ORIGINAL RESEARCH ARTICLE

Cardiovascular Therapeutics

Effects of the Galanin Receptor Antagonist M40 on Cardiac Function and Remodeling in Rats with Heart Failure

Ao Chen,[1] Min Li,[1] Lei Song,[1] Yunhe Zhang,[1] Zhangyuan Luo,[2] Wenzan Zhang,[2] Yingmin Chen[1] & Ben He[1]

1 Department of Cardiology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China
2 Jie Sheng (Shanghai) Biological Technology Co., Ltd, Shanghai, China

Keywords
Antagonist; Cardiac function; Galanin; Heart failure; M40; Remodeling.

Correspondence
Y. Chen, M.D., Ph.D., Department of Cardiology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, NO. 1630 Dongfang Road, Shanghai 200127, China.
Tel.: +86-136-81980901;
Fax: +86-021-34611387;
E-mail: 13681980901@139.com

doi: 10.1111/1755-5922.12144

SUMMARY

Background: Sympathetic activation and parasympathetic withdrawal are important characteristics of heart failure. Recent studies demonstrate that galanin reduces the discharge of acetylcholine and inhibits vagal bradycardia by acting on galanin receptor type 1 (GalR1). We speculated that blocking GalR1 is beneficial for heart failure. **Methods:** Rats with heart failure were induced by myocardial infarction. The rats were injected intraperitoneally with galanin receptor antagonist M40 solution (30 nmol/kg) or saline for 4 weeks. Cardiac function was assessed by echocardiography and brain natriuretic peptide (BNP) in plasma. The ratio of heart weight to body weight (HW/BW), hematoxylin–eosin (HE), and Masson trichrome stain was used to evaluate cardiac remodeling. Tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) in plasma, and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2) in heart tissue were detected to confirm the mechanism of the cardioprotection effect. **Results:** Compared with rats injected with saline, M40 effectively improved cardiac function of contraction; decreased BNP, IL-6, and HW/BW (all $P < 0.05$); attenuated cardiac fibrosis; and upregulated SERCA2 ($P < 0.05$). **Conclusion:** M40 improves cardiac function and attenuates remodeling, suggesting that galanin receptor antagonist may be a potential therapeutic agent for HF.

Introduction

Heart failure (HF) is a huge medical and public health problem which afflicts more than 5 million people in the United States. The morbidity and mortality of HF remain high in spite of the increasing costs of health care and the improvement in medical technology and therapies [1]. The imbalance of the autonomic nervous system (ANS), characterized by sympathetic activation and parasympathetic withdrawal, is one of the most important pathophysiological processes of HF [2]. Sympathetic activation increases the release of norepinephrine which may reduce cardiac function, promote cardiac remodeling, and worsen HF [3,4]. β-Blockers have been an effective strategy for treating HF through reducing sympathetic nerve system activity [5], indicating that restoring the balance of ANS is beneficial to improving HF.

Adenosine triphosphate (ATP), neuropeptide Y (NPY), and galanin are all sympathetic cotransmitters, in addition to norepinephrine, the main neurotransmitter of sympathetic nerves [6,7]. It is generally accepted that galanin is involved in central cardiovascular control [8]. Nevertheless, a previous study has demonstrated that both galanin and NPY released by sympathetic nerves may attenuate vagal neurotransmission [9]. Further study confirms that galanin released from sympathetic nerves may reduce the discharge of acetylcholine and inhibit vagal bradycardia by acting on GalR1 localized on cardiac cholinergic neurons [10]. The increased sympathetic activity during HF promotes the release of galanin, which exacerbates the imbalance of ANS. Hence, we speculate that the GalR1 antagonist M40 may suppress the inhibitory effect on the vagal nerve induced by galanin in HF, thus improving the balance of ANS and cardiac function.

Methods

Animals

Adult male Sprague Dawley rats weighted 220–240 g were supplied by Sino-British Sippr/BK Lab Animal Ltd., Shanghai, China. All procedures were conducted in accordance with the Guidelines on the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research and the Guidelines of Animal Care. This study was approved by the ethical committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University.

HF Model and Grouping

HF rat model was induced by sustained coronary artery ligation. Briefly, rats were intubated and ventilated with a small animal ventilator (model SAR-830, CWE Inc., USA) after being anesthetized with pentobarbital sodium. The left anterior descending

© 2015 John Wiley & Sons Ltd

(LAD) coronary artery was identified and then ligated with 6-0 silk suture. Sham operation rats underwent the same procedure without LAD ligation. The HF models were confirmed by echocardiography (HP SONOS 5500, Hewlett-Packard, Palo Alto, CA, USA); 3 weeks later, left ventricular ejection fraction (LVEF) ≤50% was defined as HF. The survived rats were randomly divided into following 3 groups: HF rats treated with M40 (M40 group, n = 9), HF rats treated with saline (Saline group, n = 8), and sham operation rats (Sham group, n = 10).

Drug Administration

The galanin receptor antagonist M40 (Tocris, Bristol, UK) was dissolved into saline. M40 group rats were injected intraperitoneally with M40 solution (30 nmol/kg) for 4 weeks. Saline and Sham group rats were injected with saline solution in the same way.

Echocardiography

Rats were anesthetized with isoflurane, and transthoracic echocardiography was performed after drug administration as previously described [11]. Left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVEDS) were measured from M-mode images, and subsequently, LVEF and fractional shortening (FS) were calculated.

Enzyme-linked Immunosorbent Assay

At the end of drug administration, the blood samples were collected from medial canthus into heparin tube and then centrifuged at 3000 × *g* for 10 min at 4°C to prepare plasma. Plasma samples were stored at −80°C. Plasma brain natriuretic peptide (BNP), tumor necrosis factor-α (TNF-α), and interleukin 6 (IL-6) were detected by enzyme-linked immunosorbent assay (ELISA) with ELISA kits (HCB, Catharines, Canada) following the manufacturer's instructions.

Tissue Preparation and Histopathologic Analysis

After the preparation of plasma samples, the rats were weighted and then killed by overdose of pentobarbital sodium. The heart was excised and wet-weighted. The ratio of the heart weight to body weight (HW/BW) was calculated as an indicator of cardiac hypertrophy. All the heart tissues were stored in liquid nitrogen for Western blotting analysis or fixed in 4% paraformaldehyde for histopathologic analysis.

Each heart tissue was cut into three thick slices from the apex to base along the long axis. After standard paraffin embedding, two 5-μm-thick sections were obtained from each slice and then were stained with hematoxylin–eosin (HE) and Masson trichrome, respectively. Histopathologic images were obtained from the light microscope and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). The fibrosis area and left ventricular area were measured by Image-Pro Plus 6.0, and the fibrosis ratio was calculated as the fibrosis area divided by total left ventricular area. The ratios of three slices of each tissue were calculated, and the mean ratio was obtained as the fibrosis ratio of the tissue.

Western Blotting

Western blotting was used to detect the effect of M40 on sarco (endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2). In short, total protein was extracted, and SERCA2 protein was separated by gel electrophoresis (40 μg total protein was loaded) and then transferred to cellulose acetate membrane. The membrane was incubated with primary antibody anti-SERCA2 ATPase antibody (Abcam, Cambridge, UK, diluted 1:1000) overnight at 4°C after handled with blocker buffer. The reference protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. After incubation with horseradish peroxidase (HRP)-linked secondary antibody, chemiluminescence was used to detect protein bands by scanning with an ImageQuant LAS 4000 scanner (GE Healthcare, Fairfield, CT, USA).

Statistical Analysis

All values are presented as mean ± standard error of the mean. Statistical difference between groups was analyzed using one-way ANOVA with a Bonferroni *post hoc* test for multiple comparisons. $P < 0.05$ was considered statistically significant. The data analysis was performed by SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

Results

Effects of M40 on Cardiac Function

LVEF was significantly increased in the M40 group compared with the Saline group (34.8 ± 2.1 vs. 27.5 ± 1.7%; $P < 0.05$; Figure 1A), and a significant decrease in LVEDD and LVEDS was detected in the M40 group (9.0 ± 0.2 vs. 10.6 ± 0.4 mm; 7.5 ± 0.3 vs. 9.1 ± 0.4 mm; all $P < 0.05$; Figure 1B and C). However, no significant difference was found between the M40 and Saline group in FS (Figure 1D).

Effect of M40 on Plasma BNP

At 4 weeks after drug administration, the M40 group and Saline group revealed significant increase in plasma BNP compared with the Sham group (331.43 ± 38.26, 406.26 ± 68.25 vs. 102.06 ± 28.89 pg/mL; $P < 0.05$; Figure 2A). Plasma BNP was significantly lower in the M40 group than in the Saline group (331.43 ± 38.26 vs. 406.26 ± 68.25; pg/mL; $P < 0.05$; Figure 2A).

Effects of M40 on Plasma Inflammatory Factors

The inflammatory factors TNF-α and IL-6 were also detected. The results of ELISA indicated that plasma TNF-α and IL-6 were significantly higher in the Saline group than in the Sham group (Figure 2B and C). Plasma level of IL-6 of the M40 group was significantly reduced compared with the Saline group (71.71 ± 9.36 vs. 93.82 ± 19.18 pg/mL; $P < 0.05$; Figure 2B). Although there was also a decrease by 12.4 pg/mL of TNF-α in the M40 group, as compared to the Saline group, the difference did not reach statistical significance (51.39 ± 15.04 vs. 63.79 ± 20.70 pg/mL; $P > 0.05$; Figure 2C).

© 2015 John Wiley & Sons Ltd

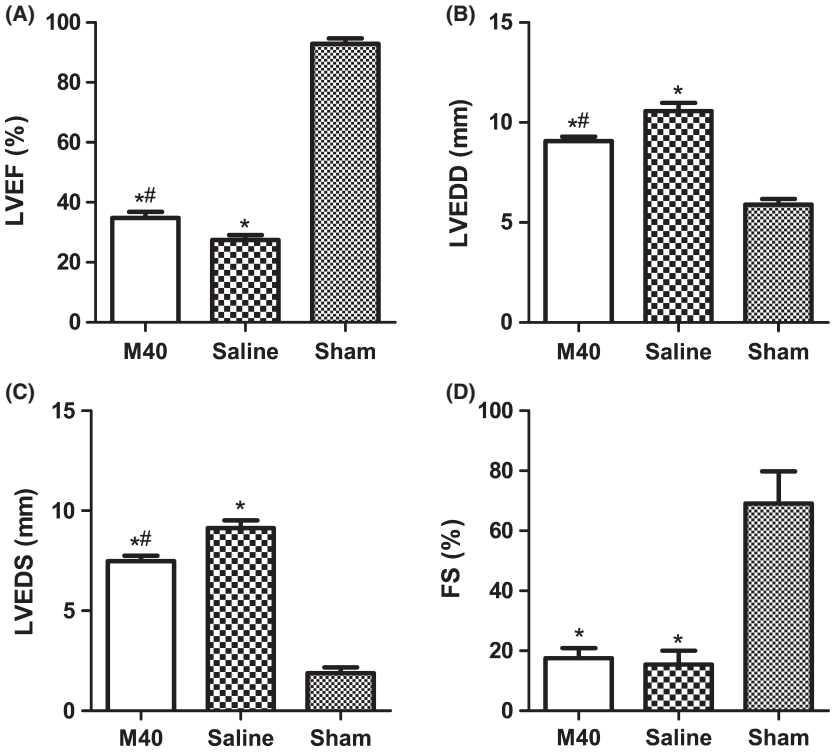

Figure 1 Effect of M40 on cardiac function assessed by echocardiography, including LVEF (**A**), LVEDD (**B**), LVEDS (**C**), and FS (**D**). Results are presented as mean ± standard error of the mean. $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. Saline. LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic diameter; LVEDS, left ventricular end-systolic diameter; FS, fractional shortening.

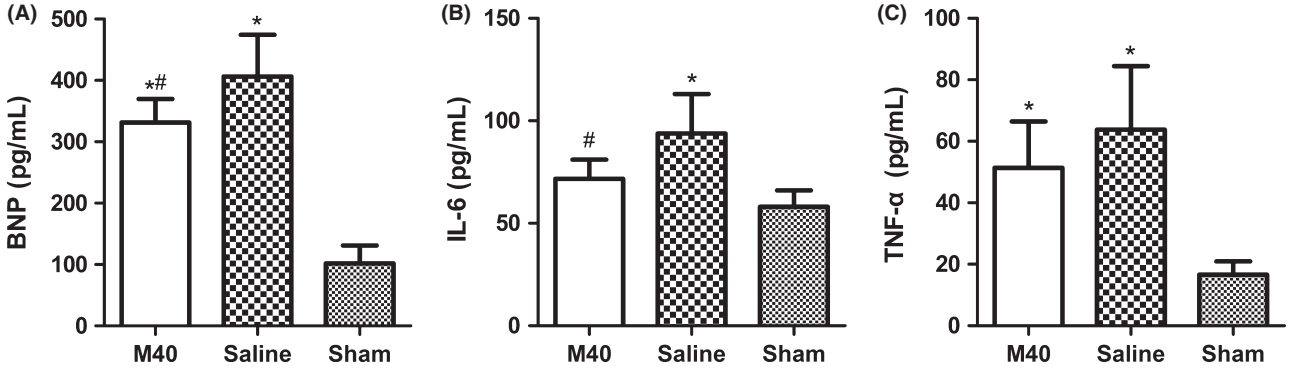

Figure 2 Effect of M40 on plasma BNP (**A**), IL-6 (**B**), and TNF-α (**C**) assessed by ELISA. Results are presented as mean ± standard error of the mean. $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. Saline. BNP: brain natriuretic peptide. IL-6: interleukin 6. TNF-α: tumor necrosis factor-α.

Effects of M40 on Cardiac Morphology

No significant difference in BW was detected among groups; however, the HW/BW value of the M40 and Saline group were significantly increased compared with the Sham group (Figure 3D). Meanwhile, significant decrease in HW/BW of the M40 group was observed compared with the Saline group (0.34 ± 0.024 vs. 0.39 ± 0.026; $P < 0.05$; Figure 3D). HE stain of the Saline group revealed obvious myofiber necrosis, disarray, and broken sarcomere which were attenuated by M40 in the M40 group (Figure 3A). Masson stain of the Saline group demonstrated severe increased fibrosis ratio which was also ameliorated by M40 treatment (0.32 ± 0.092 vs. 0.44 ± 0.110; $P < 0.05$; Figure 3B and C).

Effects of M40 on SERCA2

Compared with the Sham group, levels of SERCA2 in M40 and Saline group were both decreased significantly; nonetheless, by comparing the M40 and Saline group, M40 treatment may effectively enhance the expression level of SERCA2 (Figure 4).

 © 2015 John Wiley & Sons Ltd

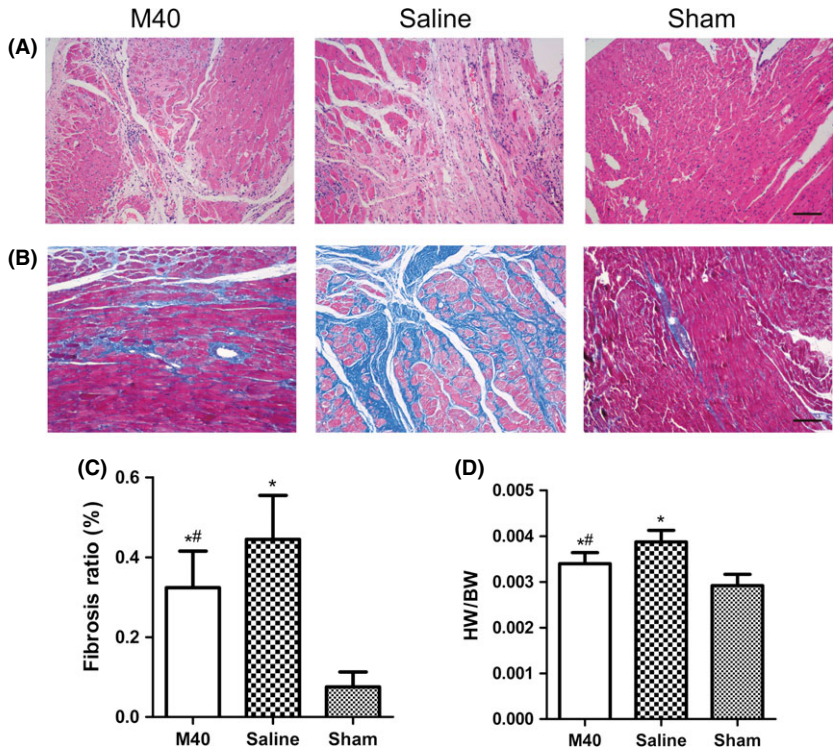

Figure 3 Effects of M40 on cardiac morphology. The morphological changes of cardiomyocytes were observed by HE staining (**A**, scale bar = 500 μm). The degree of cardiac fibrosis was assessed by Masson trichrome staining (**B**, scale bar = 500 μm). Fibrosis ratio was compared among the three groups (**C**). HW/BW also was calculated and analyzed (**D**). Results are presented as mean ± standard error of the mean. $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. Saline. HE: hematoxylin–eosin. HW/BW: heart weight/body weight.

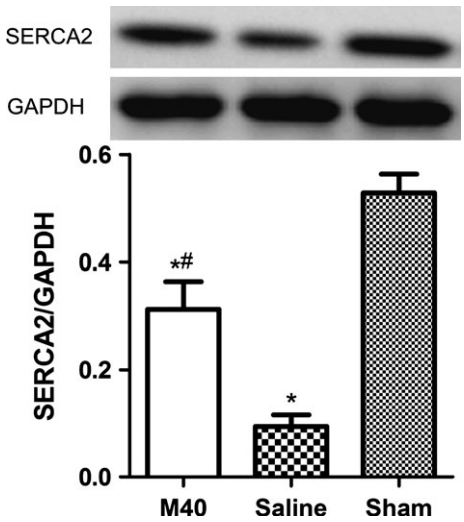

Figure 4 Effect of M40 on the expression level of cardiac SERCA2. Results are presented as mean ± standard error of the mean. $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. Saline. SERCA2: sarco(endo)plasmic reticulum Ca^{2+}-ATPase.

Discussion

The present study aimed to investigate the cardioprotection effect of galanin receptor antagonist M40 on HF, and the underlying mechanism. We established postinfarction HF rat model and administered M40 *in vivo* to explore the effects of M40 on cardiac function and remodeling. The results showed that M40 could enhance heart function and ameliorate cardiac remodeling. Next, we demonstrated that M40 decreased plasma inflammatory factors and regulated the expression of SERCA2, which might be the mechanism of cardioprotection, although the exact mechanisms are still needed to be clarified.

The dysregulation of ANS is closely related to the development of HF; high levels of sympathetic drive releases more norepinephrine which was demonstrated to cause deterioration of cardiac function and be a negative prognostic indicator of HF [12,13]. β-Blockers prevent such adverse outcomes and ameliorate cardiac remodeling by inhibiting sympathetic activity [12]. Interestingly, direct vagal nerve stimulation or using cholinesterase inhibitor can also be beneficial for HF [14,15]. This illustrates that reducing sympathetic drive or increasing vagal drive can both improve HF.

Galanin, a 29 amino acid neuropeptide isolated from porcine intestine initially, is widely distributed throughout the central and peripheral nervous system and some other tissues [16]. Most of the biological functions of galanin remain unclear; however, galanin is mainly involved in modulating cognition, feeding, memory, neuropathic pain, and so on [17]. Up to now, researchers have identified three types of galanin receptors: galanin receptor type 1 (GalR1), type 2 (GalR2), and type 3 (GalR3), which are all part of the G protein-coupled receptor superfamily. The role of galanin as

© 2015 John Wiley & Sons Ltd

a cotransmitter of sympathetic nerve in regulating ANS has drawn much attention. Previous study demonstrated that the expression of galanin in ventricular tissue was obviously increased in rats with LAD coronary artery ligation [18–20]. Several studies have provided evidence that galanin may reduce cholinergic transmission in the heart of mice [9,21]. Herring et al. [10] found that sympathetic nerve stimulation still reduced the release of acetylcholine and inhibited vagal bradycardia after blocking sympathetic transmitter receptors except the galanin receptors, indicating that high level of sympathetic activity might inhibit vagal nerve through galanin. They further demonstrated that GalR2 and GalR3 were not expressed in heart tissue; however, GalR1 was highly expressed [10].

Although the inhibition effect of galanin released from sympathetic nerves on vagal activity by acting on GalR1 has been demonstrated, the effect of GalR1 blocking on HF has not been reported. In this study, we examined the effect of galanin receptor antagonist M40 on heart function in a rat model of coronary ligation. Echocardiography results revealed significant improvement in LVEF, LVEDD, and LVEDS, which represented the enhancement of cardiac systolic function. BNP is a considerable neurohormonal predictor of left ventricular function [22,23]. We also demonstrated a significant decrease in BNP after M40 treatment, providing support for our viewpoint that M40 might improve cardiac function. Furthermore, we explored the change of SERCA2 after administration of M40 to confirm the mechanism. SERCA2 is a kind of Ca^{2+}-ATPase which plays an important role in maintaining cardiac calcium handling and pump function [24]; thus, it is closely related to cardiac function and contractility [25]. The expression of SERCA2 decreases during HF, inducing reduced sarcoplasmic reticulum Ca^{2+} content and impaired cardiac function [26]. Our study showed a significant enhanced activity of SERCA2 after M40 treatment, which might be the underlying mechanism responsible for the cardioprotective effect.

The effect of M40 on attenuating cardiac remodeling was shown by cardiac morphology results in the present study. It is well known that sympathetic overactivity during HF is responsible for cardiac remodeling [27]. M40 may enhance vagal activity through blocking the inhibition effect from sympathetic nerve, and vagal activity further ameliorates cardiac remodeling by counteracting sympathetic activity. Recently, the cholinergic antiinflammatory pathway reveals that acetylcholine is able to modulating inflammatory response and the production of inflammatory factors by acting on α7 subunit of the nicotinic acetylcholine receptor [28]. Interestingly, we found that M40 effectively decreased IL-6 which promoted cardiac remodeling by inducing the expression of intercellular adhesion molecule-1 [29]. It is reasonable to speculate that the enhanced vagal activity by M40 may release more acetylcholine which attenuates the inflammatory response and cardiac remodeling.

Conclusions

Administration of the galanin receptor antagonist M40 improved cardiac function and attenuated remodeling in rats with HF. The upregulation of cardiac SERCA2 and decrease in plasma IL-6 might be the potential mechanism involved in the cardioprotective effect. It implies that targeting of galanin receptor could be a promising way to treat HF.

Funding

This work was supported by the Shanghai Committee of Science and Technology (grant number: 124119b1700).

Author Contributions

Ao Chen, Min Li, Lei Song, and Yunhe Zhang contributed to study design, literature research, and experimental studies. Ao Chen, Yunhe Zhang, and Zhangyuan Luo contributed to data acquisition and statistical analysis. Ao Chen and Min Li contributed to manuscript preparation. Wenzan Zhang, Yingmin Chen, and Ben He contributed to manuscript revision.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Go AS, Mozaffarian D, Roger VL, et al. Heart disease and stroke statistics–2014 update: A report from the American Heart Association. *Circulation* 2014;**129**:e28–e292.
2. Schwartz PJ, De Ferrari GM. Sympathetic-parasympathetic interaction in health and disease: Abnormalities and relevance in heart failure. *Heart Fail Rev* 2011;**16**:101–107.
3. Mann DL, Kent RL, Parsons B, Cooper GT. Adrenergic effects on the biology of the adult mammalian cardiocyte. *Circulation* 1992;**85**:790–804.
4. Floras JS. Clinical aspects of sympathetic activation and parasympathetic withdrawal in heart failure. *J Am Coll Cardiol* 1993;**4**(Suppl A):72A–84A.
5. Hjalmarson A, Goldstein S, Fagerberg B, et al. Effects of controlled-release metoprolol on total mortality, hospitalizations, and well-being in patients with heart failure: The Metoprolol CR/XL Randomized Intervention Trial in congestive heart failure (MERIT-HF). MERIT-HF Study Group. *JAMA* 2000;**283**:1295–1302.
6. Kummer W. Galanin- and neuropeptide Y-like immunoreactivities coexist in paravertebral sympathetic neurones of the cat. *Neurosci Lett* 1987;**78**:127–131.
7. Burnstock G. Autonomic neurotransmission: 60 years since sir Henry Dale. *Annu Rev Pharmacol Toxicol* 2009;**49**:1–30.
8. Diaz-Cabiale Z, Parrado C, Narvaez M, Millon C, Puigcerver A, Fuxe K, Narvaez JA. Neurochemical modulation of central cardiovascular control: The integrative role of galanin. *EXS* 2010;**102**:113–131.
9. Potter EK, Smith-White MA. Galanin modulates cholinergic neurotransmission in the heart. *Neuropeptides* 2005;**39**:345–348.
10. Herring N, Cranley J, Lokale MN, et al. The cardiac sympathetic co-transmitter galanin reduces acetylcholine release and vagal bradycardia: Implications for neural control of cardiac excitability. *J Mol Cell Cardiol* 2012;**52**:667–676.
11. Yang XP, Liu YH, Rhaleb NE, Kurihara N, Kim HE, Carretero OA. Echocardiographic assessment of cardiac function in conscious and anesthetized mice. *Am J Physiol* 1999;**277**:H1967–H1974.
12. Sabbah HN. The cellular and physiologic effects of beta blockers in heart failure. *Clin Cardiol* 1999;**22**(Suppl 5):V16–V20.
13. Swedberg K, Eneroth P, Kjekshus J, Wilhelmsen L. Hormones regulating cardiovascular function in patients with severe congestive heart failure

 © 2015 John Wiley & Sons Ltd

and their relation to mortality. CONSENSUS Trial Study Group. *Circulation* 1990;**82**:1730–1736.
14. Premchand RK, Sharma K, Mittal S, et al. Autonomic regulation therapy via left or right cervical vagus nerve stimulation in patients with chronic heart failure: Results of the ANTHEM-HF trial. *J Card Fail* 2014;**20**:808–816.
15. Li M, Zheng C, Kawada T, Inagaki M, Uemura K, Shishido T, Sugimachi M. Donepezil markedly improves long-term survival in rats with chronic heart failure after extensive myocardial infarction. *Circ J* 2013;**77**:2519–2525.
16. Tatemoto K, Rokaeus A, Jornvall H, McDonald TJ, Mutt V. Galanin – a novel biologically active peptide from porcine intestine. *FEBS Lett* 1983;**164**:124–128.
17. Mechenthaler I. Galanin and the neuroendocrine axes. *Cell Mol Life Sci* 2008;**65**:1826–1835.
18. Alston EN, Parrish DC, Hasan W, Tharp K, Pahlmeyer L, Habecker BA. Cardiac ischemia-reperfusion regulates sympathetic neuropeptide expression through gp130-dependent and independent mechanisms. *Neuropeptides* 2011;**45**:33–42.
19. Habecker BA, Gritman KR, Willison BD, Van Winkle DM. Myocardial infarction stimulates galanin expression in cardiac sympathetic neurons. *Neuropeptides* 2005;**39**:89–95.
20. Ewert TJ, Gritman KR, Bader M, Habecker BA. Post-infarct cardiac sympathetic hyperactivity regulates galanin expression. *Neurosci Lett* 2008;**436**:163–166.
21. Smith-White MA, Iismaa TP, Potter EK. Galanin and neuropeptide Y reduce cholinergic transmission in the heart of the anaesthetised mouse. *Br J Pharmacol* 2003;**140**:170–178.
22. Troughton RW, Frampton CM, Yandle TG, Espiner EA, Nicholls MG, Richards AM. Treatment of heart failure guided by plasma aminoterminal brain natriuretic peptide (N-BNP) concentrations. *Lancet* 2000;**355**:1126–1130.
23. Richards AM, Nicholls MG, Yandle TG, et al. Plasma N-terminal pro-brain natriuretic peptide and adrenomedullin: New neurohormonal predictors of left ventricular function and prognosis after myocardial infarction. *Circulation* 1998;**97**:1921–1929.
24. Ericsson M, Andersson KB, Amundsen BH, et al. High-intensity exercise training in mice with cardiomyocyte-specific disruption of Serca2. *J Appl Physiol* 2010;**108**:1311–1320.
25. Ericsson M, Sjaland C, Andersson KB, Sjaastad I, Christensen G, Sejersted OM, Ellingsen O. Exercise training before cardiac-specific Serca2 disruption attenuates the decline in cardiac function in mice. *J Appl Physiol* 2010;**109**:1749–1755.
26. Bers DM. Altered cardiac myocyte Ca regulation in heart failure. *Physiology (Bethesda)* 2006;**21**:380–387.
27. Levick SP, Murray DB, Janicki JS, Brower GL. Sympathetic nervous system modulation of inflammation and remodeling in the hypertensive heart. *Hypertension* 2010;**55**:270–276.
28. Tracey KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. *J Clin Invest* 2007;**117**:289–296.
29. Kobara M, Noda K, Kitamura M, et al. Antibody against interleukin-6 receptor attenuates left ventricular remodelling after myocardial infarction in mice. *Cardiovasc Res* 2010;**87**:424–430.

© 2015 John Wiley & Sons Ltd