Sympathetic Nervous System Modulation of Inflammation and Remodeling in the Hypertensive Heart

Scott P. Levick, David B. Murray, Joseph S. Janicki, Gregory L. Brower

Abstract—Chronic activation of the sympathetic nervous system is a key component of cardiac hypertrophy and fibrosis. However, previous studies have provided evidence that also implicate inflammatory cells, including mast cells (MCs), in the development of cardiac fibrosis. The current study investigated the potential interaction of cardiac MCs with the sympathetic nervous system. Eight-week-old male spontaneously hypertensive rats were sympathectomized to establish the effect of the sympathetic nervous system on cardiac MC density, myocardial remodeling, and cytokine production in the hypertensive heart. Age-matched Wistar Kyoto rats served as controls. Cardiac fibrosis and hypertension were significantly attenuated and left ventricular mass normalized, whereas cardiac MC density was markedly increased in sympathectomized spontaneously hypertensive rats. Sympathectomy normalized myocardial levels of interferon-γ, interleukin 6, and interleukin 10, but had no effect on interleukin 4. The effects of norepinephrine and substance P on isolated cardiac MC activation were investigated as potential mechanisms of interaction between the two. Only substance P elicited MC degranulation. Substance P was also shown to induce the production of angiotensin II by a mixed population of isolated cardiac inflammatory cells, including MCs, lymphocytes, and macrophages. These results demonstrate the ability of neuropeptides to regulate inflammatory cell function, providing a potential mechanism by which the sympathetic nervous system and afferent nerves may interact with inflammatory cells in the hypertensive heart. (Hypertension. 2010;55:270-276.)

Key Words: sympathectomy ■ fibrosis ■ mast cell ■ cytokines ■ inflammation ■ substance P ■ sympathetic nervous system

Unregulation of the sympathetic nervous system (SNS) is involved in numerous cardiovascular disease processes and is responsible for alterations in normal myocardial structure and function, including the induction of arrhythmias, alterations in contractile properties, and cardiac remodeling.1 In an experimental setting, there is evidence that the SNS mediates hypertension-induced cardiac fibrosis and hypertrophy through α- and β-adrenergic receptors, respectively.2 However, concomitant with elevated SNS activity, induction of an inflammatory infiltrate into the heart also occurs, which appears to mediate the development of hypertension-induced cardiac fibrosis but not hypertrophy. This inflammatory component includes mast cells (MCs),3-6 macrophages,7,8 and possibly T cells.9,10 MCs maintain a close anatomic association with nerves,11,12 with sympathetic neurons able to alter rat basophilic leukemia (RBL-2H3) MC membrane resistance.13 In addition, other inflammatory cells express adrenergic receptors, with modulation of cytokine production being either enhanced or repressed by stimulation of these receptors.14 Alternatively, we have shown that cardiac MCs can also be activated by neuropeptides, such as substance P,15 which is found mainly in afferent sensory nerves. Given the prominent involvement of both the SNS and inflammation in myocardial remodeling associated with hypertension, we sought to determine whether SNS-mediated regulation of inflammation is a mechanism involved in myocardial remodeling secondary to hypertension and whether afferent nerves also have the potential to regulate inflammation in the hypertensive heart.

Methods

All of the in vivo experiments were performed using adult male spontaneously hypertensive rats (SHRs; n = 18) and Wistar Kyoto rats (WKYs; n = 13) housed under standard environmental conditions and maintained on commercial rat chow and tap water ad libitum. SHRs were chosen for this study because they have been documented to have elevated SNS activity.16 All of the studies conformed to the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In addition, the protocol was approved by our institution’s animal care and use committee. All of the rats were anesthetized by IP injection of sodium pentobarbital (50 mg/kg).

Experimental Protocol and Sympathectomy Surgery

Rats were divided into 4 groups: (1) untreated WKYs; (2) sympathectomized WKYs (WKY+Symp); (3) untreated SHRs; and (4)
sympathectomized SHRs (SHR+Symp). Data reported herein for untreated WKYs and SHRs have been published previously. Bilateral sympathectomy was performed at 8 weeks of age, and all of the rats were euthanized at 20 weeks of age. This time point was chosen because it represents a steady state of hypertension, as well as established left ventricular (LV) hypertrophy and fibrosis in the SHR. The sympathectomy surgery was conducted by the attending veterinarian at Charles River Laboratories using the following procedure. A ventral midline incision was made in the neck, allowing the exposed omohyoid muscle to be retracted laterally to expose the carotid artery internal and external branch regions. The superior cervical ganglion lying within this bifurcation was carefully excised. This procedure was repeated on the contralateral side. A successful sympathectomy was evidenced by the occurrence of Horner’s syndrome. Adrenalectomy was not performed in these animals because the aim was to examine the specific interactions between the SNS and MCs, not the role of circulating norepinephrine.

### Hemodynamic Parameters

At the experimental end point, animals were anesthetized, and measurements of systolic, diastolic, and mean arterial pressures were made using a high fidelity Millar catheter inserted into the carotid artery and advanced into the aortic arch. Catheter data were analyzed using PVAN 3.5 software.

### Histological Analysis

MC number and density were determined from pinacynol erythrosine–stained LV sections as described previously (please see the online Data Supplement). LV interstitial collagen volume fraction was determined by analysis of picrosirius red–stained sections as described previously (please see the online Data Supplement).

### Cytokine Characterization

Interferon (IFN)-γ, interleukin (IL) 4, IL-6, and IL-10 were quantified from frozen LV tissue (please see the online Data Supplement) to determine the effects of sympathectomy, using commercial ELISA kits (Alpco Diagnostics).

### Cardiac Inflammatory Cell Isolation Procedure

Cardiac inflammatory cells were isolated as described previously. Briefly, a thoracotomy exposed the intact pericardial sac. The tip of a Teflon catheter sleeve, attached to a sterile syringe, was then inserted into the pericardial sac, and the sac filled with Hanks’ balanced salt solution (7.4 pH) which was composed of the following: (1) Hanks’ calcium- and magnesium-free salt solution; (2) HEPES (13 mmol/L); (3) 607 μM of deoxyribonuclease I (Sigma Chemical Co); and (4) an antibiotic-antimycotic mixture of penicillin G sodium, streptomycin sulfate, and amphotericin B (Gibco-BRL, Life Technologies). The buffer was then aspirated into another sterile syringe. The cells were pelleted and resuspended in Hyclone buffer (Hanks’ balanced salt solution containing magnesium sulfate [1.1 mmol/L], calcium chloride [1.3 mmol/L], and phenol red; Hyclone) before undergoing treatment.

### Isolated Cardiac MC Studies

Cardiac MCs were obtained during the cardiac inflammatory cell isolation procedure described above. Cardiac MC responses were examined by incubating 4x10^5 MCs per treatment tube with norepinephrine or substance P at specific concentrations: 0, 1x10^-12, 1x10^-9, 1x10^-6, and 1x10^-3 M. The samples were incubated at 37°C for 20 minutes, and the posttreatment supernatants and pellets were separated for subsequent analysis of histamine as a marker of MC degranulation. The percentage of histamine release was determined by dividing the histamine value from the supernatant by total histamine (supernatant plus pellet). Histamine concentration was measured using a Neogen Veratox Histamine ELISA.

### Substance P–Mediated Production of Angiotensin II by Isolated Cardiac Inflammatory Cells

Isolated cardiac inflammatory cells (2x10^5 per well) were maintained in DMEM containing 10% FBS and treated with substance P (1x10^-4 M) for 20 hours. The medium was collected and assayed for angiotensin II using a commercial enzyme immunoassay kit (SPBio), which has cross-reactivities of 4%, 36%, and 33% with angiotensin I, angiotensin III, and angiotensin 3-8, respectively.

### Immunofluorescent Staining of α-Adrenergic Receptors

Please see the online Data Supplement for details.

### Statistical Analysis

All of the grouped data were expressed as mean ± SD or SEM, as appropriate. Group comparisons were made by 1-way ANOVA using SPSS 11.5 software (SPSS Inc). When a significant F test (P<0.05) was obtained, intergroup comparisons were analyzed using the Fisher protected least-significant difference post hoc testing.

### Results

**Biometrics**

The Table displays grouped biometric data. Although no differences in body weight were observed between WKYs, SHRs, and WKY+Symps, a significant increase was observed in sympathectomized SHRs. The Table also includes LV weight and LV weight indexed to body weight as measures of hypertrophy. SHRs developed significant LV hypertrophy in comparison with WKYs by both measures, with sympathectomy preventing this hypertrophic response. There were no weight differences between WKYs and WKY+Symp. Lung weights showed no significant differences. SHRs showed marked increases in systolic, diastolic, and mean arterial pressures compared with WKYs.

**Hemodynamic Parameters**

At the experimental end point, animals were anesthetized, and measurements of systolic, diastolic, and mean arterial pressures were made using a high fidelity Millar catheter inserted into the carotid artery and advanced into the aortic arch. Catheter data were analyzed using PVAN 3.5 software.
blood pressure parameters, whereas sympathectomy had no effect on blood pressure in the WKY group.

Collagen Volume Fraction
Collagen volume fraction results are displayed in Figure 1. LV collagen volume fraction in the SHRs was markedly increased in comparison with that in WKYs. However, this increase was significantly attenuated in the sympathectomized SHR group. Collagen volume fraction was not affected by sympathectomy in the WKYs.

Cardiac MC Number and Density
A significant increase in the number of MCs occurred within the left ventricle of SHRs (WKY: 73.3±10.3 versus SHR: 105.7±14.8 cells per section; *P<0.05). However, because of the larger area of the SHR left ventricle, there was no difference when expressed as MC density (SHR+Symp: 316.3±68.6 cells per section; †P<0.05 versus WKYs and SHRs). In contrast, sympathectomy had no effect on MC number or density in WKYs (WKY+Symp: 84.2±12.1 cells per section).

Myocardial Cytokine Profile
Elevated levels of IFN-γ in the SHR (Figure 3A) were normalized by sympathectomy. Sympathectomy had no effect in the WKY. Although myocardial IL-4 levels were also elevated in the SHR (Figure 3B), sympathectomy did not change these levels. However, sympathectomy in the WKY did cause a significant increase in IL-4. Myocardial IL-6 (Figure 3C) was found to be markedly lower in the SHR than in the WKY, but was normalized by sympathectomy. Sympathectomy did not change IL-6 levels in the WKY. IL-10 was also found to be decreased in the SHR myocardium (Figure 3D). Sympathectomy resulted in increased amounts of IL-10 in both WKYs and SHRs.

Effect of SNS Products on Cardiac MC Activation and Angiotensin II Production
Incubation of isolated cardiac MCs with norepinephrine at concentrations of 1×10⁻¹², 1×10⁻⁹, 1×10⁻⁶, and 1×10⁻⁴ M did not induce degranulation (Figure 4A). In contrast, while no effects of substance P were seen at lower concentrations, 1×10⁻⁴ M did stimulate cardiac MC degranulation (Figure 4B). Also, this concentration of substance P stimulated the production of angiotensin II by a mixed population of isolated cardiac inflammatory cells (Figure 4C).

Identification of α-Adrenergic Receptors on Cardiac MCs
Isolated cardiac MCs did not possess α-adrenergic receptors (Figure S2).

Discussion
Chronic elevation of SNS activity in the myocardium is a key component of the altered signaling pathways that accompany cardiac remodeling resulting from hypertension. Although there is evidence that the SNS mediates cardiac fibrosis in hypertension via α-adrenergic receptor stimulation, the exact mechanisms have not been fully elucidated. However, it is likely to be more complex than direct activation of α-adrenergic receptors on cardiac fibroblasts. To this end,
inflammation is also an important component in the development of hypertension-induced cardiac fibrosis.\textsuperscript{3–5,7,8,18} More specifically, we recently demonstrated a causal role for MCs in regulating myocardial cytokines, macrophage recruitment, and development of fibrosis in the hypertensive heart.\textsuperscript{6} Given the critical roles of both SNS upregulation and MCs in hypertension-induced myocardial remodeling, the current study aimed to explore the effects of surgical sympathectomy on cardiac MCs, as well as specific cytokines, in the hypertensive heart.

After sympathectomy in SHRs, we observed a dramatic 5-fold increase in cardiac MC density. Although surgical removal of the superior cervical ganglion likely does not result in complete denervation of the heart, nerve fibers from this ganglion do project to the heart,\textsuperscript{19} and this striking change in MC density clearly demonstrates an impact of its removal on the heart. This same relationship is not present in the normal heart, because such a change did not occur in sympathectomized WKYs. Facoetti et al\textsuperscript{20} also found cardiac MC density to be unchanged in normal Sprague-Dawley rats after 10 weeks of chemical sympathectomy induced by 6-hydroxydopamine. These disparate effects on MC density are likely indicative of underlying differences in the local environment of normal versus hypertensive hearts. That

**Figure 3.** Changes in LV levels of (A) IFN-\(\gamma\), (B) IL-4, (C) IL-6, and (D) IL-10 in WKYs (n=4), SHRs (n=4), WKY+Symps (n=4), and SHR+Symps (n=4). All of the values are mean\(\pm\)SEM. *P<0.05 vs WKYs; †P<0.05 vs SHRs.

After sympathectomy in SHRs, we observed a dramatic \(\approx\)5-fold increase in cardiac MC density. Although surgical

**Figure 4.** Concentration-responses for histamine release by isolated cardiac MCs in response to norepinephrine (n=4; A) and substance \(\text{P}\) (n=4; B). Angiotensin II was produced in response to stimulation with substance \(\text{P}\) (1 \(\times\) 10\(^{-4}\) M) by a mixed population of isolated cardiac inflammatory cells, including MCs, lymphocytes, and macrophages (C). *P<0.05 vs control.
fibrosis was attenuated in sympathectomized SHRs despite MC density being elevated suggests that either sympathectomy altered MCs to those of a nonfibrotic phenotype or, alternatively, MCs signal the SNS, but can no longer induce a profibrotic response from the damaged SNS. Interestingly, in our isolated cardiac MC experiments, incubation with norepinephrine failed to induce degranulation, and we were unable to detect α-adrenergic receptors on cardiac MCs using flow cytometry. This is consistent with a previous report that norepinephrine was not the mechanism by which contact with sympathetic neurons decreased membrane resistance in immortalized RBL-2H3 cells, a widely used model for MCs. However, some afferent nerve fibers projecting from the heart travel through the superior cervical ganglia, and substance P, which is primarily located in afferent nerve fibers, has been demonstrated to be present in superior cervical ganglia. Substance P–containing nerves are associated with numerous areas of the heart, including the ventricle, atria, valves, and connective tissue lining. Here we showed that substance P was a strong inducer of isolated cardiac MC degranulation, consistent with what we have demonstrated previously and suggestive of afferent activation of cardiac MCs.

Suzuki et al. using a coculture system, showed that RBL-2H3 MCs activated sympathetic neurons after IgE stimulation. Activation of sympathetic neurons did not occur if RBL-2H3 cells were not present. The most obvious mechanism by which MC activation of neurons could occur is through release of MC tryptase acting on protease activated receptor 2 (PAR-2). Steinhoff et al. recently detected PAR-2 on a large number of neurons containing substance P, and activation of these receptors by tryptase in dorsal horn slices initiated the release of substance P. However, cardiac MCs are also capable of producing renin and are assumed to be involved in the local generation of angiotensin II in the heart. Silver et al. postulated that MC-generated angiotensin II could then activate nerves through angiotensin receptors. We isolated a mixed population of inflammatory cells, including lymphocytes, macrophages and MCs, from rat hearts and found that incubation with substance P resulted in angiotensin II production. Thus, while it appears that the SNS is regulating cardiac MC density in sympathectomized SHRs, MCs may in actuality be upstream of SNS activation and, therefore, responding to an underlying stimulus (eg, increased wall stress) by unsuccessfully attempting to up-regulate SNS activity. That is, the increase in MC density may be an attempt to achieve increased SNS activity that cannot be elicited because of the sympathectomy. Therefore, we speculate that a self-perpetuating relationship may exist where cardiac MC tryptase could activate PAR-2 on afferent nerve fibers causing the release of substance P, which, in turn,
induces angiotensin II production by inflammatory cells that then acts on fibroblasts and also sympathetic nerves to release norepinephrine (Figure 5). Angiotensin II has been shown to induce myocardial necrosis through the stimulation of sympathetic neurons. In fact, a similar situation has been described in the rat knee joint, where it appears that sensory nerve activation of MCs led to stimulation of the SNS and, subsequently, plasma extravasion. Elaboration of norepinephrine by the SNS in response to stimulation by MCs would explain how α-adrenergic receptors mediate fibrosis when cardiac MCs do not degranulate in response to norepinephrine in our study. This may also explain why sympathectomy does not result in increased cardiac MC density in the normal heart where there is no underlying pathological stimulus driving MCs to upregulate sympathetic activity.

With this clear SNS-MC interaction, we sought to determine the effect of sympathectomy on selected myocardial cytokines that we have shown previously to be regulated, either directly or indirectly, by MCs in the hypertensive heart. Both IFN-γ and IL-4 were elevated in the SHR, with sympathectomy normalizing IFN-γ but not IL-4. IFN-γ is a marker of T-helper 1 T-cell phenotype, and its elevation in hypertensive myocardium compared with the WKY, sympathectomy increased IL-4 levels in the WKY. Combined with our previous studies, it would appear that the SNS has a role in regulating IL-4 in the normal heart, but that role is shifted to MCs in the hypertensive heart. Although the levels of IL-6 and IL-10 were lower in the SHR myocardium compared with the WKY, sympathectomy returned them to normal. What is intriguing about these results is that, with the exception of IL-4, these changes are almost identical to what we have observed previously in the SHR after MC stabilization. This similarity, together with the close spatial relationship between the nervous system and MCs, is more evidence of an interplay between the SNS and MCs in the regulation of the inflammatory process in the hypertensive heart.

In addition to attenuating cardiac fibrosis, sympathectomy in the SHR also prevented myocardial hypertrophy. However, our previous finding that MC stabilization did not prevent hypertrophy in the hypertensive heart indicates that MCs are not involved in modulation of hypertrophy. Rather than acting via inflammatory cells to induce hypertrophy, the SNS most likely has direct effects on cardiomyocytes via β-adrenergic receptors. Although it cannot be ruled out that the changes in these parameters and the assayed cytokines were the result of the sympathectomy-related attenuation of blood pressure, we believe that the following observations support the argument that the changes in cytokines and fibrosis were related to direct interactions between the SNS and MCs. First, attenuation of hypertrophy by sympathectomy has been shown to be independent of systolic blood pressure, and further demonstrated that chemical sympathectomy in hypertensive rats prevented cardiac fibrosis independent of blood pressure. Second, the increase in cardiac MC density was dramatic with sympathectomy in the SHR but not in the normal nonhypertensive heart. Third, our in vitro studies demonstrate that substance P, found in afferent nerves, can stimulate cardiac MC activation and angiotensin II production from a mixed population of cardiac inflammatory cells. Finally, the cytokine profile reported herein after sympathectomy in the SHR is almost identical to that after MC stabilization in the SHR, where blood pressure was unaffected.

**Perspectives**

The results of this study reiterate the importance of the SNS in the development of myocardial remodeling and provide evidence of a link between the neurohormonal and inflammatory paradigms of hypertension by demonstrating the importance of SNS interactions with cardiac MCs and cytokine production. Furthermore, although a number of inflammatory cell types have been implicated in the development of fibrosis, our findings are consistent with the hypothesis that MCs not only trigger and orchestrate inflammatory responses but are also responsible for cardiac fibrosis. However, it is important to note that not all of the effects of the SNS cardiac remodeling are mediated via MCs. Clearly hypertrophy does not appear to involve SNS interactions with MCs, but instead is likely the result of direct stimulation of cardiomyocyte β-adrenergic receptors. The findings herein indicate the need for further investigation regarding the mechanisms mediating SNS activation, as well as inflammatory cell and afferent nerve interactions in the hypertensive heart, with particular focus on the role of substance P.

**Acknowledgments**

We thank Purnima Jani for her excellent technical assistance.

**Sources of Funding**

This study was supported in part by an American Heart Association postdoctoral fellowship 0825510E (to S.P.L.), as well as National Heart, Lung, and Blood Institute grants R01-HL-62226 (to J.S.J.) and R01-HL-073990 (to J.S.J.).

**Disclosures**

None.

**References**


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Hypertension. 2010;55:270-276; originally published online January 4, 2010;
doi: 10.1161/HYPERTENSIONAHA.109.142042
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/55/2/270

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SYMPATHETIC NERVOUS SYSTEM MODULATION OF INFLAMMATION AND REMODELING IN THE HYPERTENSIVE HEART

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Materials and Methods

Mast Cell Number and Density

At the end of the 12 week study period, anesthetized rats were euthanized by excision of the heart, which was then rinsed in ice-cold saline prior to separating and weighing the LV plus septum and RV. A slice of the LV from its equatorial region was fixed with Telly’s fixative (100 mL 70% ethanol; 5 mL glacial acetic acid; 10 mL formaldehyde) for histological analysis, while the apical portion was snap-frozen in liquid nitrogen and stored at -80°C for subsequent use. To determine mast cell density, 5 µm thick paraffin embedded LV cross-sections were stained with the mast cell specific stain, pinacynol erythrosinate. Mast cell counts from the entire LV section were carried out in a blinded fashion at 400x magnification, with mast cell density determined by dividing the total number of mast cells by the area of the respective LV cross-section as determined from the digitized image (ImageQuant, Molecular Dynamics).

Collagen Volume Fraction

Paraffin-embedded LV cross-sections cut to 5 µm in thickness were incubated with phosphomolybdic acid (0.2%) to reduce background before staining with picrosirius red (0.1% Sirius Red F3BA in picric acid). Tissue sections were analysed in a blinded fashion using a Biorad MRC-1024 confocal laser-scanning microscope with a 200x magnification. Twenty representative fields per LV cross-section were imaged. Image analysis was performed using Scion Image software (National Institutes of Health, USA). Briefly, a pixel count for the amount of collagen was obtained for each field. This was then expressed as a percentage of the overall number of pixels present in the field giving a percentage area of interstitial collagen. Perivascular areas were excluded from the analysis.
Protein Extraction from LV tissue

100mg of LV tissue from each heart was maintained on ice and cut into pieces approximately 1 mm². This tissue was then placed into the homogenization tubes and immersed in 800 µL of PBS/protease inhibitor solution. Tissues were homogenized on ice before being sonicated. 50 µL of 10 % Triton-X 100 was then added to each sample and vortexed. The samples were then allowed to sit on ice for 30 minutes. Samples were again vortexed after 15 of the 30 minutes. Following Triton-X treatment the samples were centrifuged @ 16,000 rpm (4°C) for 10 min and the supernatant collected and frozen at -80°C.

Flow Cytometry

Isolated cells were washed and blocked against non-specific staining with PBS/1% BSA for 30 min before incubation for 45 min at room temperature with either AR32AA4 (MC; mouse monoclonal, 1:100; BD Pharmingen), anti-CD4-PE (mouse monoclonal, 1:100; BD Pharmingen), anti-CD8-PE (mouse monoclonal, 1:100; BD Pharmingen), anti-CD68 (macrophage; mouse monoclonal, 1:100; Serotec) and anti-α1 adrenergic receptor (rabbit monoclonal, 1:500; Abcam). AR32AA4 and anti-CD68 were conjugated with Alexa Fluor 488 secondary antibody for the inflammatory cell profiling experiments (supplemental figure 1). AR32AA4 was conjugated with PE secondary antibody for the α1 adrenergic receptor experiments. α1 adrenergic receptor was conjugated with Alexa Fluor 488 secondary antibody. Cell preparations were then analyzed using an Epics XL FACS system equipped with a 15mW argon ion laser operating at 488nm and data analyzed with EXPO 32 software.
Reference List

Figure S1. Representative flow cytometric analysis of the distribution of inflammatory cells isolated from normal rat hearts using the isolation technique described in Methods. (A) mast cells; (B) CD4$^+$ T cells; (C) CD68$^+$ macrophages; and (D) CD8$^+$ T cells.
Figure S2. Flow cytometric analysis of α-adrenergic receptors and cardiac mast cells in the isolated cardiac inflammatory cell preparation. The analysis indicates that cardiac mast cells do not possess α-adrenergic receptors.