among the four groups were examined using one-way ANOVA and the post-hoc Tukey test. Survival data are presented as Kaplan–Meier curves, and the effect of treatment on 280-day survival was analysed using a log-rank test. The differences were considered significant for $P < 0.05$.

Results

Telemetric haemodynamic measurements

The averaged HR decreased significantly from the second week of treatment in both groups, but to a greater degree in the DLT group than in the LT group (*Figure 2A*). The difference in HRs between the DLT and LT groups reached ~30 b.p.m. at the sixth week of treatment (315 ± 6 vs. 341 ± 6 b.p.m., $P < 0.05$), while the mean BP was not lower in the DLT than in the LT group [74 ± 6 vs. 68 ± 3 mmHg, *P*, non-significant (NS); *Figure 2B*].

Anaesthetic haemodynamics and cardiac remodelling

The haemodynamic and remodelling parameters measured at the eighth week after extensive MI are shown in *Table 1*. Although there were no significant differences between the two groups in the body weight, normalized biventricular weight, infarct size, mean arterial pressure, and HR, the DLT rats had a significantly higher cardiac index and maximum and minimum d*P*/d*t* of LV pressure. Right atrial pressure and LV end-diastolic pressure were lower in the DLT than in the LT rats. The prevention of cardiac dysfunction in the DLT rats

Table 1 Haemodynamic and remodelling parameters after 6 weeks of treatment in rats with chronic heart failure

	LT group ($n = 13$)	DLT group ($n = 14$)	*P*-value
BW, g	442 ± 15	457 ± 10	NS
HW, g/kg	2.60 ± 0.09	2.51 ± 0.06	NS
Infarct size, %	46 ± 1	47 ± 2	NS
MBP, mmHg	69 ± 3	75 ± 2	NS
HR, b.p.m.	289 ± 7	299 ± 8	NS
CI, mL/min/kg	127 ± 4	145 ± 6	<0.05
LVEDP, mmHg	22 ± 2	17 ± 2	<0.05
LV dP/dt_{max}, mmHg/s	3430 ± 117	4064 ± 170	<0.01
LV dP/dt_{min}, mmHg/s	2657 ± 123	3076 ± 137	<0.05
RAP, mmHg	2.01 ± 0.29	1.16 ± 0.27	<0.05

Values are means ± SEM.
BW, body weight; CI, cardiac index; DLT, donepezil + losartan; HR, heart rate, HW, biventricular weight normalized by body weight; LT, losartan alone; LVEDP, left ventricular end-diastolic pressure; LV dP/dt_{max}, maximum dP/dt of left ventricular pressure; LV dP/dt_{min}, minimum dP/dt of left ventricular pressure; MBP, mean arterial pressure; RAP, right atrial pressure; SEM, standard error of the mean.
P-value, significance of difference between LT and DLT groups assessed by Student's *t*-test. NS, not significant ($P > 0.05$).

was accompanied by a significantly decreased cardiac fibrosis index in the non-infarct septum ($0.97 \pm 0.09\%$ vs. $1.22 \pm 0.07\%$, $P < 0.05$; *Figure 3C*).

Response of neurohumoral levels to treatments

The plasma levels of norepinephrine, epinephrine, and BNP were significantly lower in the DLT than in the LT group (*Table 2*). Although plasma AVP and Ang II did not differ significantly between the two groups, the cardiac Ang II concentration in the DLT group was reduced by two-thirds compared with the LT group.

Microvascular immunohistochemistry

The immunohistochemical study showed a greater degree of neovascularization in the DLT group than in the LT group (*Figure 4A*). Quantitative analysis demonstrated that capillary density was significantly higher in the DLT than in the LT group (*Figure 4B*, 107 ± 5 vs. 58 ± 3 vessels/field, $P < 0.01$). Further immunohistochemical double staining with AT_1 and SMA showed less AT_1 receptor activity in the DLT than in the LT group (*Figure 4C*).

Angiotensin II type 1 and type 2 receptor expression

Cardiac AT_1 receptor expression (*Figure 5A*) was detected in the 43 and 85 kDa putative bands. The 85 kDa band is a dimer or a different *N*-glycosylation state of the AT_1 receptor.[23] Rats with MI showed no change in AT_1 43 kDa but had significantly greater AT_1 85 kDa expression than normal rats. LT treatment significantly suppressed AT_1 43 kDa without any effect on AT_1 85 kDa compared with untreated MI. DLT treatment significantly reduced AT_1 85 kDa expression but not AT_1 43 kDa (*Figure 5C*) compared with LT. Cardiac AT_2 receptor expression (*Figure 5B*), detected in the 48 and 62 kDa bands, did not differ significantly among the four groups (*Figure 5D*).

Long-term survival study

Rats with healed extensive MI ($n = 51$) were used in the long-term survival study. As shown in the Kaplan–Meier curves for 280-day all-cause mortality (*Figure 6*), the survival rate of CHF rats was markedly higher in the DLT group than in the LT group from day 140 (92% vs. 64%, $P = 0.014$) to the end of the observation period. Eight rats (31%) in the DLT group vs. only 2 rats (8%) in the LT group survived until 280 days ($P = 0.022$). The median survival was 167 days in the LT group and 227 days in the DLT group. The cause of death was further analysed. Pump failure accounted for 30% of deaths in the DLT group and 81% of deaths in the LT group, indicating that DLT therapy had a significant effect in preventing pump failure death (Supplementary material online, *Figure S1A*, 30% vs. 81%, $P = 0.009$). However, the incidence of sudden cardiac death did not differ between the DLT (57%) and LT (56%) groups (Supplementary material online, *Figure S1B*, $P = 0.506$). Even after the rats that survived until the end of the study period were excluded, the LT and DLT groups showed no significant difference in either normalized biventricular weight (3.04 ± 0.09 vs. 2.98 ± 0.09 g/kg) or infarct size ($48\% \pm 3\%$ vs. $48\% \pm 1\%$).

Discussion

This study has shown that treatment with the DLT combination in severe CHF rats (i) markedly improved 280-day survival; (ii) ameliorated cardiac dysfunction; and (iii) decreased HR without significantly altering mean BP, compared with the LT group. The DLT combination also (iv) significantly suppressed plasma catecholamine, BNP, and cardiac Ang II levels and (v) improved histological features relative to the LT group. These findings suggest that pharmacological restoration of vagal tone by an acetylcholinesterase inhibitor, donepezil, and inhibition of RAS by losartan may work co-operatively to enhance cardioprotective beneficial effects in CHF rats with extensive MI.

Previously obtained knowledge

The study design and protocol of the present study are in line with those of our previous report,[22] the results of which are summarized in brief. We randomized MI-induced CHF rats to donepezil treatment (DT) or no treatment (UT) groups 14 days after the induction of MI. Donepezil was administered dissolved in drinking water (5 mg/kg/day). Compared with the UT group, DT therapy significantly prevented LV remodelling and dysfunction, and reduced neurohumoral activation. This prevention was associated with a significant improvement in survival at 140 days in the DT

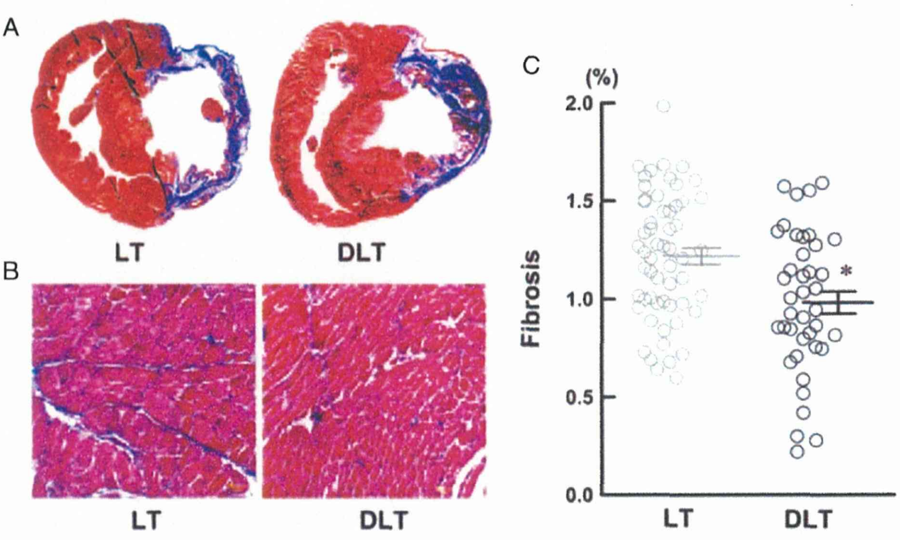

Figure 3 Masson's trichrome staining on ventricle slides of rats with chronic heart failure. (*A*) Representative biventricular sections of extensive myocardial infarction. (*B*) Representative image of the myocardial fibrosis in the non-infarct septum. (*C*) Myocardial fibrosis index in the septum area [1.22 ± 0.07%; losartan alone (LT), $n = 7$, 56 fields vs. 0.97 ± 0.09%; donepezil + losartan (DLT), $n = 5$, 40 fields]. Values are means ± SEM. *$P < 0.05$ vs. LT by Student's *t*-test.

group (54%) compared with the UT group (29%, $P = 0.03$). These values will be referred to in the following discussion.

Possible treatment mechanisms involved in donepezil + losartan therapy

We assessed stress-free haemodynamics of CHF rats by telemetry. DLT therapy reduced ambulatory HR compared with LT therapy, while the mean BP in the DLT group was similar to that in the LT group (*Figure 2*). These results are partly consistent with those of a previous study, in which parasympathetic activation by donepezil significantly reduced HR but did not affect mean BP.[22] Prevention of cardiac dysfunction, as evidenced by the increased cardiac index (*Table 1*), may also have contributed to the maintenance of mean BP, yielding arterial baroreflex-mediated inhibition of sympathetic nerve activity and reduction of HR.[24] A prolonged cardiac cycle is beneficial in enhancing and maintaining cardiac function, by decreasing myocardial oxygen consumption, increasing coronary flow, and increasing ventricular filling volume.[25] Clinical studies also indicate that HR is an independent risk factor in CHF,[13] and antiadrenergic treatment by beta-blockers is a routine prescription for most CHF patients.[26] In a recent clinical trial, SHIFT, HR reduction with ivabradine improved clinical outcomes in CHF patients.[27] Therefore, the bradycardic effect seen in the DLT group may be one of the important factors for preventing cardiac dysfunction.

While both the DLT combination and beta-blockers exert bradycardic effects, the former may have a potential advantage compared with the latter. Because adrenergic receptors exist on both the sinus node and cardiac myocytes, beta-blockers not only decrease HR but also suppress ventricular contractility. Hence, due to these negative inotropic effects, beta-blockers may not be suitable for patients with decompensated conditions or pre-existing myocardial dysfunction, since the maintenance of cardiac output in such patients depends in part upon sympathetic drive. On the other hand, because of the scarce distribution of acetylcholine-dependent potassium channels in the ventricles, vagal activation shows less direct negative inotropic effects.[28,29] Moreover, because pre-ganglionic fibres are cholinergic, donepezil

Table 2 Neurohumoral variables in plasma and tissue after 6 weeks of treatment in rats with chronic heart failure

	LT group ($n = 13$)	DLT group ($n = 14$)	*P*-value
Plasma norepinephrine, pg/mL	1522 ± 273	931 ± 104	<0.05
Plasma epinephrine, pg/mL	2375 ± 386	1187 ± 138	<0.01
Plasma BNP, pg/mL	391 ± 8	358 ± 10	0.01
Plasma AVP, pg/mL	293 ± 19	278 ± 22	NS
Plasma Ang II, pg/mL	34 ± 4	50 ± 8	NS
Cardiac Ang II, pg/g protein	121 ± 34	42 ± 18	<0.05

Values are means ± SEM.
Ang II, angiotensin II; AVP, arginine vasopressin; DLT, donepezil + losartan; LT, losartan alone; SEM, standard error of the mean.
P-value, significance of difference between LT and DLT groups assessed by the Mann–Whitney U-test. NS, not significant ($P > 0.05$).

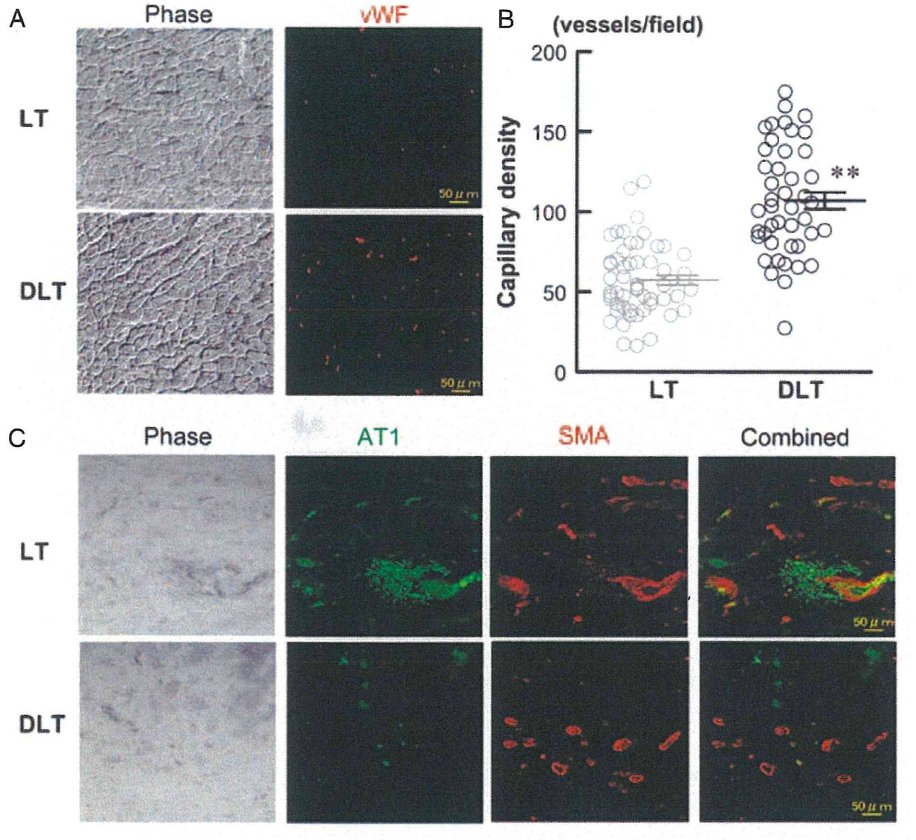

Figure 4 Immunohistochemical and microvessel analysis in the peri-infarct area of rats with chronic heart failure. (*A*) Representative micrographs of von Willebrand factor (vWF, red) immunostaining. Phase: phase-contrast image. (*B*) Quantitative analysis of capillary density. [58 ± 3; losartan alone (LT), $n = 6$, 54 fields vs. 107 ± 5 vessels/field; donepezil + losartan (DLT), $n = 5$, 44 fields]. Values are means ± SEM. **$P < 0.01$ vs. LT by Student's *t*-test. (*C*) Immunofluorescent expression of cardiac angiotensin II type 1 (AT_1) receptor (green) and smooth muscle actin (SMA, red) in the ischaemic border zone. The vascular AT_1 receptor appeared more active in the LT ($n = 3$) than in the DLT ($n = 3$) group.

can theoretically increase synaptic transduction in both sympathetic and parasympathetic nerves.[30,31] Therefore, donepezil may increase synaptic acetylcholine and reduce HR, with less negative effects on ventricular contractility. In the DLT group, the finding of decreased HR without significant effects on mean BP may partly support this interpretation; this phenomenon is similar to the effects of ivabradine treatment that showed HR reduction without negative inotropism in CHF.[27]

In addition to the above-mentioned haemodynamic-related mechanisms, the cholinergic anti-inflammatory pathway[32,33] may be another important mechanism for cardiac protection in DLT therapy.[34–36] In a previous study, we found that DT rats had lower levels of inflammatory cytokines in the heart, liver, and lung tissue compared with UT rats,[22] and this effect of donepezil may partly account for the suppression of myocardial interstitial fibrosis in the DLT group compared with the LT group (*Figure 3C*). Furthermore, the cholinergic angiogenic pathway[37] might lead to increased capillary density in the peri-infarct region (*Figure 4B*). Abundant vascularization is the basis or indicator of elastic myocardium,[38] and may have partially improved or preserved cardiac function, resulting in the improved outcomes in the DLT group compared with the LT group.

Effects of donepezil + losartan therapy on cardiac remodelling and neurohumoral factors

Normalized biventricular weight, an indicator of cardiac hypertrophy, was not significantly lower in the DLT group than in the LT group. Because losartan and other ARB drugs exhibit potent anticardiac remodelling effects[39–42] with respect to cardiac hypertrophy, there may not have been much room for further improvement by the addition of donepezil. However, histological and neurohumoral assays might provide new insights into the understanding of DLT therapy, as follows. The DLT group showed lower Ang II levels in cardiac tissue compared with the LT group (*Table 2*). Donepezil inhibits local Ang II production through a

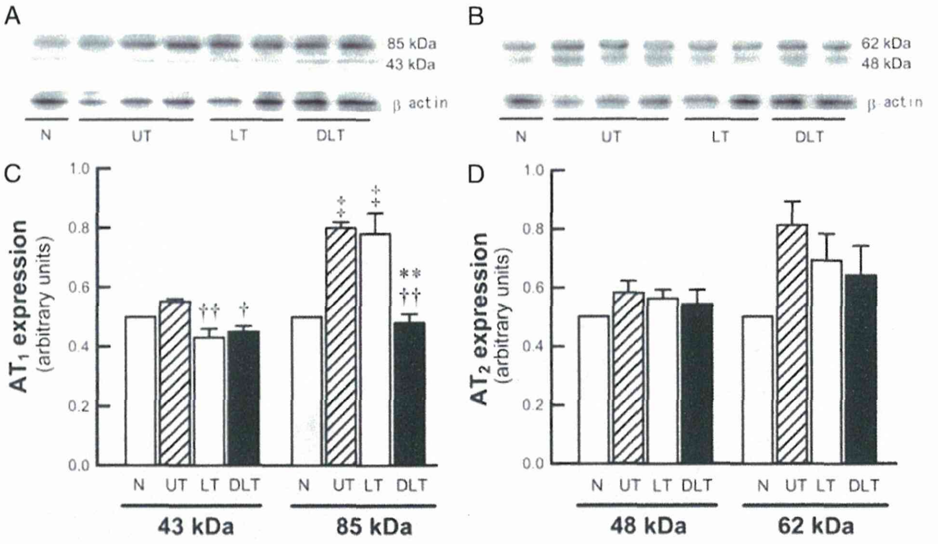

Figure 5 Western blotting analyses. (*A*) Representative immunoblots of homogenates of the left ventricle from rats with chronic heart failure (CHF) probed with polyclonal angiotensin II type 1 (AT_1) receptor antibody. (*B*) Polyclonal angiotensin II type 2 (AT_2) receptor antibody. (*C*) Densitometry analysis of AT_1 and (*D*) AT_2 receptors obtained from normal controls (N, $n=5$), untreated CHF (UT, 8 weeks post-myocardial infarction, $n=5$), losartan alone (LT; $n=7$) and donepezil + losartan (DLT; $n=7$) rats. Values are means ± SEM. †$P<0.05$, ††$P<0.01$ vs. UT; ‡$P<0.01$ vs. N; **$P<0.01$ vs. LT by one-way ANOVA and the post-hoc Tukey test.

heart-specific chymase-dependent pathway.[43] Along with increasing capillary density, DLT therapy decreased vascular AT_1 receptor activity (*Figure 4C*), which may indicate that this receptor is directly involved in angiogenesis.[23] Further, the results of the protein detection assay showed that, although UT rats had no change in AT_1 43 kDa, they had significantly greater AT_1 85 kDa expression than normal rats; this finding is consistent with a previous report.[23] Losartan treatment significantly decreased the AT_1 43 kDa receptors compared with UT, a result similar to that of a previous report indicating that long-term ARB treatment caused down-regulation of AT_1 receptors.[41] Addition of donepezil induced down-regulation of the AT_1 85 kDa receptors (*Figure 5C*) compared with the LT group. The AT_1 85 kDa receptor is a dimer or a different *N*-glycosylation state that is necessary for the adequate delivery of the receptor from the endoplasmic reticulum to the plasma membrane.[23,44] Whether donepezil modulates this process directly or indirectly is unknown.

DLT therapy significantly reduced plasma catecholamine and BNP levels compared with LT therapy (*Table 2*). Therefore, this combination therapy would effectively retard the vicious circle of maladaptation in CHF, by augmenting vagal tone and suppressing chronic overactivity of the sympathetic nervous system and the RAS.

Effects of donepezil + losartan therapy on survival

In the present study, CHF rats with extensive MI were enrolled and followed for up to 280 days (294 days post-MI). We found

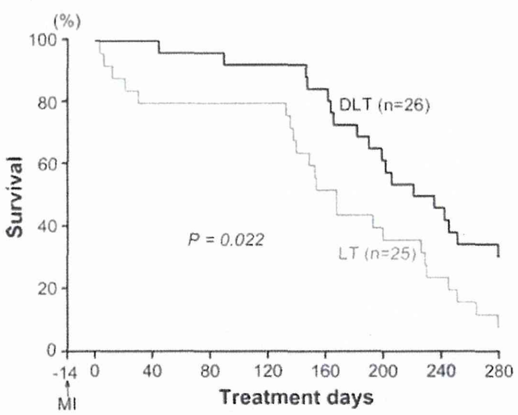

Figure 6 Kaplan–Meier survival curves of animals treated with losartan alone (LT) and donepezil + losartan combination (DLT). Treatment was started 14 days after myocardial infarction (MI). DLT significantly improved the 280-day survival rate compared with LT (31% vs. 8%, $P=0.022$, by log-rank test). The median survival for the LT group was 167 days, compared with 227 days for the DLT group.

that the median survival was 227 days in the DLT group and 167 days in the LT group. A significant difference in survival was detected from day 140 (*Figure 6*; 92% vs. 64%, $P=0.014$) to the end of the observation period (*Figure 6*; 31% vs. 8%, $P=0.022$). This result may indicate that DLT therapy conferred better prognostic benefits, not only than losartan alone, but also than donepezil alone (DT, 54%; UT, 29%, at day 140).[22] Further analysis of the cause of death (Supplementary material online, *Figure S1*) demonstrated that adding donepezil reduced pump failure death (Supplementary material online, *Figure S1A*; 30% vs. 89%, $P = 0.009$) but not sudden cardiac death (Supplementary material online, *Figure S1B*; 57% vs. 56%, $P = 0.506$). This may suggest that the beneficial effect of donepezil treatment which started 2 weeks after MI was mainly exerted through the improvement of cardiac function, but the effects on arrhythmias and sudden cardiac death should be interpreted carefully with further investigation. From the viewpoint of 140-day survival, DLT therapy (92%) provided an equivalent treatment effect to that attained by VNS (86%) in CHF rats;[15] however, the differences in the protocols prevent us from inferring that the beneficial effects of DLT therapy are the same as those of VNS therapy.

Beta-blockers, ACE inhibitors, or ARBs have been reported to improve the prognosis of CHF rats with a small to moderate infarct size, but had no or less effect on long-term survival in rats with a large infarct size.[39,45,46] In agreement with these reports, only two rats (8%) in the LT group survived until 280 days in the present study. In contrast, eight rats (31%) in the DLT group survived until 280 days, demonstrating the additional beneficial effects obtained from the DLT combination for the treatment of severe CHF induced by a large MI.

Possible clinical implications

To the best of our knowledge, this study is the first to demonstrate the therapeutic efficacy of a combination of parasympathetic activation by donepezil and RAS inhibition by losartan in the treatment of CHF. In fact, since RAS inhibition by ACE inhibitors and/or ARBs has been established as a first-line therapy to prevent the progression of heart failure,[47] and donepezil has been clinically approved for the treatment of Alzheimer's disease, this drug combination could be considered as a novel therapeutic strategy for patients with severe CHF. Furthermore, a recent cohort study reported that use of a cholinesterase inhibitor reduced the risk of MI and death in subjects with Alzheimer's disease.[48] Future studies are warranted to evaluate this promising therapy in clinical settings. Based on its different mechanism of HR reduction, donepezil may be an alternative for those patients who are intolerant of beta-blocker therapy, or for those whose HR does not decrease adequately in response to beta-blockers. In addition, whether donepezil can be combined with the beta-blocker therapy for CHF should be examined in a further study.

Limitations

Although the drugs used in this study are clinically available, their mode of administration during clinical use in humans and in animal studies is different. In this study, the drugs were dissolved in drinking water (donepezil) or admixed in the diet (losartan). Because of their nocturnal habits, rats usually feed and drink frequently during the night (e.g. drinking 4–16 times daily). Several circulatory surges might have occurred due to donepezil absorbed from the drinking water. Therefore, the dynamics of drug metabolism in this animal model would be different from the clinical situation in which donepezil is taken once a day. In rat studies, the drug is usually dissolved in drinking water at a concentration of 50–150 times the clinical dose.[39,45] We selected the daily dose of donepezil for the rats to be almost 50 times the clinical dose used for Alzheimer's disease. It may be necessary to clarify whether this difference will influence the therapeutic effects of the drug.

Conclusions

This study demonstrates that a combination of donepezil and losartan has a marked protective effect against cardiac dysfunction, and improves long-term survival in CHF rats with extensive MI. We propose the pharmacological inhibition of acetylcholinesterase concomitant with ARB treatment as a novel therapeutic strategy for CHF patients.

Supplementary Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary methods and results.

Figure S1. Survival rates were classified by the cause of death as either pump failure or sudden cardiac death.

Table S1. Weekly averaged hemodynamic measurements in rats with chronic heart failure at baseline and following treatment

Conflict of interest: none declared.

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