Cardiac Sympathetic Afferent Denervation Attenuates Cardiac Remodeling and Improves Cardiovascular Dysfunction in Rats With Heart Failure

Han-Jun Wang, Wei Wang, Kurtis G. Cornish, George J. Rozanski, Irving H. Zucker

Abstract—The enhanced cardiac sympathetic afferent reflex (CSAR) contributes to the exaggerated sympathoexcitation in chronic heart failure (CHF). Increased sympathoexcitation is positively related to mortality in patients with CHF. However, the potential beneficial effects of chronic CSAR deletion on cardiac and autonomic function in CHF have not been previously explored. Here, we determined the effects of chronic CSAR deletion on cardiac remodeling and autonomic dysfunction in CHF. To delete the transient receptor potential vanilloid 1 receptor–expressing CSAR afferents selectively, epicardial application of resiniferatoxin (50 μg/mL), an ultrapotent analog of capsaicin, was performed during myocardial infarction surgery in rats. This procedure largely abolished the enhanced CSAR, prevented the exaggerated renal and cardiac sympathetic nerve activity and improved baroreflex sensitivity in CHF rats. Most importantly, we found that epicardial application of resiniferatoxin largely prevented the elevated left ventricle end-diastolic pressure, lung edema, and cardiac hypertrophy, partially reduced left ventricular dimensions in the failing heart, and increased cardiac contractile reserve in response to β-adrenergic receptor stimulation with isoproterenol in CHF rats. Molecular evidence showed that resiniferatoxin attenuated cardiac fibrosis and apoptosis and reduced expression of fibrotic markers and transforming growth factor-β receptor I in CHF rats. Pressure–volume loop analysis showed that resiniferatoxin reduced the end-diastolic pressure volume relationships in CHF rats, indicating improved cardiac compliance. In summary, cardiac sympathetic afferent deletion exhibits protective effects against deleterious cardiac remodeling and autonomic dysfunction in CHF. These data suggest a potential new paradigm and therapeutic potential in the management of CHF. (Hypertension. 2014;64:745-755.) • Online Data Supplement

Key Words: autonomic nervous system • heart failure

Chronic heart failure (CHF) is a serious and debilitating condition with poor survival rates and an increasing level of prevalence. The exaggerated sympathoexcitation that is a hallmark of CHF is a critical factor in the development and progression of the CHF state. Sympathoexcitation targets multiple organs and causes long-term effects that contribute to disease progression. Therapeutic targeting to block excessive sympathetic activation in CHF has become an accepted modality. The mechanism(s) by which sympathetic excitation occurs in the CHF state are not completely understood. Previous studies from this laboratory have shown that the enhanced cardiac sympathetic afferent reflex (CSAR), a sympathoexcitatory reflex originating in the heart, is a critical contributor to the elevated sympathetic tone in CHF.1,2

It is well known that myocardial ischemia causes the production and release of several metabolites including bradykinin, prostaglandins, and protons that stimulate sympathetic afferent nerve endings, leading to increases in arterial pressure (AP), heart rate (HR), and sympathetic nerve activity.1,3,4 In CHF, the CSAR is sensitized, and cardiac sympathetic afferents are tonically activated.1–3 Recent evidence indicates that transient receptor potential vanilloid 1 (TRPV1)–expressing cardiac afferent fibers are necessary for sensing and triggering the activation of the CSAR and that desensitization of these afferents by systemic administration of resiniferatoxin (RTX), an ultrapotent analog of capsaicin capable of inducing rapid degeneration of TRPV1-expressing afferent neurons and fibers,5–7 can almost completely abolish CSAR activation in adult rats. Given that a strategy for abolishing the enhanced CSAR-induced sympathoexcitation may be beneficial for preventing the deleterious progression of CHF, we performed epicardial application of RTX at the time of myocardium infarction (MI) surgery in rats to (1) investigate potential beneficial effects of selective deletion of TRPV1-expressing CSAR afferents on autonomic dysfunction and (2) determine whether the CSAR control of sympathetic nerve activity (especially cardiac sympathetic nerve activity [CSNA]) plays a critical role in deleterious cardiac remodeling in CHF.

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Furthermore, we investigated the effects of CSAR afferent desensitization on cardiac morphological and hemodynamic function in rats with CHF.

Methods
Experiments were performed on male Sprague–Dawley rats weighing 400 to 500 g. An experimental timeline is shown in Figure S1 in the online-only Data Supplement. In brief, terminal experiments were performed 9 to 11 weeks after MI in rats treated with either RTX or vehicle. These experiments were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center and performed under the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Model of CHF and Cardiac Sympathetic Afferent Desensitization
Heart failure was produced by coronary artery ligation as previously described.8–9 Cardiac function in all experimental animals was measured 2 and 6 weeks after MI by echocardiography (VEVO 770, Visual Sonics, Inc) as previously described.8–10 To desensitize cardiac sympathetic afferents, RTX (1 mg; Sigma Aldrich) was dissolved in 2 mL of ethanol and mixed with 18 mL of Tween in isotonic saline. RTX (50 μg/mL) was painted twice on the entire left and right ventricles with a small brush just before coronary ligation. The dose of RTX was determined by our preliminary experiments in which we found nearly complete ablation of TRPV1-expressing cardiac nerve endings on the surface of the rat heart for >10 weeks (Figure S2).

General Surgical Preparation for Acute Experiments
At the acute terminal experiments that were performed 9 to 11 weeks after MI, rats were anesthetized with urethane (800 mg/kg IP) and α-chloralose (40 mg/kg IP). Renal sympathetic nerve activity (RSNA) and CSNA were recorded for evaluating basal renal and cardiac sympathetic tones. A detailed surgical description is available in the online-only Data Supplement.

At the end of the experiment, the rat was euthanized with an overdose of pentobarbital sodium. The maximum cardiac or renal nerve activity (Max) occurred 1 to 2 minutes after the rat was euthanized as previously described.1 The background noise for sympathetic nerve activity was recorded 15 to 20 minutes after the rat was euthanized. Using the unit conversion function of Powerlab Chart system, baseline nerve activity was determined as a percent of Max after the noise level was subtracted.

Activation of the CSAR
Epicardial application of capsaicin and bradykinin was used to stimulate the cardiac sympathetic afferents effectively. The time interval between capsaicin and bradykinin was 15 to 20 minutes to allow the AP, HR, and RSNA to return to and stabilize at their control levels.

Construction of Arterial Baroreflex Curves
Baroreflex curves were generated by measuring the HR and RSNA responses to decreases and increases in AP by intravenous administration of nitroglycerin (25 μg) followed by phenylephrine (10 μg) as previously described.2

Measurement of Left Ventricular Performance
Left ventricular (LV) function was assessed using a Millar PV loop System (Millar Instruments, Houston, TX). A detailed method description is available in the online-only Data Supplement.

Histopathology
The heart was fixed in 10% phosphate buffered formalin, embedded in paraffin, and cut into 10 μm serial sections from apex to base. Picrosirius red staining was performed to detect myocardial fibrosis. A detailed method description is available in the online-only Data Supplement.

Immunohistochemistry staining of cleaved caspase 3 was also performed in paraffin sections from the same heart tissues, which were used for picrosirius red staining experiments. A detailed method description is available in the online-only Data Supplement.

Terminal Deoxynucleotidyl Transferase 2'-Deoxyuridine 5'-Triphosphate–Mediated Nick-End Labeling Staining
Terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate–mediated nick-end labeling (TUNEL) staining and analysis were performed to evaluate the effect of RTX on cardiac apoptosis. A detailed method description is available in the online-only Data Supplement.

Western Blot Analysis
Protein expressions of α-smooth muscle actin, fibronectin, transforming growth factor-β (TGF-β) receptor 1, cleaved caspase 3, caspase 3, and β1-adrenergic receptors (β1-AR) in the heart from sham+vehicle, sham+RTX, CHF+vehicle, and CHF+RTX rats were assessed by Western blot technique. A detailed method description is available in the online-only Data Supplement.

Statistical Analysis
All values are expressed as mean±SEM. Differences between groups were determined by a 2-way ANOVA followed by the Tukey post hoc test. P<0.05 was considered statistically significant.

Results
RTX Causes Loss of TRPV1-Expressing Cardiac Afferents
We examined TRPV1 protein expression in cardiac nerve endings from vehicle and RTX-treated rats using immunofluorescence. In vehicle-treated rats, TRPV1 immunoreactivity was primarily located to the epicardial surface and coexpressed with protein gene product 9.5 (a nerve terminal marker; Figure S2A). In RTX-treated rats, TRPV1 immunoreactivity was absent in the epicardium when it was measured 1 week, 4 to 6 weeks, and 9 to 11 weeks after epicardial application of RTX (50 μg/mL), respectively (Figure S2A), suggesting effective depletion of TRPV1-expressing cardiac afferent during these periods. In another parallel set of experiments, we further confirmed the efficiency of RTX-induced TRPV1-expressing CSAR afferent ablation functionally by epicardial application of capsaicin and bradykinin (10 μg/mL) in sham+RTX rats treated with various doses (0.5 μg/mL, 5 μg/mL, and 50 μg/mL) and at various time points (1 week, 4–6 weeks, 9–11 weeks, and 6 months after RTX). These data show that both epicardial application of 5 μg/mL and 50 μg/mL RTX largely abolished the cardiovascular responses to acute epicardial application of capsaicin and bradykinin 1 week after RTX in sham rats (Figure S2B). However, in the preliminary experiment, we observed that 40% of sham rats that were treated with epicardial application of RTX at a dose of 5 μg/mL showed partial recovery of the cardiovascular responses to acute epicardial application of capsaicin and
Sham+Vehicle

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CHF+vehicle rats exhibit exaggerated mean AP, HR, and

shown in Figure 1A–1C, compared with sham+vehicle rats,

and CHF rats 9 to 11 weeks after RTX were performed. As

6 months after RTX (Figure S2C).

responses to capsaicin and bradykinin were largely recovered at

and bradykinin for >10 weeks, whereas these cardiovascular

vascular responses to acute epicardial application of capsaicin

and CHF+RTX rats. Values are means±SE. *P<0.05 vs sham+vehicle; †P<0.05 vs CHF+vehicle. ABP indicates arterial blood pressure; CSNA, cardiac sympathetic nerve activity; HR, heart rate; MAP, mean arterial pressure; and RSNA, renal sympathetic nerve activity.

E, n=6/each group) in sham+vehicle, sham+RTX, chronic heart failure (CHF)+vehicle, and CHF+RTX rats. Values are mean±SE. *

Heart weight, mg 1438±28 1430±30 2239±61* 1650±49*†

Table. Hemodynamic and Morphological Data in Sham and CHF Rats After Vehicle or RTX Treatment

Table. Hemodynamic and Morphological Data in Sham and CHF Rats After Vehicle or RTX Treatment

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<th>CHF+Vehicle (n=23)</th>
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Values are mean±SE. BW indicates body weight; CHF, chronic heart failure; DAP, diastolic arterial pressure; HR, heart rate; HW, heart weight; LVEDP, left ventricle end-diastolic pressure; MAP, mean arterial pressure; RTX, resiniferatoxin; SAP, systolic arterial pressure; and WLW, wet lung weight.

*P<0.05 vs sham+vehicle.
†P<0.05 vs CHF+vehicle.

bradykinin 10 weeks after RTX, whereas all rats treated with 50 μg/mL RTX were still insensitive to capsaicin and bradykinin at this time point. To achieve a long-lasting CSAR ablation, we chose to use 50 μg/mL RTX in all the following experiments. Data from our time course experiments demonstrated that epicardial application of this dose of RTX abolished the cardiovascular responses to acute epicardial application of capsaicin and bradykinin for >10 weeks, whereas these cardiovascular responses to capsaicin and bradykinin were largely recovered at 6 months after RTX (Figure S2C).

Functional experiments comparing RTX-treated sham and CHF rats 9 to 11 weeks after RTX were performed. As shown in Figure 1A–1C, compared with sham+vehicle rats, CHF+vehicle rats exhibit exaggerated mean AP, HR, and RSNA responses to epicardial application of either capsaicin or bradykinin. However, the cardiovascular and sympathetic activation in response to epicardial capsaicin in both sham and CHF rats was almost completely abolished by epicardial pretreatment with RTX. RTX also greatly attenuated the bradykinin-induced CSAR activation in both sham and CHF rats. Effect of RTX on Body Weight, Organ Weight, and Hemodynamics in Sham and CHF Rats

Morphological and hemodynamic measurements of all groups of rats are summarized in the Table. The heart weight and lung weight-to-body weight ratios were significantly higher in CHF+vehicle rats compared with sham+vehicle rats, suggesting cardiac hypertrophy and pulmonary congestion in
the CHF state. However, after pretreatment with epicardial RTX, there was a significant decrease in both the heart weight and lung weight-to-body weight ratios in CHF rats compared with CHF+vehicle rats, suggesting a protective effect of RTX on cardiac hypertrophy and pulmonary congestion. Importantly, there was no significant difference in infarct size between CHF+vehicle and CHF+RTX rats (Table; Figure S3), which excludes the possibility that the variability in infarct size caused the difference in the heart weight or lung weight between CHF+vehicle and CHF+RTX rats.

Hemodynamic and echocardiographic measurements point to several other important differences between CHF+vehicle and CHF+RTX rats. CHF+vehicle rats exhibited elevated LV end-diastolic pressure (LVEDP) and time-dependent decline in ejection fraction and fractional shortening compared with sham+vehicle rats (Table; Table S1), indicating decreased ventricular function. However, the increased LVEDP observed in CHF+vehicle rats was largely prevented in CHF+RTX rats, which is consistent with the finding that lung congestion was also reduced in CHF+RTX rats. MI-induced cardiac dilation in CHF rats, indicated by increased LV systolic and diastolic diameters and volumes, was significantly reduced by RTX treatment compared with sham rats (Table S1). Interestingly, although LVEDP and cardiac dilation were reduced by RTX, there was no significant difference in ejection fraction or fractional shortening 2 weeks and 6 weeks after MI between CHF+RTX and CHF+vehicle rats (Table S1). $dP/dt$ measurements (Table; Figure 2) further show differences between CHF+vehicle and CHF+RTX rats. Compared with sham+vehicle rats, CHF+vehicle rats exhibited decreased $dP/dt_{max}$ and $dP/dt_{min}$, suggesting both systolic and diastolic dysfunction in the hearts of CHF rats (Table). However, the blunted $dP/dt_{min}$ in CHF+vehicle rats was significantly increased in CHF+RTX rats, potentially indicating improved diastolic function in CHF+RTX rats. Although there was no significant difference in $dP/dt_{max}$ at rest between CHF+RTX and CHF+vehicle rats, the $dP/dt_{max}$ response to isoproterenol stimulation in CHF+RTX rats was significantly greater compared with CHF+vehicle rats (Figure 2). At the molecular level, the decreased protein expression of $\beta_1$-AR in myocardium of CHF+vehicle rats was partially reversed in CHF+RTX rats (Figure 3D). There were no significant differences in cardiac morphological, echocardiographic, or hemodynamic parameters between sham+vehicle and sham+RTX rats.

**Effects of RTX on Cardiac Fibrosis in Sham and CHF Rats**

Considering that the RTX-mediated reduction in LVEDP in CHF rats may be related to an improvement in cardiac

![Figure 2](http://hyper.ahajournals.org/)

*Figure 2. Representative tracings (A) and mean data (B) showing basal $dP/dt_{max}$ and diastolic $dP/dt_{min}$, as well as the dose-dependent responses to $\beta$-adrenergic receptor stimulation with isoproterenol (ISO; 0.01, 0.1, and 1.0 μg/kg, 0.2 mL). Values are means±SE. n=6/group. *P<0.05 vs sham+vehicle; †P<0.05 vs chronic heart failure (CHF)+vehicle. ABP indicates arterial blood pressure; HR, heart rate; LVP, left ventricular pressure; and RTX, resiniferatoxin.*
compliance, we further evaluated cardiac fibrosis using picrosirius red staining and protein expression of 2 fibrotic markers (α-smooth muscle actin and fibronectin), as well as their upstream activator, TGF-β. RTX reduced the expression of all these markers in the noninfarcted LV and IVS walls (Figure 3). RTX did not cause any significant change in either picrosirius red staining or protein expressions of fibrotic markers and TGF-β receptor I in noninfarcted LV and IVS walls in sham rats.

**Effects of RTX on Basal Sympathetic Tone and Arterial Baroreflex Function in Sham and CHF Rats**

Baseline RSNA and CSNA were significantly higher in CHF+vehicle rats compared with sham+vehicle rats, indicating increased renal and cardiac sympathetic tone in CHF. RTX lowered basal RSNA and CSNA in CHF rats (Figure 1D; Figure S4; Figure S5). Furthermore, urinary norepinephrine excretion was significantly reduced in CHF+RTX rats compared with vehicle-treated rats (Figure 1E). In addition, RTX significantly increased the average slope and Gainmax and decreased the minimum HR and RSNA of the arterial baroreflex control of both HR and RSNA (Figure 4; Table S2). In contrast to the CHF rats, there were no significant effects on the average slope, Gainmax, and minimum RSNA or HR of arterial baroreflex control in sham rats (Figure 4; Table S2).

**In Vivo Pressure–Volume Loop Analysis in Sham and CHF Rats Treated With Vehicle or RTX**

Using LV pressure–volume loop analysis (Figure 5; and Table S3), basal load-independent contractile function, as measured by the slope of the preload recruitable stroke work relationship and end-systolic pressure volume relationship, was significantly reduced in CHF+vehicle rats compared with sham+vehicle rats. Load-sensitive measures of systolic and diastolic function such as the maximum and minimum
rates of pressure change ($dP/dr_{max}$ and $dP/dr_{min}$) and ejection fraction were also significantly lower in CHF+vehicle compared with sham+vehicle rats (Table S3). Furthermore, compared with sham+vehicle rats, higher Tau and LVEDP were found in CHF+vehicle rats, indicating compromised LV active and passive diastolic function. In addition, compared with sham+vehicle rats, CHF+vehicle rats presented with significant LV chamber enlargement, as shown by increased end-systolic and diastolic volume (Table S3). These data are consistent with the data obtained by echocardiographic measurements (Table S2). The end-diastolic pressure volume relationship was also significantly elevated in CHF+vehicle rats compared with sham+vehicle rats, suggesting increased ventricular stiffness in CHF (Figure 5; Table S3). Compared with the CHF+vehicle rats, structural and functional abnormalities were ameliorated by RTX, as shown by a reduction in LV chamber size, increased $dP/dr_{min}$, reduced Tau and LVEDP, as well as reduced stiffness as shown by the end-diastolic pressure volume relationship (Figure 5; Table S3). Interestingly, RTX treatment did not have a significant effect on basal contractile function such as $dP/dr_{min}$, preload recruitable stroke work, and end-systolic pressure volume relationship in CHF rats. In addition, RTX had no effect on contractile and relaxation parameters in sham rats.

**Effects of RTX on Cardiac Apoptosis in Sham and CHF Rats**

Cardiac remodeling includes hypertrophy, fibrosis, and apoptosis. Based on the beneficial effects of RTX on cardiac hypertrophy and cardiac fibrosis in CHF rats, we further investigated the effect of RTX on cardiac apoptosis in sham and CHF rats. TUNEL staining was performed, and representative photomicrographs are depicted in Figure 6A and Figure S6. We found that compared with sham+vehicle rats, there was a significant increase in TUNEL-positive cells in the LV infarct, LV peri-infarct, LV remote, and IVS regions of the myocardium in CHF+vehicle rats (Figure 6B–6E). However, TUNEL-positive cells were markedly reduced in the LV infarct, LV peri-infarct, LV remote, and IVS regions of the myocardium from CHF+RTX rats compared with CHF+vehicle rats (Figure 6). There was no significant difference in the number of TUNEL-positive cells in the heart between sham+vehicle and sham+RTX rats. Furthermore, data from immunohistochemical staining demonstrated that there were increased cleaved caspase 3-positive cardiomyocytes in the LV peri-infarct, LV remote, and IVS regions of the myocardium in CHF+vehicle rats compared with sham+vehicle rats, which was largely prevented by epicardial application of RTX after coronary artery ligation (Figure S7A). In addition, protein expression of cleaved caspase 3 in LV remote and IVS regions of the myocardium was significantly increased in CHF+vehicle rats compared with sham+vehicle rats, whereas RTX treatment prevented the elevated protein expression of cleaved caspase 3 in the myocardium of CHF rats (Figure S7B and S7C).

**Discussion**

The results of the current study clearly demonstrate that epicardial application of RTX just before the creation of an MI abolished the enhanced CSAR, prevented the exaggerated renal and cardiac sympathoexcitation, and increased the blunted baroreflex in CHF rats. Importantly, we found that epicardial application of RTX prevented many of the pathological indices of the CHF state. These included elevated LVEDP, lung edema, cardiac hypertrophy, chamber dilation, and contractile reserve in response to β-adrenergic receptor stimulation with isoproterenol. RTX also ameliorated cardiac fibrosis and...
Increased CSNA is a primary characteristic of patients experiencing CHF.11,12 Prolonged stimulation of the β-adrenergic neurohormonal axis has been shown to contribute to the progression of CHF and mortality in both animal models and in humans.13,14 Measurements of cardiac norepinephrine spillover in patients with CHF reflecting CSNA indicate that it is increased earlier and to a greater extent than sympathetic nerve activity to other organs.15–17 Data based on direct CSNA recording in the present study also supports the concept that CHF rats have markedly higher basal cardiac sympathetic tone than sham rats. However, the mechanisms underlying this augmented cardiac sympathetic outflow in CHF are not fully understood. One potential explanation is an increased CSAR control of cardiac sympathetic efferent outflow (also called the cardiac–cardiac reflex) in CHF. It is well known that myocardial ischemia releases large amounts of metabolites including bradykinin, ATP, prostaglandins, and protons that stimulate cardiac sympathetic afferent nerve endings and cause an excitatory response of mean AP, HR, and sympathetic nerve activity to other organs.18–20 Previous studies from this laboratory1,2 demonstrated that acute epicardial application of lidocaine, which blocked cardiac sympathetic afferent input, decreased baseline mean AP, HR, and RSNA in anesthetized CHF dogs and rats but not in sham animals, indicating that the CSAR is tonically activated and contributes to the elevated RSNA in CHF. We further reported that the sites at which the CSAR is sensitized reside at both the afferent endings21 and in the central nervous system.19 Whether the tonic activation of the CSAR also contributes to the elevated CSNA has not been explored. In the present study, we provide direct evidence that selective cardiac sympathetic afferent deafferentation by epicardial application of RTX prevents local excessive cardiac sympathetic activation in CHF rats. After blocking the CSAR control of cardiac sympathetic activation by RTX, we also observed multiple improvements in the structure and function of the myocardium in CHF rats. These findings highlight a critical contribution of the CSAR to the enhanced cardiac sympathetic tone, as well as cardiac remodeling in CHF.

We examined multiple indices of cardiac remodeling and function in CHF rats treated with RTX. Our data show a reduction in chamber size, lower LVEDP, reduced cardiac hypertrophy, and attenuated cardiac fibrosis and apoptosis. Based on these protective effects, we think the RTX-mediated improvement in cardiac diastolic function in CHF rats to be of primary importance. CHF rats treated with RTX did not exhibit the expected increase in LVEDP and lung congestion compared with CHF+vehicle rats. Our pressure–volume loop data established a direct beneficial effect of RTX on cardiac diastolic function in the failing heart, independent of systemic vascular resistance. Furthermore, our molecular data suggest that RTX attenuated cardiac fibrosis in noninfarcted areas of the LV and IVS of CHF rats by reducing activation of the classic TGF-β receptor fibrotic signaling pathway. Taken together, these data suggest that the lower LVEDP observed in CHF+RTX rats is, at least in part, because of an RTX-mediated improvement of cardiac compliance. Because peripheral venous...
beds are highly innervated by sympathetic nerves and RTX-induced CSAR attenuation reduced the exaggerated systemic sympathoexcitation in CHF, another possibility is a reduction in venoconstriction and thus reduced cardiac filling, which would contribute to lower LVEDP in RTX-treated CHF rats.

With regard to the effect of RTX on cardiac systolic function in CHF, it is important to emphasize that the favorable effects of RTX on dP/dt max were not evident in the basal (unstimulated) state but rather in response to β-adrenergic receptor stimulation with isoproterenol, indicating that the myocardial contractile reserve was substantially increased after RTX treatment. This finding is consistent with the observation that the downregulation of β1-AR in the myocardium of CHF rats was partially prevented by RTX. The underlying mechanisms by which RTX partially restored the β1-AR expression in CHF remain to be identified. A reasonable speculation is that by removing the influence of the CSAR on sympathetic outflow to the heart and thereby reducing excessive myocardial norepinephrine release, an upregulation of the β1-AR takes place. However, this hypothesis awaits confirmation.

RTX treatment affected the remodeling process in other ways. In CHF+RTX rats, LV chamber size was smaller as reflected by reduced LV end-systolic and end-diastolic diameters and volumes compared with CHF+vehicle rats even when ejection fraction and infarct size were similar. It is known that during the progression of CHF, persistent ischemia and inflammation in the ischemic zone impair granulation tissue, extracellular matrix formation, and ischemic myocyte survival, resulting in extension and expansion of the failing myocardium. The current study provides strong evidence for the fact that epicardial RTX applied at the time of MI creation reduces this vicious cycle and delays progression of the CHF state. In a previous study by White et al, it was clearly shown that LV end-systolic volume rather than ejection fraction was the major determinant of survival in patients who recovered from an MI. Therefore, the RTX-mediated reduction of cardiac volume may potentially contribute to enhanced survival for patients with MI.

RTX significantly ameliorated cardiac apoptosis in both infarcted and noninfarcted areas of LV in CHF rats. It is well known that cell death is an important determinant of cardiac remodeling because it causes a loss of contractile tissue, compensatory hypertrophy of myocardial cells, and reparative fibrosis. Therefore, the RTX-mediated reduction in cardiac apoptosis may play an important role in these multiple RTX-mediated cardiac protective effects in CHF rats. The mechanisms underlying the reduction of cardiac apoptosis remain unknown. Considering that (1) excessive β-AR stimulation has been reported to induce cardiac myocyte apoptosis both in vivo and in vitro and (2) RTX prevents the exaggerated cardiac sympathetic tone in CHF, it is reasonable to speculate that RTX may reduce cardiac
apoptosis through the CSNA–β-adrenergic neurohormonal axis. Further studies will be needed to explore the molecular mechanisms underlying the RTX-induced protection against cardiac apoptosis in CHF.

The CSAR is a sympathoexcitatory reflex and contributes to the elevation in sympathetic tone. The latter can antagonize baroreflex function in the CHF state. In a previous study, we reported that in anesthetized rats with CHF, acute epicardial application of lidocaine decreased baseline RSNA and improved the impaired baroreflex control of RSNA, suggesting that tonic cardiac sympathetic afferent input plays an important role in blunting the baroreflex in CHF. In a more recent study, we further reported that acute stimulation of cardiac sympathetic afferents by epicardial application of capsaicin significantly decreased basal discharge of barosensitive nucleus of the solitary tract (nucleus tractus solitarius) neurons in anesthetized rats, indicating that the nucleus tractus solitarius plays an important role in processing the interactions between the CSAR and the baroreflex. However, because of the acute nature of the experiments in these previous studies, it is not known whether the interaction between the CSAR and the baroreflex occurs in more chronic pathological conditions such as CHF.

In this study, we further found that epicardial application of RTX at the time of MI creation prevents the blunted baroreflex control of HR and RSNA 9 to 11 weeks after MI, suggesting a chronic interaction between the CSAR and the baroreflex in CHF. In addition, the effects of lidocaine and RTX on the improvement in baroreflex function seem to be specific to the CHF state because in sham-operated rats, we failed to find any change in RSNA and baroreflex parameters after epicardial application of either lidocaine or RTX, indicating that there is little, if any, tonic cardiac sympathetic afferent input in either the regulation of sympathetic nerve activity or interaction with the baroreflex in the normal state.

In the current study, RTX was selectively delivered to the epicardium to ablate the TRPV1-expressing cardiac afferents. A previous study by Zahner et al showed that sympathetic vasomotor tone is intact after systemic treatment with RTX. Another study by Szolcsányi et al reported that bradycardia and the fall in blood pressure evoked by electric stimulation of the peripheral vagus were not impaired after systemic RTX injection, suggesting that cardiac parasympathetic tone remained unaltered after RTX pretreatment. In addition, another earlier study by Barber et al reported that epicardial application of phenol (a nonselective neurotoxin) interrupted sympathetic afferents but not vagal afferents in a canine model, suggesting that vagal afferents are mainly located in deeper LV layers and are less affected by epicardial application of drugs. This finding is important for the current work since epicardial application of RTX was used because it makes epicardial application of RTX more specific in targeting cardiac sympathetic afferents even if a small subpopulation of vagal afferents also express TRPV1 receptors. Therefore, it seems that RTX selectively targets cardiac sympathetic afferents without impairing cardiac sympathetic and parasympathetic efferent nerves and vagal afferent nerves. Using this selective cardiac afferent desensitization strategy, we demonstrated multiple beneficial effects of RTX-induced inhibition of the CSAR on cardiac and autonomic function in CHF rats. In contrast to the current study, genetic knockout techniques to systematically delete TRPV1 receptors in mice have reported conflicting findings concerning either the beneficial or deleterious effects of global deletion of TRPV1 on cardiac function. There are several major differences between selective TRPV1-expressing cardiac afferent ablation and systemic pharmacological antagonism or genetic deletion of TRPV1 receptor strategies. The greatest difference between the current study and those mentioned above is that RTX not only deletes TRPV1 receptors in cardiac afferents but also damages the TRPV1-expressing nerve endings rather than only blocking TRPV1 receptors. Considering that TRPV1-expressing cardiac sympathetic afferent endings also express many other sensory receptors such as tachykinin and purinergic receptors, RTX also impairs these sensory receptors after damage of TRPV1-expressing cardiac afferent nerve endings. This explains why epicardial RTX application blocks both epicardial capsaicin and bradykinin-induced cardiovascular responses, whereas TRPV1 receptor blockade using epicardial ido-RTX only abolishes epicardial capsaicin-induced responses without any effect on the bradykinin-induced cardiovascular response. In addition, it should be pointed out that knockout animals have systemic TRPV1 deletion from birth, which raises the issue of developmental and compensatory changes. Similar issues may be at play in other models of global TRPV1 deletion such as in a neonatal rat model that was treated with a large dose of capsaicin. There is clear evidence showing that adult rats that are treated with capsaicin as neonates exhibit reductions in both basal coronary flow and cardiomyocyte size. This effect may result in an increase in cardiac damage when the heart is exposed to ischemia or pressure overload. Finally, global TRPV1 deletion using either genetic knockout or systemic pharmacological techniques will affect the central and peripheral nervous system, as well resulting in the loss of both protective and deleterious effects of TRPV1 activation. Therefore, in our opinion, selective ablation of TRPV1-expressing sensory afferents may or may not achieve the same cardiovascular effects in MI rats compared with those studies using pharmacological antagonism or genetic deletion of TRPV1 receptors.

Limitations

In this study, epicardial application of RTX was performed at the time of MI rather than several days or weeks after MI. Therefore, beneficial effects of RTX on cardiac and autonomic function indicate an early protective effect against the progress of deleterious cardiac remodeling in post-MI rats. However, whether epicardial application of RTX several days or weeks after MI has similar beneficial effect on cardiac dysfunction in post-MI rats remains unknown. We speculate that epicardial application of RTX during MI or early post-MI in rats should have greater protective effects on cardiac dysfunction than during the mid or late post-MI period. For example, with regard to the current finding that epicardial application of RTX during MI largely prevents cardiac fibrosis in post-MI rats, it is less likely that epicardial application of RTX at the mid or late post-MI period would have the same beneficial effect because cardiac fibrosis would already be well developed during that period. A previous study by Judd and...
Wexler\textsuperscript{33} reported that myocardial hydroxyproline content (an amino acid primarily found in collagen) was increased by day 7 after MI and increased further by \( \approx 2 \) weeks after MI. This may suggest a potentially ideal therapeutic window for RTX-mediated protective effect against cardiac fibrosis caused by MI. Once myocardial collagen accumulation is well established, the potential protective effect of RTX against cardiac fibrosis observed in the current study may be reduced. Clearly further studies are needed to address this issue.

Another potential limitation of this study relates to potential reinnervation after RTX-induced cardiac afferent ablation. A previous study by Karai et al\textsuperscript{34} reported that either subcutaneous or intrathoracic injection of RTX (6 \( \mu \)g/mL, 30 \( \mu \)L) attenuates thermal nociception and inflammatory hyperalgesia in rats. Interestingly, the beneficial effects of subcutaneous RTX injection on inflammatory hyperalgesia only lasted \( \approx 20 \) days, whereas the effect of intrathoracic injection of RTX lasted much longer (\( \approx 1 \) year). In the current study, when applying a higher concentration (50 \( \mu \)g/mL) of RTX to the surface of heart, we found that this treatment abolished the CSAR for \( \geq 9 \) to 11 weeks. However, we did observe a recovery of CSAR sensitivity at 6 months after epicardial application of RTX, indicating that this intervention if performed in a clinical setting may need to be repeated. However, based on the study by Karai et al.,\textsuperscript{34} intrathoracic RTX injection at thoracic segments to ablate TRPV1-expressing cardiac sympathetic afferent neurons may be an alternative for longer ablation of the CSAR in patients with MI. Clearly further studies are needed to address this issue. In addition, we acknowledge a limitation of this study is that all the terminal experiments were performed under anesthesia. Some of hemodynamic data such as basal blood pressure and HR should be further confirmed by conscious telemetry recording. Finally, we acknowledge a limitation that the current study did not answer the question of whether epicardial application of RTX during coronary ligation could reduce infarct size because we excluded animals with small MIs in both CHF+vehicle and CHF+RTX groups to rule out a possible contribution of surgical variability to cardiac function. Additional studies should be performed to address this important issue.

In summary, the present study provides the first evidence demonstrating that selective cardiac sympathetic afferent deafferentation by epicardial application of RTX during MI prevents cardiac and autonomic dysfunction in rats 9 to 11 weeks after MI. These findings demonstrate the importance and therapeutic potential of targeted deletion of CSAR in ventricular remodeling after MI. Figure S8 summarizes the potential role of the CSAR in the regulation of sympathetic outflow and how interruption of this reflex may be used in sympathoexcitatory disease states.

**Perspectives**

RTX, an ultrapotent analog of capsaicin and capable of inducing rapid degeneration of TRPV1-expressing afferent neurons and fibers,\textsuperscript{35–37} has been widely explored for its SANalgesic properties.\textsuperscript{35–37} The current study reports a novel use of RTX for potential intervention in the cardiac remodeling process in patients after an MI. We think it is possible to deliver RTX to the surface of the heart using pericardial centesis and thereby selectively ablate cardiac sympathetic afferents in patients with CHF. Therefore, our current findings that RTX-induced CSAR deafferentation prevents cardiac remodeling and reduces cardiovascular dysfunction in CHF rats have high translational potential. Ongoing studies from this laboratory are further investigating the potential beneficial effects of epicardial application of RTX on cardiac dysfunction in larger animal models.

**Acknowledgments**

We thank Johnnie F. Hackley, Pam Curry, Kaye Talbitzer, and Richard Robinson for their expert technical assistance. We also thank Dr David Wert, as well as the University of Nebraska Medical Center Tissue Sciences Facility, for their help with the histopathology, terminal deoxynucleotidyl transferase 2’-deoxyuridine 5’-triphosphate-mediated nick-end labeling, and cleaved caspase 3 staining experiments.

**Sources of Funding**

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**Disclosures**

None.

**References**


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Supplemental Materials

Cardiac Sympathetic Afferent Denervation attenuates cardiac remodeling and improves cardiovascular dysfunction in rats with heart failure

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Running Title: Cardiac Afferent Denervation in Heart Failure

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Model of CHF and cardiac sympathetic afferent desensitization

Heart failure was produced by coronary artery ligation as previously described. Briefly, the rat was ventilated at a rate of 60 breaths/min with 3% isoflurane in oxygen during the surgical procedure. A left thoracotomy was performed through the fifth intercostal space, the pericardium was opened, the heart was exteriorized, and the left anterior descending coronary artery was ligated. Sham-operated rats were prepared in the same manner but did not undergo coronary artery ligation. If the infarct size was less than 15%, the rat was considered as having an MI too small to significantly impact cardiac function and was excluded from data analyses.

In order to desensitize cardiac sympathetic afferents, resiniferatoxin (RTX, 1 mg; Sigma Aldrich) was dissolved in 2 ml of ethanol and mixed with 18 ml of Tween in isotonic saline [2 ml of Tween 80 (Sigma-Aldrich) dissolved in 16 ml of isotonic saline]. RTX (50 µg/ml) was painted twice on the entire left and right ventricles with a small brush just prior to coronary ligation. The dose of RTX was determined by our preliminary experiments in which we found nearly complete ablation of TRPV1-expressing cardiac nerve endings on the surface of the rat heart (Figure 2S). During acute terminal experiments, the efficiency of RTX-induced cardiac sympathetic afferent desensitization was further confirmed by examining the cardiovascular and neural responses to epicardial application of capsaicin or bradykinin (10 µg/ml). Vehicle was painted on the ventricles as a control. All rats survived from the sham surgery. There was no obvious difference in 3-day post-MI survival rate (67% vs. 65%) between CHF+RTX and CHF+Vehicle treated rats.

Cardiac function in all experimental animals was measured 2 and 6 weeks after MI by echocardiography (VEVO 770, Visual Sonics, Inc.) as previously described. In all acute terminal experiments except for pressure volume (PV) loop analysis, a Millar catheter (SPR 524; size, 3.5-Fr; Millar Instruments, Houston, TX) was advanced through the carotid artery into the left ventricle (LV) to determine LV end-diastolic pressure (LVEDP), LV systolic pressure (LVSP), the maximum first derivative of LV pressure (dp/dt_max) and minimum first derivative of LV pressure (dp/dt_min). In PV loop analysis experiments, cardiac function was measured by using a Millar PV loop System (Millar Instruments, Houston, Tex, USA). In addition, in some experiments (n=6/each group), three doses of isoproterenol (ISO, 0.01, 0.1 and 1.0 µg/kg, 0.2 ml) were injected into the left jugular vein to compare cardiac function in response to beta-adrenergic receptor stimulation in sham and CHF rats with and without treatment with RTX. Baseline LVEDP, LVSP, HR, dp/dt_max and dp/dt_min as well as their changes in response to three doses of ISO injection were recorded and compared in all four groups. At the end of these acute experiments, the rats were euthanized with an overdose of pentobarbital sodium. The hearts and lungs were removed, and the ratio of the infarct area to total LV area was measured.

General Surgical Preparation for Acute Experiments

At the acute terminal experiments that were performed 9-11 weeks post-MI, rats were anesthetized with urethane (800 mg/kg ip) and α-chloralose (40 mg/kg ip). The trachea was cannulated, and the rat was paralyzed with pancuronium bromide (1 mg/kg iv, 0.1 mg/kg thereafter as needed) and ventilated artificially with room air supplemented with 100% oxygen. A Millar catheter (SPR 524; size, 3.5-Fr; Millar Instruments, Houston, TX) was advanced
through the right common carotid artery into the left ventricle to determine basal cardiac functional parameters such as LVEDP, LVSP, dp/dt\text{max} and dp/dt\text{min}. The transducer was then pulled back into the aorta and left in place to record arterial pressure (AP) and mean arterial pressure (MAP). Heart rate was derived from the AP pulse using Chart 7.1 software and a PowerLab model 16S (ADInstruments, Colorado Springs, CO) data acquisition system. The right jugular vein was cannulated for intravenous injections. Body temperature was maintained between 37°C and 38 °C by a heating pad.

**Recording renal sympathetic nerve activity (RSNA) and cardiac sympathetic nerve activity (CSNA).**

Renal sympathetic nerve activity was recorded as previously described\(^1\). Generally, the left kidney, renal artery, and nerves were exposed through a left retroperitoneal flank incision. Sympathetic nerves running on or beside the renal artery were identified. The renal nerve was cut distally to avoid recording afferent activity. The renal sympathetic nerves were placed on a pair of platinum-iridium recording electrodes and then were covered with a fast-setting silicone (Kwik-Sil; World Precision Instruments). Nerve activity was amplified (×10000) and filtered (bandwidth: 100 to 3000 Hz) using a Grass P55C preamplifier. The nerve signal was monitored on an oscilloscope. The signal from the oscilloscope was displayed on a computer where it was rectified, integrated, sampled (1 KHz), and converted to a digital signal by the PowerLab data acquisition system.

In order to record CSNA, the chest was opened through the left second intercostal space. The left stellate ganglion was identified, and a branch innervating the heart was isolated and cut as distal as possible. The central end of cardiac sympathetic efferent nerve was placed on a pair of platinum-iridium recording electrodes and then was covered with a fast-setting silicone (Kwik-Sil; World Precision Instruments). Similar to RSNA recording, CSNA was amplified (×10000) and filtered (bandwidth: 100 to 3000 Hz) using a Grass P55C preamplifier. The nerve signal was monitored on an oscilloscope. The signal from the oscilloscope was displayed on a computer where it was rectified, integrated, sampled (1 KHz), and converted to a digital signal by the PowerLab data acquisition system.

At the end of the experiment, the rat was euthanized with an overdose of pentobarbital sodium. The maximum cardiac or renal nerve activity (Max) occurred 1-2 min after the rat was euthanized as previously described\(^1\). The background noise for sympathetic nerve activity was recorded 15-20 min after the rat was euthanized. Using the unit conversion function of Powerlab Chart system, baseline nerve activity was determined as a percent of Max after the noise level was subtracted.

**Activation of the CSAR**

Epicardial application of capsaicin and bradykinin has been demonstrated to effectively stimulate the cardiac sympathetic afferents\(^5\). The chest was opened through the fourth intercostal space. The pericardium was removed to expose the left ventricle. A square of filter paper (3 × 3 mm) saturated with capsaicin (10 μg/ml) or bradykinin (10 μg/ml) was applied to the anterior surface of the left ventricle. Hemodynamic and neural parameters were continuously recorded. After the peak response, the epicardium was rinsed three times with 10 ml of warm normal saline.
(38°C). The time interval between capsaicin and bradykinin was about 15-20 min to allow the AP, HR and RSNA to return to, and stabilize at their control levels.

**Construction of Arterial Baroreflex Curves**

Baroreflex curves were generated by measuring the RSNA responses to decreases and increases in AP by intravenous administration of nitroglycerin (25 µg) followed by phenylephrine (10 µg) as previously described. The RSNA response was normalized as a percent of baseline. A sigmoid logistic function was fit to the data using a nonlinear regression program (SigmaPlot version 8.0; SPSS). Four parameters were derived from the following equation: %RSNA or HR = A/1 + exp[B(MAP – C)] + D, where A is the RSNA or HR range, B is the slope coefficient, C is the pressure at the midpoint of the range (BP50), and D is minimum RSNA or HR. The peak slope [or maximum gain (Gainmax)] was determined by taking the first derivative of the baroreflex curve described by the equation.

**Measurement of Left Ventricular Performance**

Left ventricular (LV) function was assessed using a Millar PV loop System (Millar Instruments, Houston, Tex, USA). A microtip PV catheter (SPR-838) was inserted into the right carotid artery and advanced into the LV. A polyethylene catheter was inserted into the left jugular vein for fluid administration. After stabilization for 10–20 minutes, LV-PV signals were recorded continuously at a sampling rate of 1000Hz using an MPVS-300 conductance system (Millar Instruments) coupled to a PowerLab 8/30 (ADInstruments, Inc., USA). 50 µL of 20% saline was injected intravenously so as to establish a parallel conductance volume from the shift of PV relations, and this was used for correction of the cardiac mass volume. LV-PV loops were also captured by transiently compressing the inferior vena cava. LV parameters were computed using cardiac PV analysis software (PVAN3.2, Millar Instruments). Volume calibrations were performed with the Millar volume calibration cuvette which consists of a 1 cm deep cylindrical block with cylindrical holes of known diameters ranging from 2 to 11 mm filled with fresh heparinized whole rat blood. The linear volume-conductance regression of the absolute volume in each cylinder versus the raw signal acquired by the conductance catheter was used for the volume calibration using PVAN 3.2. In addition to the hemodynamic parameters described above the system calculated, the time constant of isovolumetric relaxation (Tau), ejection fraction (EF), end-diastolic volume (EDV), cardiac output (CO), stroke volume (SV), stroke work (SW), preload recruitable stroke work (PSRW), end systolic pressure volume relationship (ESPVR) and the end diastolic pressure volume relationship (EDPVR).

**Histopathology.**

The heart was fixed in 10% phosphate buffered formalin, embedded in paraffin, and cut into 10 µm serial sections from apex to base. Picrosirius red staining was performed to detect myocardial fibrosis. In brief, embedded paraffin heart sections were placed onto Colorfrost Plus microscope slides (Fisher Scientific), deparaffinized, dehydrated and incubated in saturated 0.1% picrosirius red for 90 min. Then sections were rinsed twice with 0.01N HCl for 1 min, dehydrated with an ethanol gradient, and prepared for histologic analysis. Using National Institutes of Health ImageJ software, the interstitial fibrotic area was calculated by measuring the collagen-positive red area (square millimeters) within the LV infarct, LV peri-infarct, LV remote
area and inter-ventricular septum (IVS). Myocardial fibrosis was expressed as percent fibrosis, the fraction of red stained area in the total myocardium from different LV regions and IVS separately, with results obtained from 10 fields per section per animal. In addition, when cutting each section that was prepared for sirus red staining, the next section was also saved and prepared for Masson's trichrome staining to evaluate the infarct size. Only the myocardial sections that had similar infarct size between CHF+vehicle and CHF+RTX rats were used for sirus red staining to analyze cardiac fibrosis so as to exclude the possibility that the variable degree of infarct size caused differences in cardiac fibrosis between CHF+vehicle and CHF+RTX rats.

For immunohistochemistry staining of cleaved caspase 3 (CC3), paraffin sections from the same heart tissues which were used for picrosirius red staining experiments were deparaffinized in xylene and rehydrated through a descending gradient of alcohol (100%, 95%, 80%, 2 minutes at each concentration) and then water. Antigen retrieval was achieved with 10 mmol/L Tris, 1 mmol/L EDTA, and 0.05% Tween 20 pH 9.0 for 20 minutes in a steamer and then cooled for 20 minutes. Individual sections were then incubated with primary antibodies to the apoptotic marker cleaved caspase-3 (1:500, Invitrogen, 700182). Immunohistochemistry was done on a DAKO Autostainer utilizing a peroxidase DAB method (Leica, Novalink RE7150-K). Human tonsil tissue served as a positive control. Using National Institutes of Health ImageJ software, data were calculated by measuring percentage of the CC3-positive cardiomyocytes within the LV peri-infarct, LV remote area and interventricular septum (IVS).

Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling staining.

Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) staining and analysis was performed to evaluate the effect of RTX on cardiac apoptosis. In brief, heart sections were deparaffinized and incubated for eight minutes in fresh permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate). After the slices were rinsed twice in 1X PBS solution, an in situ apoptotic cell death detection kit (Fluorescein, Roche Applied Biosystems) was used to detect host myocardium apoptotic nuclei. Heart sections were thereafter mounted with Anti-fade Vecta shield mounting medium containing 4, 6-diamidino-2-phenylindole (DAPI; Vector laboratories) for nuclear visualization. Sections were scanned under Leica fluorescence microscopes (DM5500) and analyzed by MetaMorph Microscopy Automation & Image Analysis Software (Molecular Devices). The percentage of total apoptotic nuclei was determined in the LV infarct, LV peri-infarct, LV remote regions and IVS from 5-6 animals/group. Percentage total apoptotic nuclei was calculated as total number apoptotic nuclei/total number of DAPI × 100%.

Western Blot Analysis

After the rats were euthanized with an overdose of pentobarbital sodium (150 mg/kg, IV), the hearts and lungs were rapidly removed on ice and infarct size was quickly measured. Then the LV infarct, LV peri-infarct, LV remote regions and IVS were quickly dissected on ice and stored on -80 °C. During the experiments, the samples were lysed with 20 mM Tris-HCl buffer, PH 8.0, containing 1% NP-40, 150 mM NaCl, 1mM EDTA, 10% glycerol, 0.1% β-mercaptoethanol, 0.5 mM dithiothreitol, and a mixture of proteinase and phosphatase inhibitors (Sigma). Protein concentration was measured by the BCA protein assay method using bovine serum albumin as standard. The proteins were loaded onto a 10% SDS-PAGE gel along with protein standards.
(Bio-Rad Laboratories) in a separate lane for electrophoresis and then transferred to polyvinylidene fluoride membrane. The membrane was probed with mouse monoclonal antibody against alpha smooth muscle actin (α-SMA, 1:500 dilutions, Sigma aldrich) or rabbit polyclonal antibody against fibronectin, Transforming growth factor beta (TGF-β) receptor 1 (1:500 dilutions, Sigma aldrich), cleaved caspase 3 (1:500, cell signaling), caspase 3 (1:1000, cell signaling) and beta 1-adrenergic receptors (β1-AR, 1:500 dilutions, Santa Cruz Biotechnology) and secondary antibody of goat anti-rabbit or anti-mouse IgG (1:5000 dilutions, Pierce Chemical). The protein signals were detected by enhanced chemiluminescence reagent (Pierce Chemical) and analyzed using UVP BioImaging Systems. GAPDH (1:1000, Santa Cruz Biotechnology) was used to verify equal protein loading, and the densitometric results of α-SMA, fibronectin, TGF-β1 and β1-AR receptors were reported as the ratio to GAPDH.

**Immunofluorescence labeling of TRPV1 receptors in cardiac nerve endings**

To determine the dose-dependent (0.5 µg/ml, 5 µg/ml and 50 µg/ml) and time course (1 week, 4-6 weeks and 9-11 weeks after RTX) effects of RTX on cardiac TRPV1-expressing nerve terminals in the heart, immunofluorescence labeling experiments were conducted in the myocardium obtained from 4 vehicle-treated and 20 RTX-treated rats (each dose or time point includes 4 rats). In another parallel set of experiments, to further confirm the efficiency of RTX-induced TRPV1-expressing CSAR afferent ablation, CSAR functional tests using epicardial application of capsaicin and bradykinin (10 µg/ml) were performed in sham+RTX rats treated with different doses (0.5 µg/ml, 5 µg/ml and 50 µg/ml) or various times post treatment (1 week, 4-6 weeks, 9-11 weeks and 6 months after RTX). Except for vehicle (n=9) and 9-11 week RTX-treated rats (n=8), each group includes 4 rats. All dose-dependent experiments (immunofluorescence labeling and functional CSAR test) were performed 1 week after epicardial application of RTX.

Rats (n=4/group) were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). The rats were perfused through the aorta, first with 100 ml heparinized saline followed by 500 ml 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (PBS, PH=7.4). The rat hearts were immediately removed and immersed in 4% paraformaldehyde in 0.1 mol/L PBS (PH=7.2) overnight at 4 °C. The tissues were then transferred to 30% sucrose in PBS and kept in the solution until they sank to the bottom. Thereafter, the blocks were rapidly frozen and 10 µm sections were cut on a Leica cryostat and thawed onto gelatin-coated slides.

For double immunostaining of TRPV1 receptors within cardiac nerve terminals, sections were stained with protein gene product 9.5 (PGP 9.5, a nerve terminal marker)\(^7\). Cardiac sections, after being pre-incubated in 10% goat serum for 60 min, were incubated with mouse anti-TRPV1 antibody (1:200 dilution, Abcam, Cambridge, MA, USA) and rabbit anti-PGP 9.5 antibody (1:200 dilution, Millipore, MA, USA) overnight at 4°C. Sections were then washed with PBS and incubated with fluorescence-conjugated secondary antibody (Alexa 488-conjugated goat anti-mouse IgG and Alexa 568-conjugated goat anti-rabbit IgG, 1:200, Invitrogen, CA, USA) for 60 min at room temperature. After three washes with PBS, the sections were mounted on pre-cleaned microscope slides. Slides were observed under a Leica fluorescent microscope with corresponding filters. Pictures were captured by a digital camera system. No staining was seen when a negative control was performed with PBS instead of the primary antibody (data not shown).
Statistical Analysis

All values are expressed as mean ± standard error of the mean (SE). Differences between groups were determined by a two-way ANOVA followed by the Tukey *post hoc* test. P <0.05 was considered statistically significant.
References


Table S1. Two-week and Six-week echocardiographic data in sham and CHF rats with vehicle or RTX treatment

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Values are mean ± SE. LVED, left ventricle end-diastolic diameter; LVSD, left ventricle end-systolic diameter; LVSV, left ventricle end-systolic volume; LVDV, left ventricle end-diastolic volume; EF, ejection fraction; FS, fractional shortening. *P<0.05 vs. 2w. † P<0.05 vs. CHF+ Vehicle.
Table S2. Summary data for baroreflex function in sham and CHF rats treated with either vehicle or RTX

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<td>CHF+RTX</td>
<td>112.6±10.5†</td>
<td>88.0±5.5</td>
<td>28.5±5.2</td>
<td>3.07±0.35†</td>
</tr>
<tr>
<td><strong>MAP-HR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham+Vehicle</td>
<td>155.0±11.5</td>
<td>91.7±5.1</td>
<td>177.2±13.8</td>
<td>3.80±0.46</td>
</tr>
<tr>
<td>Sham+RTX</td>
<td>155.0±25.0</td>
<td>108.8±8.0</td>
<td>168.3±21.9</td>
<td>3.89±0.31</td>
</tr>
<tr>
<td>CHF+Vehicle</td>
<td>68.1±5.2*</td>
<td>88.7±5.7</td>
<td>279.6±9.8*</td>
<td>1.30±0.20*</td>
</tr>
<tr>
<td>CHF+RTX</td>
<td>128.0±12.1†</td>
<td>103.8±6.1</td>
<td>207.6±8.6†</td>
<td>3.70±0.57†</td>
</tr>
</tbody>
</table>

Values are mean ± SE. n=8-10/each group. a is the RSNA or HR range, x0 is the pressure at the midpoint of the range (BP50), y0 is minimum RSNA or HR and Gmax is the maximum gain of baroreflex curve.
Table S3. Summary data for pressure volume loop experiments

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham + Vehicle (n=8)</th>
<th>Sham + RTX (n=7)</th>
<th>CHF + Vehicle (n=8)</th>
<th>CHF + RTX (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF (%)</td>
<td>70.1 ± 2.8</td>
<td>73.0 ± 2.6</td>
<td>31.1 ± 4.0*</td>
<td>36.4 ± 4.4*</td>
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<tr>
<td>SW (mmHg × μL)</td>
<td>13274± 1603</td>
<td>13150 ± 1432</td>
<td>5284± 431*</td>
<td>8000 ± 1124*†</td>
</tr>
<tr>
<td>SV (μL)</td>
<td>157.3 ± 16.0</td>
<td>168.6 ± 16.5</td>
<td>115.1 ± 9.8*</td>
<td>120.2 ± 16.7</td>
</tr>
<tr>
<td>CO (mL/min)</td>
<td>58.5 ± 5.3</td>
<td>56.9± 5.4</td>
<td>38.5 ± 3.0*</td>
<td>41.9 ± 3.3*</td>
</tr>
<tr>
<td>Afterload</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVESP (mmHg)</td>
<td>130.5 ± 8.0</td>
<td>135.1 ± 7.5</td>
<td>108.3± 7.5*</td>
<td>111.9± 6.1*</td>
</tr>
<tr>
<td>Ea (mmHg/μL)</td>
<td>0.9±0.1</td>
<td>1.0±0.1</td>
<td>1.4±0.2*</td>
<td>1.0±0.1†</td>
</tr>
<tr>
<td>Preload</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>3.4±0.6</td>
<td>4.3±0.6</td>
<td>19.8±1.8*</td>
<td>7.3 ± 1.7†</td>
</tr>
<tr>
<td>LVEDV (μL)</td>
<td>229.0 ± 18.6</td>
<td>231.2 ± 19.8</td>
<td>372.3 ± 20.9*</td>
<td>329.9 ± 26.1*</td>
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<tr>
<td>Contractility</td>
<td></td>
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<tr>
<td>dP/dt_{max} (mmHg/s)</td>
<td>8934.5 ± 431.0</td>
<td>9154.1 ± 389.7</td>
<td>5448.4± 415.1*</td>
<td>5313.3 ± 362.7*</td>
</tr>
<tr>
<td>ESPVR</td>
<td>2.0 ± 0.2</td>
<td>2.1± 0.2</td>
<td>1.1 ± 0.2*</td>
<td>1.2 ± 0.1*</td>
</tr>
<tr>
<td>PSRW (mW/μL)</td>
<td>103.1 ± 3.3</td>
<td>104.3 ± 4.3</td>
<td>57.1 ± 5.0*</td>
<td>62.1 ± 4.0*</td>
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<td>Lusitropy</td>
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<tr>
<td>dP/dt_{min} (mmHg/s)</td>
<td>-8403.6 ± 273.9</td>
<td>-8677.2 ± 258.2</td>
<td>-3477.6 ± 103.9*</td>
<td>-4746.6 ± 243.2*†</td>
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<tr>
<td>Tau-G (ms)</td>
<td>9.5 ± 0.6</td>
<td>9.8± 0.8</td>
<td>18.9 ± 1.4*</td>
<td>12.5 ± 1.1†</td>
</tr>
<tr>
<td>EDPVR</td>
<td>0.02 ± 0.002</td>
<td>0.02 ± 0.003</td>
<td>0.13± 0.02*</td>
<td>0.04 ± 0.006*†</td>
</tr>
</tbody>
</table>

Values are mean ± SE. SW, stroke work; CO, cardiac output; SV, stroke volume; Ea, arterial elastance; LVESP, LV end-systolic pressure; LVEDP, LV end-diastolic pressure; dP/dt_{max}, maximal slope of systolic pressure increment; dP/dt_{min}, maximal slope of diastolic pressure increment; Tau, time constant of isovolumetric relaxation; PSRW, preload recruitable stroke work; ESPVR, end systolic pressure volume relationship; EDPVR, end diastolic pressure volume relationship. *P<0.05 vs. sham+Vehicle. † P<0.05 vs. CHF+ Vehicle.
Figure S1. A schematic overview of the timeline in the current study. As shown in the figure, RTX (50 µg/ml) was painted on the entire left and right ventricles just prior to coronary ligation. Echographic measurements were done 2 weeks and 6 weeks post MI. Some of rats were placed into metabolic cages to collect urine 7 weeks post MI for urinary norepinephrine measurement. The terminal experiments were performed 9-11 weeks post MI, which included evaluation of baroreflex function, renal and cardiac sympathetic efferent nerve recording, isoproterenol administration and in vivo pressure volume loop analysis.
Figure S2

**Figure S2.** A, representative confocal images showing immunofluorescence double labeling of TRPV1 receptors and PGP 9.5 (a nerve terminal marker) in the left ventricle of the heart (scale bars: 180 µm) in vehicle-treated (Control) rats and RTX-treated rats with different doses and time courses. Note the dense TRPV1-positive staining on the epicardial surface of the heart in the control rat but not in the RTX-treated rat. B and C, functional CSAR tests using epicardial application of capsaicin and bradykinin in vehicle-treated rats and RTX-treated rats using various doses (0.5, 5 and 50 µg/ml) 1 week after RTX (B) and times post treatment with 50 µg/ml RTX (C). Data are expressed as mean±SE. n=4/group except for the vehicle (n=9) and 9-11 week RTX groups (n=8). *, P<0.05 vs. vehicle, †, P<0.05 vs. 1-week RTX treatment.
Figure S3. Original images showing the gross morphology of infract hearts and infarct size in rats treated with vehicle or RTX.
**Figure S4.** Representative tracings showing blood pressure and RSNA responses to euthanasia with an overdose of pentobarbital sodium in sham+vehicle, sham+RTX, CHF+vehicle and CHF+RTX rats. In each rat, the red dashed arrows point to basal RSNA, maximum RSNA and noise levels, respectively. The maximum nerve activity (Max) occurred 1-2 min after the rat was euthanized. Baseline nerve activity was taken as the percent of Max after the noise level was subtracted. Notice that maximum RSNA is similar in all 4 rats whereas the CHF rat had a higher basal RSNA level than the other rats.
Figure S5. Representative tracings showing blood pressure and CSNA responses to euthanasia with an overdose of pentobarbital sodium in sham+vehicle, sham+RTX, CHF+vehicle and CHF+RTX rats. In each rat, the red dashed arrows point to basal CSNA, maximum CSNA and noise level, respectively. Similar to maximum RSNA, the maximum CSNA (Max) also occurred 1-2 min after the rat was euthanized. However, in contrast to the RSNA response, the maximum CSNA has two bursts (a major discharge burst followed by a smaller discharge burst) after euthanasia. Baseline nerve activity was taken as percent of Max after the noise level was subtracted. Notice that maximum CSNA is similar in all 4 rats whereas the CHF rat had a higher basal CSNA level than other rats.
Figure S6. Representative 10X scanning images showing TUNEL-positive staining in LV infarct, LV peri-infarct, LV remote and IVS regions from sham+vehicle, sham+RTX, CHF+Vehicle and CHF+RTX rats. White arrows point to TUNEL-positive DNA fragments.
**Figure S7.** A, representative immunohistochemical images showing cleaved caspase 3-positive staining in LV peri-infarct, LV remote and IVS regions from sham + vehicle, sham + RTX, CHF + vehicle and CHF+RTX rats. Red arrows pointed to the cleaved caspase 3-positive staining. Solid green bar represents 30 μm. B, western blot data showing the protein expressions of cleaved caspase 3 and caspase 3 in the left ventricle (remote region) and septum in sham (S)+vehicle (V), S+RTX, CHF (C)+V and C+RTX rats. Data are expressed as mean±SE.

n=4/group. *, P<0.05 vs. sham+vehicle, †, P<0.05 vs. CHF+vehicle.
**Figure S8.** A schematic overview showing the potential mechanisms underlying the beneficial effects of epicardial application of RTX on cardiac and autonomic dysfunction in CHF. Epicardial application of RTX interrupts already activated cardiac sympathetic afferents thereby reducing a major source of sympatho-excitation in the setting of CHF. This, in turn, impacts cardiac remodeling and function. There may also be major influences on peripheral organ function such as the kidney.