

REGULATION OF SYMPATHETIC PLASTICITY IN THE HEART

BY

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This work is dedicated to my husband Brant Clarke, my parents Jacqueline Wernli and Heinz Wernli as well as to my brother Erwann Wernli for their love and support during this journey and the many years that have led to it.

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ABSTRACT

Following myocardial infarction, elevated sympathetic neuronal sprouting at the infarct border region leads to hyperexcitability which increases the risk of arrhythmias and sudden cardiac death. Surviving individuals with impaired cardiac functions are prone to develop congestive heart failure, which is also associated with sympathetic hyperexcitability. As a result, β -adrenergic receptor blockers are commonly administered to reduce adverse symptoms. Therefore our first goal was to attenuate inflammation in order to reduce sympathetic hyperinnervation in the infarct border region. Our second goal was to study the effect of β -blockers on cardiac sympathetic innervation.

Inflammatory cells in the infarct release nerve growth factor (NGF) contributing to sympathetic neuronal sprouting. To reduce inflammation, we administered dexamethasone post-infarction. Although the treatment reduced body weight, it failed to reduce infarct sympathetic hyperinnervation. However, dexamethasone is not a selective treatment and may have side effects that promote neuronal sprouting. Because our lab had previously shown that macrophages within the infarct express NGF, we depleted macrophages by clodronate liposome administration. In association with macrophage reduction, the number of myofibroblasts was slightly decreased but T-cell numbers were unchanged. Immunoblots showed that mature NGF was decreased by clodronate liposome treatment, and this change resulted

mainly from the decrease of NGF-immunoreactivity (-ir) in macrophages and to a lesser extent NGF-ir in myofibroblast, but NGF-ir in T-cells was not altered. In concert with the reduction of NGF, sympathetic hyperinnervation was suppressed. These findings suggest that macrophage NGF expression is central to the development of sympathetic hyperinnervation and may be a valuable therapeutic target.

Secondly, α or β -blockers were administered post-infarction. While border region sympathetic hyperinnervation was unchanged, β -blockers substantially increased sympathetic innervation in non-ischemic regions of the heart. Control hearts displayed similar hyperinnervation and administration of metoprolol showed that this response was selective for β 1-blockade. While two days after β -blocker treatment withdrawal, adrenergic receptors were not supersensitized, ventricular functions were enhanced by neuronal norepinephrine store release with tyramine, consistent with increased sympathetic neuroeffector functions. To determine if β -adrenoceptor activation modulates sympathetic innervation density via cardiac NGF synthesis, norepinephrine release was suppressed by chemical sympathectomy. Sympathetic depletion upregulated the translation of some proNGF isoforms but total NGF was unchanged. Consistently, adrenergic receptor activation did not modulate cardiomyocyte NGF synthesis *in vitro*. To examine potential direct effects of β -blockers on sympathetic neurons, cultures were incubated with propranolol or metoprolol, and these treatments

resulted in elevated outgrowth. Similarly, the inhibition of norepinephrine synthesis by α -methyl-p-tyrosine enhanced sprouting. Simultaneous incubation in dobutamine reversed outgrowth confirming that sympathetic plasticity is regulated by β 1 adrenergic auto-receptors.

In conclusion, targeting macrophage NGF synthesis may provide a valuable strategy to reduce border region sympathetic hyperinnervation. In addition, despite the wide clinical use of β -blockers, cardiac sympathetic hyperinnervation resulting from the treatment can alter ventricular functions and may contribute to severe adverse effects upon withdrawal.

CHAPTER 1. GENERAL INTRODUCTION

Background

Cardiovascular disease (CVD) is the leading cause of death in the United States, accounting for approximately 35% of all deaths. Currently, ~80,000,000 Americans have one or more types of CVD and the cost associated with the disease is estimated at \$475.3 billion for 2009 (Lloyd-Jones, et al., 2009). The prevalence of CVD increases with age and with a lower education level, however a healthy life style including no smoking, maintaining a healthy weight, consuming 5 fruits and vegetables per day as well as performing regular physical activities can reduce chances of developing CVD (Lloyd-Jones, et al., 2009). Coronary heart disease alone accounts for ~ 21% of all CVD (Lloyd-Jones, et al., 2009) and can lead to myocardial infarction (MI) which is caused by thrombosis in a coronary artery. In 2009, it is estimated that ~785 000 Americans will have a new MI and ~470,000 will have a recurrent MI, including ~21% silent MIs. On average, MIs reduce life span by approximately 15 years (Lloyd-Jones, et al., 2009).

The main cause of CVD is atherosclerosis, a syndrome characterized by accumulation of fatty deposits and inflammatory cells within the wall of the arteries. These plaques can cause hardening of the arteries leading to their enlargement in order to compensate for the reduced blood flow. The stress on the artery wall eventually results in either plaque rupture which can lead to the formation of a thrombus that obstructs the artery, or to aneurysm (Fuster, et al., 2007).

The inflammatory cascade post-MI

Complete closure of a coronary artery by a thrombus leads to ischemia and necrosis of the ventricle. Ventricular functions are impaired and can result in immediate organ failure. When the damage is less extensive, a complex inflammatory cascade is triggered in order to repair the necrosed area. Infarct healing is divided into three overlapping phases: the inflammatory phase, the proliferative phase, and the maturation phase (Frangogiannis, 2006).

During the inflammatory phase, secreted chemokines and cytokines attract neutrophils into the infarcted area. This is followed by the recruitment and the proliferation of other inflammatory cells including macrophages which phagocytose matrix debris and dead cells. During this process, macrophage become activated leading to the upregulation of many secreted factors (Adams, et al., 1984, Caroleo, et al., 2001). The next phase is characterized by the development of a microvascular network and of sympathetic axonal sprouting. Proliferation of fibroblasts and endothelial cells is suppressed. Myofibroblasts which are thought to transdifferentiate from different cell types (Schmitt-Graff, et al., 1994, Hasan, et al., 2000) secrete extracellular matrix proteins (DiPietro LA., 2003). During the maturation phase, inflammatory cells undergo apoptosis and a collagen-based scar is formed (Frangogiannis, 2006).

Remodeling of sympathetic innervations post-MI

Post-infarction ventricular remodeling induces extensive modifications of the sympathetic nervous system. This system plays a critical role in the heart by enhancing conduction through the heart which increases chronotropy, inotropy and electrical conduction velocity. Normal conduction through the heart starts at the pacemaker cells in the sino-atrial (SA) node on the right atrium. The impulse is passed along cardiomyocytes through gap junctions inducing their contraction. The internodal pathway rapidly conducts the impulse to the atrio-ventricular (AV) node located at the junction between the atria and the ventricle. The impulse is then conducted from the bundle of His to the Purkinje fibers down the septal wall to the apex of the heart. It is subsequently dispersed through the ventricle wall back to the base of the heart. Cardiomyocyte contraction induces blood to be pumped out of the ventricles and of the atria (Fuster, et al., 2007).

Myocardial infarction is believed to interfere with the electrical conduction through the heart two fold, first by causing a necrosed area disrupting cardiomyocyte impulses and by promoting the degeneration of the axons traveling through that lesion, second, by causing sympathetic hyperinnervation and hyperexcitability in the border region of the infarct (Martins, et al., 1980, Zipes, 1990).

These finding have been demonstrated by performing chemical or electrical stimulations of the ventricle and measuring responses. Contraction

of the ischemic region was improved by exogenous norepinephrine administration while sympathetic nerve stimulation had no effect (Martins, et al., 1980). Further, to determine the viability of the fibers above and below the infarct, electrodes were positioned and the effective refractory period (ERP) was measured during left and right sympathetic nerve stimulation of the stellate ganglion. The results demonstrated that ERP was shortened in sites above the infarct 1 to 3 weeks after infarction, unlike in regions below the infarct, which presented shortened ERP prior to infarction (Zipes, 1990). Moreover, norepinephrine from tissue biopsies of sites with shortened ERP were elevated (Zipes, 1990). Because non-uniform excitations of the ventricle can provoke arrhythmias (Han, et al., 1964, Kralios, et al., 1975), post-infarction modifications of the sympathetic innervations could cause arrhythmias.

Additionally, changes in the pattern of sympathetic nerves were observed in concert with post-infarction adverse symptoms. Regenerating nerve fibers stained with S-100 were observed besides spindle-shaped cells oriented along the axis of the myocytes. Both populations were found to move sequentially toward the center of each lesion following infarction in rats (Vracko, et al., 1990). Neuronal proliferation was also observed at the periphery of the infarct in human hearts (Vracko, et al., 1991) and this sympathetic sprouting was correlated with previous arrhythmogenesis (Cao, et al., 2000b). Together, these studies demonstrated that post-infarction

cardiac nerve sprouting is associated with serious adverse effects. To understand the mechanisms causing neuronal sprouting, we and others studied its relationship with nerve growth factor (NGF) (Hiltunen, et al., 2001, Zhou, et al., 2004, Hasan, et al., 2006).

The role of NGF post-MI

NGF is a potent sympathetic neurotrophin and is critical for proper target innervation (Levi-Montalcini, 1987). Neurotrophins bind to members of the Trk family receptor with a high affinity, and p75 receptor can strengthen this interaction. Activation of the Trk receptors induces neuronal differentiation and survival (Levi-Montalcini, 1987). In the absence of Trk receptors, neurotrophins bind to p75 which can lead to apoptosis (Lee, et al., 2001, Fahnstock, et al., 2004). NGF has a number of different molecular weight isoforms. RNA splice variants are translated into proteins that can be glycosylated and sulfonated. Those can then be cleaved intracellularly and extracellularly by furin (Boutilier, et al., 2008) or extracellular matrix metalloproteinases (MMPs) and by serine protease plasmin (Lee, et al., 2001). The higher molecular weight proNGFs have a higher affinity for p75 receptor while the mature ~14 kD isoform preferentially binds to TrkA receptor (Lee, et al., 2001). The various NGF isoforms have been implicated in a variety of developmental and physiological processes (Shelton, et al., 1984, Levi-Montalcini, 1987, Young, et al., 2004), which are mediated by

intracellular NGF taken up at the periphery and retrogradely transported to the cell body (Stoeckel, et al., 1975).

Enhanced cardiac sympathetic innervation by infusion of NGF to the left stellate ganglion increased ventricular tachycardia, ventricular fibrillation and sudden cardiac death (Chen, et al., 2001) implying that NGF may lead to those adverse effect following myocardial infarction. Macrophages and myofibroblasts were found to express high levels of NGF (Hasan, et al., 2006), which peaked 7 days after myocardial infarction causing sympathetic innervation to peak at the same time (Zhou, et al., 2004, Hasan, et al., 2006). In explant co-cultures, infarcted tissue was most neuritogenic 7 days post-infarction, inducing enhanced sympathetic neuronal sprouting from the ganglion (Hasan, et al., 2006). Moreover, neutralizing NGF with anti-NGF antibody in those cultures suppressed hyperinnervation confirming the direct relationship between NGF and hyperinnervation (Hasan, et al., 2006). Thus, NGF appears central to the development of sympathetic hyperinnervation in the infarct border region.

Reducing border region hyperinnervation by decreasing inflammation

The inflammatory environment is known to promote sympathetic neuronal sprouting by releasing NGF, thus we administered the anti-inflammatory glucocorticoid dexamethasone to reduce inflammation and we studied sympathetic hyperinnervation. As a more selective approach, we

identified the role of macrophage depletion on sympathetic hyperinnervation by injecting clodronate liposomes (Van Rooijen, et al., 1994). Macrophages were previously found to synthesize NGF in the border region and their proliferation coincides with cardiac nerves sprouting (Hasan, et al., 2006) . The activation of macrophages promotes NGF overexpression *in vitro* (Caroleo, et al., 2001) suggesting that the inflammatory environment of the infarct may be prone to releasing high NGF levels as a result of macrophage recruitment and activation. Therefore, reducing macrophage NGF may provide a selective therapeutic target to reduce hyperinnervation.

The role of β -blockers on cardiac sympathetic innervation

While sympathetic hyperinnervation and hyperactivity after myocardial infarction are compensatory mechanisms to prevent heart failure due to loss of active myocardium, they have been shown to induce arrhythmias (Cao, et al., 2000b, Zhou, et al., 2001). Moreover, impaired cardiac functions in surviving individuals can lead to congestive heart failure (CHF), which is also characterized by hyperactivity of the sympathetic nervous system in an attempt to restore blood pressure (Ferrari, et al., 1998, Hunt, 2005). Therefore, after myocardial infarction, β -blockers are used to reduce a number of adverse effects including chest pain, arrhythmias and the probability of reinfarction and mortality (Yusuf, et al., 1985, Viskin, et al., 1995). In addition, they are also used to decrease the effects of sympathetic

hyperactivity during CHF (Hunt, 2005). Therefore, we studied their role on cardiac innervation post-infarction and in uninjured hearts. While they are widely used, withdrawal from β -blocker has long been known to induce adverse effects including worsening of angina, myocardial infarction and ventricular dysrhythmias (Miller, et al., 1975, Mizgala, et al., 1976, Nattel, et al., 1979, Hoeks, et al., 2007). The adverse symptoms are thought to result mainly from receptor supersensitivity which begins a couple of days after withdrawal, peaks after 6 days and is followed by a progressive decrease for about a week until the response returns to control levels (Nattel, et al., 1979, Hedberg, et al., 1980). However our results, suggest that sympathetic innervation may contribute to those adverse effects.

Additionally, adrenergic receptor activation has been shown to promote NGF synthesis in many cell types (Dal Toso, et al., 1988, Colangelo, et al., 1998), therefore we studied whether β -adrenergic receptor activation promotes neuronal remodeling by modifying cardiac NGF production.

Collectively, the findings presented in this dissertation identify a selective method to downregulate NGF in the infarct border region, providing insights for a possible therapeutic approach. In addition we identified that β -blocker treatment induces sympathetic sprouting which contributes to abnormal neuroeffector function following treatment withdrawal.

**CHAPTER 2. POSSIBLE FACTORS MODULATING SYMPATHETIC
HYPERINNERVATION FOLLOWING MYOCARDIAL INFARCTION**

- a. ROLE OF SYMPATHETIC INNERVATION IN REGULATING
VENTRICULAR NGF LEVELS**

- b. ROLE OF DEXAMETHASONE ON BORDER REGION
SYMPATHETIC HYPERINNERVATION**

ABSTRACT

The abrupt necrosis of the ventricle as the result of a thrombus, leads to an extensive tissue remodeling. Myocyte apoptosis triggers an inflammatory cascade which is characterized by the secretion of cytokines, chemokines and growth factors. The release of nerve growth factor (NGF) induces sympathetic sprouting at the infarct border region which can lead to arrhythmias and sudden cardiac death. The objective of this study was to identify potential ways to selectively reduce border region sympathetic hyperinnervation; first, by reducing cardiomyocytes NGF synthesis via reduction of adrenoceptor activation, second, by reducing inflammation with dexamethasone. To induce chemical sympathectomy, rats were given guanethidine or saline injections. Sympathectomy was confirmed by immunohistochemical stainings. Ventricle NGF synthesis was determined by immunoblots, revealing that sympathectomy increased the 37 and 44kD proNGF isoforms in the lateral wall and in the base of the heart respectively compared to control heart samples, but total NGF proteins were unchanged between groups. Alternatively, rats received coronary artery ligation and were treated with dexamethasone or with saline for 7 days. Dexamethasone induced a significant weight loss compared to saline treated rats. Border region sympathetic innervation was quantified and no difference was found between groups. Together these results show that the modulation of cardiac NGF synthesis via adrenoceptors or the administration of dexamethasone

after infarction are strategies that fail to reduce sympathetic hyperinnervation. Future studies should target a more selective aspect of the neurotrophic inflammatory response.

INTRODUCTION

Infarct border region is associated with sympathetic hyperinnervation, which can lead to arrhythmias and sudden cardiac death (Cao, et al., 2000a, Cao, et al., 2000b, Chen, et al., 2001). Amongst many factors that are involved in the inflammatory cascade (Frangogiannis, 2004, Frangogiannis, et al., 2005), nerve growth factor (NGF) is a central neurotrophin causing sympathetic hyperinnervation after myocardial infarction (Zhou, et al., 2004, Hasan, et al., 2006). In explant studies, we found that border region infarcted tissue induced maximal sprouting from superior cervical ganglia at 7 days post-coronary artery ligation. Moreover, this outgrowth was suppressed by the addition of anti-NGF antibodies in the conditioned medium, suggesting that NGF is required for border region sympathetic hyperinnervation (Hasan, et al., 2006). Reducing NGF could therefore provide a decrease of sympathetic hyperinnervation leading to a reduced risk of developing arrhythmias after myocardial infarction. However, NGF is expressed by many cell types (Hasan, et al., 2000) including cardiomyocytes (Lockhart, et al., 2000) and inflammatory cells (Hasan, et al., 2006) and is important in a number of

physiological functions. Therefore, we studied different ways to promote downregulation of border region NGF.

A. Role of sympathetic innervation in regulating ventricular NGF levels

First, we studied if blocking β adrenergic receptors (AR) on cardiomyocytes at the border region could lead to the selective downregulation of NGF. The activation of β 2-AR has been found to induce robust NGF expression in many cell types including fibroblasts and astrocytoma cells (Dal Toso, et al., 1988, Colangelo, et al., 1998). Because β -AR are present on cardiomyocytes (Rohrer, et al., 1999, Devic, et al., 2001), downregulating the activation of these receptors may also reduce NGF expression. To reduce β -AR activation we first reduced their agonist, norepinephrine by inducing chemical sympathectomy with guanethidine and we quantified changes in ventricular NGF expression.

B. Role of dexamethasone on border region sympathetic hyperinnervation

Inflammatory cells are a robust source of NGF in the border region (Hasan, et al., 2006), therefore reducing inflammation could selectively decrease NGF leading to a reduction of sympathetic hyperinnervation.

Dexamethasone is a synthetic glucocorticoid that is commonly used to reduce inflammation. Its reasonable cost and availability make it a good

candidate as a potential drug treatment. Dexamethasone mediates its action primarily by inhibiting phospholipase A2, leading the reduction of prostaglandin, thromboxane and leukotriene (Boyanovsky, et al., 2009). Besides these actions, it is also known to downregulate NGF production by peripheral organs which could lead to reduced sympathetic innervation (Lindholm, et al., 1994). However, it has been shown to promote neurite outgrowth of a neuronal cell line (Sheehan, et al., 2006). Therefore, we assessed the effect of dexamethasone treatment on border region sympathetic hyperinnervation.

MATERIAL AND METHODS

Guanethidine treatment

Because cardiovascular disease is more prevalent in post-menopausal women (Lloyd-Jones, et al., 2009), twelve female Sprague Dawley rats (Harlan Breeding Laboratories; Indianapolis, IN) were obtained at 60 days of age (~200g), housed 2-3 per cage in a climate and light (12L:12D) controlled environment with food and water *ad libitum*. All experimental protocols conformed to NIH guidelines and were approved by the University of Kansas Medical Center Animal Care and Use Committee.

Rats were anesthetized by intraperitoneal injection of KAX (i.p. injection of 60 mg/kg ketamine HCl Keta-Ject, Phoenix Scientific, St. Joseph, MO; 0.4 mg/kg atropine sulfate, Healthcare corporation, Deerfield, IL; 8 mg/kg

xylazine Xyla-Ject, Phoenix Scientific, St. Joseph, MO) and ovaries were removed by flank incisions (Zoubina, et al., 2002). A week later, six rats were given intraperitoneal injections of 25mg/kg guanethidine in saline solution twice a day at least 10 hours apart and six rats were given saline injections, at that same time. After ten days, rats were anesthetized as above and hearts were harvested.

For immunohistochemistry analysis, hearts were embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) and snap frozen in isopentane cooled in liquid nitrogen and stored at -80°C (N=2 per group). For western blots analysis, the base and the lateral wall of the heart were dissected into 3mm² samples (N=4 per group). Samples were homogenized in 200µl RIPA lysis buffer (Chemicon, Temecula, CA) with 10% protease inhibitor (Sigma, St. Louis, MO) and 1% phosphatase inhibitor (Sigma, St. Louis, MO) using Metal Rotor Stator Generator probes.

Innervation assessment after guanethidine

Hearts were cryosectioned serially at 10 µm thickness perpendicular to the apical-basal axis. Sections were fixed in 4% paraformaldehyde, blocked with normal goat serum supplemented with 1% bovine serum albumin, and immunostained overnight for the selective sympathetic axon markers dopamine β-hydroxylase (DBH, 1:200, rabbit IgG, Immunostar, Hudson, WI), or vesicular monoamine transporter 2 (VMAT2, 1:100, rabbit IgG, Chemicon,

Temecula, CA) followed by a 2 h incubation in goat anti-rabbit IgG conjugated to cy3 (1:100, Jackson Immunoresearch Laboratories, West Grove, PA). Images were captured with a 40x objective using a Nikon Eclipse TE300.

NGF western blots

Proteins were separated by SDS-PAGE (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk in TBST (10mM Tris HCl, 150mM NaCl, pH 7.4 and 0.05% Tween solution-20) followed by overnight incubation at 4°C in 1:1000 rabbit anti-NGF (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS and in goat anti-rabbit 1:5000 AP-conjugated (Jackson, West Grove, PA).

Immunoreactive proteins were detected using Immunostar substrate (BioRad, Hercules, CA) and visualized using a ChemiDoc XRS (BioRad, Hercules, CA). NGF proteins were normalized to total protein, which was determined by incubating the membranes in 7% acetic acid, 10% methanol and floated in SYPRO Ruby stain reagent (Molecular Probes, Carlsbad, CA). Proteins were visualized using an FX ProPlus (BioRad, Hercules, CA).

Dexamethasone treatment

Within a week of arrival, seven rats were anesthetized with KAX as above and ovariectomized (Zoubina, et al., 2003). A week later, rats were reanesthetized, respired mechanically, and the left anterior descending coronary artery was ligated with a silk suture (Hasan, et al., 2006). Rats received daily injections of 2mg/kg/day dexamethasone (Spectrum, Gardena, CA) in saline (N=4) or injections of saline alone (N=3). After seven days, rats were anesthetized and hearts were harvested and snap-frozen as above.

Infarct size

Due to variation in the vasculature, obstruction of a coronary artery can result in variable infarct size. Thus, this study was designed to eliminate any effects caused by variability of infarct extension.

To establish infarcts physical dimensions, one set of 6 sections taken at ~2mm intervals through the entire ventricle was stained with Masson's Trichrome (Diagnostic BioSystems, Pleasanton, CA). Sections were fixed for 10 minutes in 4% paraformaldehyde and 20 minutes at 50 °C in Bouin's solution (Electron Microscopy Sciences, Hatfield, PA). Sections were incubated in Weigert's iron hematoxin, bieberich scarlet-acid fuchsin, phosphomolybdic-phosphotungstic acid and aniline blue. Images of the infarct were captured using a Nikon SMZ 1500 microscope with a 1x objective at 0.75 optical zoom, and the area of the infarction was measured (AnalySis,

Soft Imaging System), multiplied by the distance between sampled sections, and these values summed to provide an estimate of the infarct size for each heart.

Sympathetic innervation after dexamethasone treatment

Adjacent to Masson's Trichrome sections, six sections were fixed at 4°C in methanol for 10 min, blocked with normal goat serum supplemented with 1% bovine serum albumin and stained overnight for dopamine- β -hydroxylase (DBH: 1:600, rabbit IgG, Immunostar, Hudson, WI). Secondary antibody goat anti-rabbit IgG conjugated to Alexa 555 (1:500, Invitrogen, Carlsbad, CA) was applied for 2 h. Images of 6 fields evenly distributed in the border region located in the left lateral posterior area of the heart were taken with a 40x objective using a Nikon Eclipse TE300. Nerve density was calculated by superimposing a stereological grid (8 μ m line interval) over the captured image, counting grid intersections over nerves and dividing by the total number of intersections over the sampled tissue.

Statistical Analyses

Data were analyzed using Student T-test (SigmaStat 3.11, Systat Software, Inc., San Jose, CA). Data are expressed as mean \pm SEM. Statistical significance was accepted at $P \leq 0.05$.

RESULTS

Effects of sympathectomy

To confirm that guanethidine treatment induced chemical sympathectomy, sections were stained with two sympathetic neuronal markers, DBH and VMAT2. Control hearts displayed sympathetic fibers running along cardiomyocytes with both DBH-ir (Fig. 1A) and VMAT2-ir (Fig. 1C) and guanethidine suppressed sympathetic innervation visible by a marked reduction of both DBH-ir (Fig. 1B) and VMAT2-ir (Fig. 1D).

Six bands were visible on immunoblots of NGF from heart samples with molecular weights of 73, 44, 37, 25, 23 and the mature isoform at 14kD (Fig. 2A). Sympathectomy increased the 37kD NGF isoform by 34% in the lateral wall of the heart relative to control (from $1.1 \pm 0.04 \times 10^{-2}$ after saline to $1.48 \pm 0.08 \times 10^{-2}$ INT*mm² after guanethidine). However, total NGF proteins were unchanged in that region ($7.52 \pm 1.20 \times 10^{-2}$ INT*mm² and $7.99 \pm 0.82 \times 10^{-2}$ INT*mm² after saline and guanethidine, respectively). Similarly, the 44kD NGF isoform was increased by 67% in the base of the heart after sympathectomy relative to control (from $0.36 \pm 0.042 \times 10^{-2}$ INT*mm² after saline to $0.61 \pm 0.089 \times 10^{-2}$ INT*mm² after guanethidine). However, sympathectomy did not modify total NGF protein in that region of the heart ($5.16 \pm 0.83 \times 10^{-2}$ INT*mm² after saline to $5.15 \pm 0.48 \times 10^{-2}$ INT*mm² after guanethidine).

Effect of dexamethasone

To confirm that the administered dose of dexamethasone had a significant effect, we monitored rat weight, as previously reported treatment with dexamethasone reduced total body weight compared to controls (Shafagoj, et al., 1992). During seven days, control rats gained 15 ± 3 g, while dexamethasone treatment induced a weight loss of 29 ± 5 g (Fig 3). To eliminate a non-specific effect of the treatment on sympathetic innervation caused by variation of infarct extension, we insured that infarct size was comparable between groups. Following the exclusion of one heart from the saline group due to the lack of transmural infarct, infarct sizes were 114 ± 5 mm³ in controls and 104 ± 19 mm³ after dexamethasone treatment (Fig. 4). Border region sympathetic hyperinnervation was similar to our previous findings (Wernli, et al., 2009) and was unchanged after dexamethasone treatment $1.9 \pm 0.3\%$ compared to control 1.8 ± 0.3 .

DISCUSSION

Thrombus in a coronary artery can result in ischemia followed by necrosis of the supplied ventricular tissue (Fishbein, et al., 1978, Frangogiannis, 2006). Cardiomyocyte apoptosis triggers a complex inflammatory cascade (Frangogiannis, et al., 2002). Recruited inflammatory cells promote cellular differentiation and proliferation (Garaci, et al., 1999,

Micera, et al., 2003) by secreting a number of factors including nerve growth factor (NGF). Besides contributing to wound repair (Li, et al., 1980), NGF promotes sympathetic neuronal sprouting (Levi-Montalcini, 1987, Hasan, et al., 2006). Excessive norepinephrine released by these axons at the infarct border region can induce arrhythmias (Cao, et al., 2000b). As a result, the risk of sudden cardiac death is increased (Cao, et al., 2000a). Therefore, in the present study, we evaluated two possible ways to reduce border region sympathetic hyperinnervation.

A. Role of sympathetic innervation on ventricular NGF levels

Sympathetic innervation releases norepinephrine, which activates adrenergic receptors. Since, the activation of β -AR increases NGF synthesis in various cell types (Dal Toso, et al., 1988, Colangelo, et al., 1998), this implies that by reducing their activation in the heart, border region NGF may be decreased. In order to reduce cardiac adrenergic receptor activation, we induced chemical sympathectomy with guanethidine.

We found that chemical sympathectomy increased the 37kD and 44kD proNGF isoforms in the lateral wall and the base of the heart respectively while mature NGF was hardly visible. Because proNGF isoforms tend to be pro-apoptotic (Lee, et al., 2001, Fahnstock, et al., 2004) and mature NGF appeared absent, this suggests that a positive feedback loop may take place further depleting innervations in those regions. Conversely, sympathetic

innervation may be enhanced and could thus contribute to border region hyperinnervation. Nonetheless, total NGF was unchanged in both areas.

Although transcription and translation may be increased by sympathectomy, because specific isoforms are upregulated, it is more probable that the increase of pro-NGF reflects changes in post-translational processing. NGF is subject to a number of post-translational modifications including sulfonation, glycosylation and cleavage (Lee, et al., 2001, Boutilier, et al., 2008); it is therefore possible that changes of norepinephrine activation of adrenergic receptors on cardiomyocytes modifies some of those intracellular pathways. The activation of β 1-adrenergic receptors on cardiomyocytes results in the activation of the G_s protein followed by the production of the second messenger cAMP (Wang, et al., 2004) and by the activation of protein kinase A (Lefkowitz, 2004). This cascade provokes calcium influx leading to increased force of contraction. In addition, G_s protein activation can also lead to a number of other intracellular pathways (Selbie, et al., 1998). Activation of these pathways may converge on NGF synthesis, thereby modifying the level of production of its isoforms. Alternatively, degradation may be reduced by these intracellular modifications leading to the increase of some pro-NGF isoforms.

Sympathectomy has been shown to induce changes of NGF expression in cardiac ganglia, supporting the fact that sympathetic neurons

modulate NGF intracellular pathways of other cell types in the ventricle (Hasan, et al., 2009).

The depletion of sympathetic innervation induces complex responses and has been shown to modulate NGF levels in some models but not in others. For example, surgical denervation significantly reduced NGF in the mesenteric artery (Liu, et al., 1996) suggesting that the decrease of NGF was caused by the loss of nerve plexus (Liu, et al., 1996). In another study, ciliarectomy and sympathectomy did not modify NGF levels in the iris while denervation including all sensory axons induced an increase of NGF levels (Ebendal, et al., 1983), suggesting that sensory neurons regulate NGF in that environment. Finally, chemical sympathectomy induced by 6-hydroxydopamine for one week after birth reduced norepinephrine content by 82% but did not significantly alter NGF mRNA in the heart (Clegg, et al., 1989).

Together, the absence of change of total NGF in the ventricle after removing sympathetic nerves suggests that border region cardiomyocytes may not contribute to reducing sympathetic hyperinnervation following myocardial infarction. Therefore, reducing inflammation may provide a better potential therapeutic treatment.

B. Role of dexamethasone on border region sympathetic hyperinnervation

Dexamethasone is a member of the glucocorticoid family, which is extensively used for its anti-inflammatory, immunosuppressive and antiproliferative functions (Verderame, 1986). Anti-inflammatory steroids are administered to treat a wide variety of conditions including arthritis, dermatitis, autoimmune diseases, asthma or to prevent rejection of organ transplant. However, because they interact with the naturally occurring adrenal steroids, which regulate electrolyte balance and glucose metabolism, they can cause many adverse effects. Notably, prolonged use can provoke muscle weakness and degeneration, osteoporosis, inhibition or arrest of growth in children, skin fragility and atrophy, hyperglycemia or cataract (Verderame, 1986).

Following myocardial infarction, inflammatory cells are recruited to repair the ischemic area. During this process, NGF levels are upregulated leading to sympathetic hyperinnervation. Because in addition to its anti-inflammatory properties, dexamethasone is known to reduce NGF levels in peripheral organs (Lindholm, et al., 1994), we studied its effect on border region sympathetic innervation.

We found that the dexamethasone dose administered caused a significant weight loss, consistent with previous findings (Shafagoj, et al., 1992); however, our results demonstrated that infarct border region hyperinnervation was unchanged by dexamethasone treatment.

Contrary to our findings, a study found a significant reduction in neurofilament M-ir fibres in the infarct associated with a decrease of NGF (El-Helou, et al., 2007). There may be several reasons for the discrepancy, such as drug dosage or methodological procedures; in that study, infarct size was not reported to be identical between the two groups, moreover quantification was the result of two random sections per animal. Finally neurofilament M-ir stains many neuronal populations and it is therefore not selective for sympathetic fibers.

The failure of dexamethasone to attenuate sympathetic innervation in our study is most likely a result of its pluripotent and sometimes contradicting action. Thus, in spite of the anti-inflammatory properties of dexamethasone which could reduce infarct inflammation and NGF production, dexamethasone has been shown to potentiate neurite outgrowth *in vitro* and to be associated with increased neuronal recovery after spinal cord injury (Sheehan, et al., 2006). Moreover, dexamethasone was found to enhance macrophage colony stimulating factor (M-CSF), which is the major growth factor controlling survival, proliferation and differentiation of macrophages (Lloberas, et al., 1998) and could therefore enhance macrophage NGF production in the border region (Hasan, et al., 2006). The conflicting actions of dexamethasone on neuronal outgrowth could thus explain the lack of a decrease of sympathetic innervation density after infarction following dexamethasone treatment.

In spite of the adverse effects induced by synthetic glucocorticoids they remain highly useful to treat clinical disease. Moreover, nonsteroidal anti-inflammatory drugs (NSAID) can also cause problems such as ulcers, edema, kidney failure or renal failure. Additionally, the COX-2 inhibitor sold by Merck under the name Vioxx which was thought to have potent analgesic and anti-inflammatory properties, was found to increase the risk of myocardial infarction (Warner, et al., 2008).

Thus, reducing inflammation has proven to be difficult as drug treatments can have generalized actions leading to secondary adverse effects. For this reason, the development of anti-inflammatory drugs will certainly become more refined to target a specific aspect of the inflammatory cascade. As an attempt to refine our strategy to reduce border region sympathetic hyperinnervation, we used clodronate liposomes in our next study to selectively deplete macrophages.

Conclusion

Collectively these results show that the inflammatory cascade induced to repair the necrotic tissue after coronary obstruction promotes robust sympathetic sprouting. Depleting sympathetic innervation leads to the alteration of some post-translational NGF isoforms, but total NGF remains unchanged between groups. Therefore, modifying the crosstalk between

sympathetic innervation and cardiomyocyte may not be the best target to decrease sympathetic hyperinnervation.

On the other hand, reducing the inflammation with dexamethasone did not affect hyperinnervation either. While dexamethasone is extensively used for its anti-inflammatory, immunosuppressive and antiproliferative functions, its actions are broad and can be conflicting, explaining the absence of change of innervation. Future studies will need to use a more selective anti-inflammatory approach in order to reduce NGF and sympathetic hyperinnervation without promoting outgrowth via other pathways.

FIGURES

Figure 1.

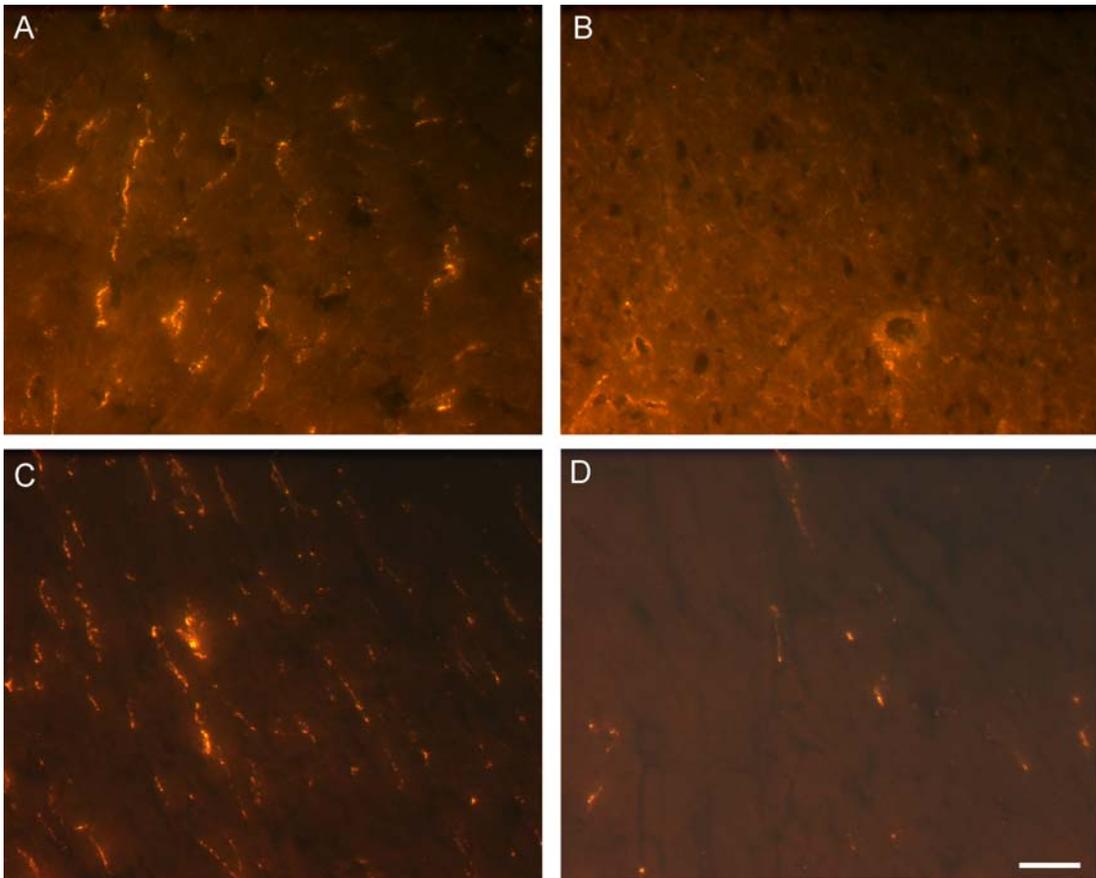


Figure 1. The reduction of sympathetic innervation after guanethidine treatment is reflected by multiple markers. Sections from control hearts were immunostained for the noradrenergic markers DBH (A) and VMAT2 (C) and sympathetic fibers are running along cardiomyocytes. After guanethidine treatment, DBH (B) and VMAT2 (D) were reduced showing that the treatment depletes ventricular sympathetic innervation. Scale=50 μ m.

Figure 2.

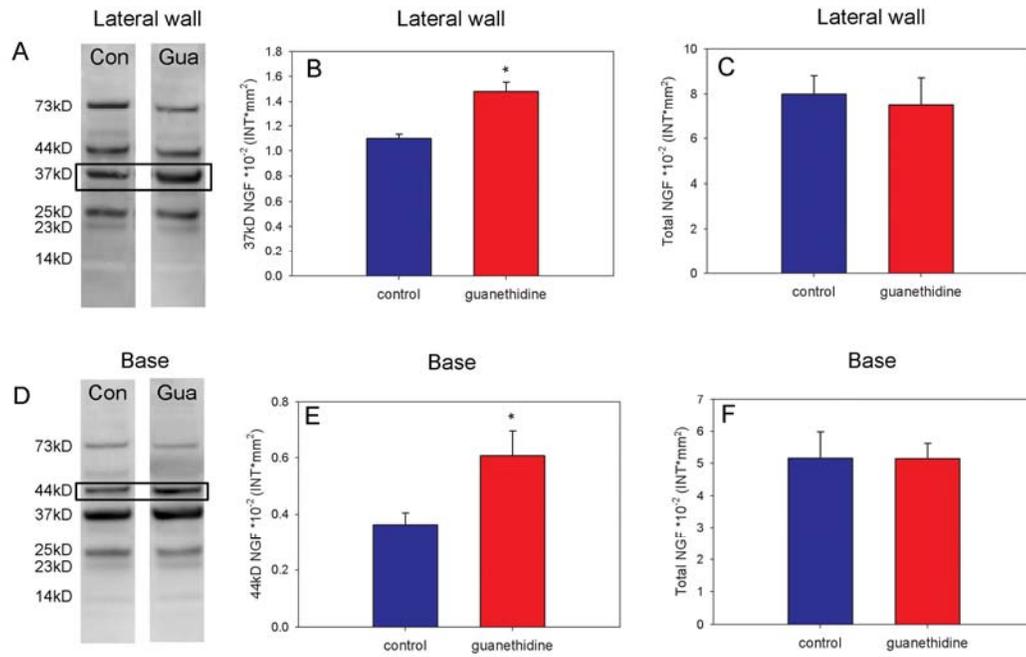


Figure 2. Effect of guanethidine on ventricular NGF levels. A shows that at least six NGF isoforms are present in the lateral wall of the heart and that guanethidine treatments increases synthesis of the 37kD isoform (box). Quantitative analysis of the 37kD NGF isoforms (B) and of total NGF protein (C). D, shows that the same isoforms are present in the base of the heart and that the synthesis of the 44kD isoform (box) is increased in that region. Quantitative analysis of the 44kD isoform (E) and of total NGF protein (F).

Figure 3.

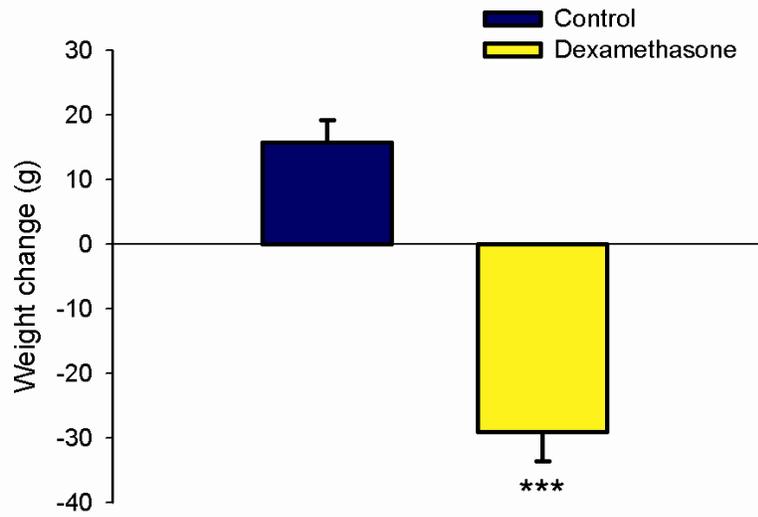


Figure 3. Effect of dexamethasone on weight. Treatment for 7 days post-infarction with dexamethasone induced weight loss while control rats given saline for the same time gained weight. *** $P < 0.001$

Figure 4.

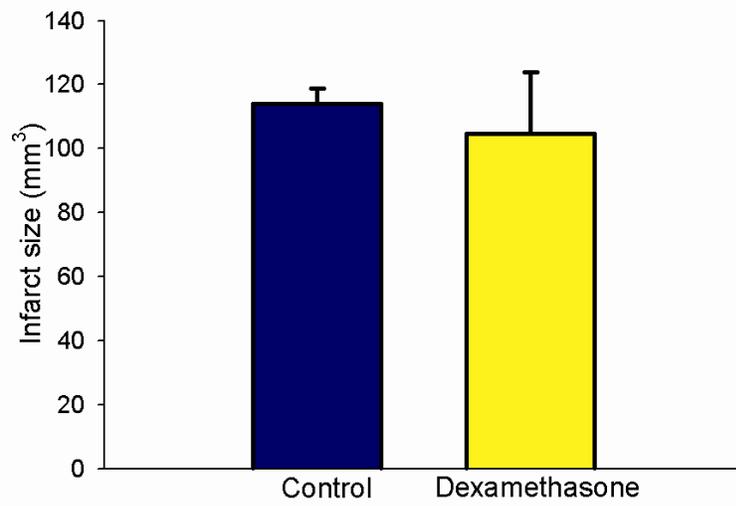


Figure 4. Infarct size of rats given dexamethasone or saline. To insure that sympathetic innervation was not influenced by a variation of infarct extension, infarct size was measured and was comparable after dexamethasone and saline treatment.

Figure 5.

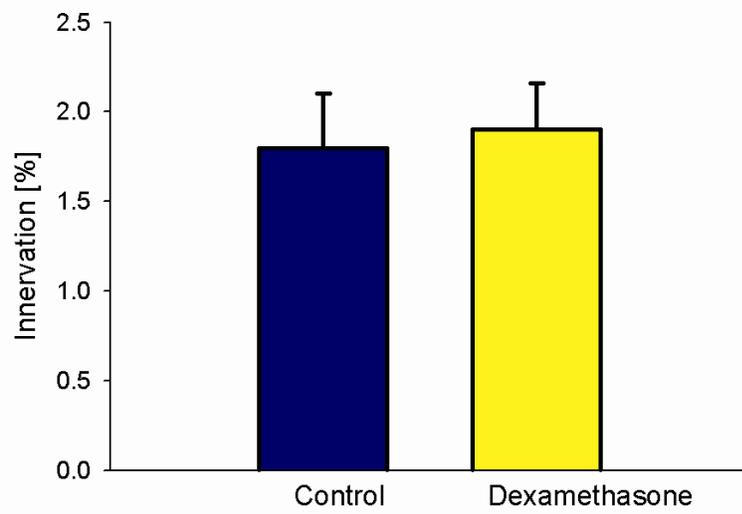


Figure 5. Effect of dexamethasone on border region sympathetic innervation density. Sympathetic innervation was quantified in the border region of six equally spaced sections. These regions were hyperinnervated in both groups and dexamethasone treatment did not influence sympathetic innervation density compared to control.

**CHAPTER 3. MACROPHAGE DEPLETION SUPPRESSES SYMPATHETIC
HYPERINNERVATION FOLLOWING MYOCARDIAL INFARCTION**

ABSTRACT

Myocardial infarction induces sympathetic axon sprouting adjacent to the necrotic region, and this has been implicated in the etiology of arrhythmias resulting in sudden cardiac death. Previous studies showed that nerve growth factor (NGF) is essential for enhanced post-infarct sympathetic sprouting, but the cell types necessary to supply this neurotrophic protein are unknown. The objective of the present study was to determine whether macrophages, which are known to synthesize NGF, are necessary for post-infarct cardiac sympathetic sprouting. Ovariectomized female rats received left coronary artery ligation or sham operation, followed by intravenous injection of liposomes containing saline vehicle or clodronate, which kills macrophages. Sham-operated myocardium contained some sympathetic axons, few myofibroblasts and T-cells and no CD-68-positive macrophages. In rats receiving saline liposomes through 7 days post-ligation, the posterolateral infarct border contained numerous myofibroblasts, macrophages and T-cells, and sympathetic innervation at the border region was increased two-fold. Treatment with clodronate liposomes reduced macrophage numbers by 69%, while myofibroblast area was reduced by 23% and T-cell number was unaffected. Clodronate liposome treatment reduced sympathetic axon density to levels comparable to the uninfarcted heart. NGF protein content measured in western blots was reduced to 33% of that present in infarcts where rats received saline-containing liposomes. Tissue morphometry

confirmed that NGF immunostaining was dramatically reduced, and this was attributable primarily to reduced macrophage content. These results show that macrophage destruction markedly reduces post-infarction levels of NGF and that the presence of elevated numbers of macrophages is obligatory for development of sympathetic hyperinnervation following myocardial infarction.

Key words: Myocardial infarction, sympathetic hyperinnervation, macrophages, nerve growth factor, inflammation

INTRODUCTION

Sudden cardiac death is a major clinical problem, accounting for more than 300,000 deaths annually in the United States (Myerburg, et al., 1992b). The risk of sudden cardiac death is especially high after myocardial infarction (MI) as 75% of victims have had a previous MI (Myerburg, et al., 1992a), generally in the 30 days prior to death (Solomon, et al., 2005). The direct cause of sudden cardiac death is most often ventricular tachycardia leading to ventricular fibrillation (Cao, et al., 2000a). Although the etiology of these post-MI ventricular arrhythmias remains incompletely understood, an increase in sympathetic nerve density at the infarct border zone has been implicated (Cao, et al., 2000b). We and others have shown that sympathetic hyperinnervation peaks 7 days after occlusion of the left coronary artery and decreases gradually through 28 days post-MI in rodents and dogs (Zhou, et

al., 2004, Hasan, et al., 2006) and this time course is consistent with the highest risk of sudden cardiac death observed clinically in humans (Solomon, et al., 2005).

Several lines of evidence suggest that nerve growth factor (NGF), a potent sympathetic neurotrophic protein (Levi-Montalcini, 1987), may play a role in post-MI sympathetic sprouting (Zhou, et al., 2004, Chen, et al., 2007). In explant cultures of infarcted tissue, NGF antibody neutralization blocked neurite outgrowth from sympathetic ganglia, demonstrating that NGF is required for development of sprouting at the infarct border zone (Hasan, et al., 2006). Similarly, *in vivo* studies where synthetic glucocorticoids were administered following MI showed reductions in infarct border zone sympathetic hyperinnervation (El-Helou, et al., 2007), and it is well established that these hormones generally suppress NGF production (Lindholm, et al., 1994). Accordingly, NGF synthesis within the infarcted region is likely to play a causal role in inducing local sympathetic sprouting.

Despite evidence that NGF synthesis is required for sympathetic hyperinnervation, the cellular mechanisms leading to elevated NGF synthesis following myocardial infarction remain unclear. In the normal rat heart, sympathetic innervation density correlates with NGF mRNA levels (Shelton, et al., 1984) suggesting that NGF upregulation in cardiac myocytes after injury could be a contributing factor. However, the border zone between damaged and intact myocardium also contains increased numbers of

macrophages and myofibroblasts (Hasan, et al., 2006). Wounds such as those created by ischemic injury are characterized by an influx of macrophages deriving from vascular monocytes that assist healing by phagocytosing cellular debris and secreting a variety of cytokines and growth factors. Cytokines present in inflammatory environments are known to promote activation of macrophages (Adams, et al., 1984) and *in vitro* macrophage activation induces NGF expression (Caroleo, et al., 2001), suggesting that macrophages could contribute to sympathetic hyperinnervation. Myofibroblasts are thought to transdifferentiate from fibroblasts, pericytes, and possibly macrophages (Schmitt-Graff, et al., 1994, Hasan, et al., 2000), and to promote wound healing through contraction and collagen deposition. Myofibroblasts, as well as another inflammatory wound cell, the T-cell, are also known to synthesize NGF (Hasan, et al., 2006), making them possible contributors to the induction of sympathetic hyperinnervation.

In the present study, we assessed the role of macrophages in the development of post-MI sympathetic hyperinnervation. We administered clodronate-containing liposomes, which are phagocytosed by macrophages and cause selective macrophage apoptosis and depletion (van Rooijen, et al., 1996). Our findings show that reductions in the macrophage population dramatically reduce sympathetic hyperinnervation and NGF content following experimental MI.

MATERIAL AND METHODS

Experimental Preparations

All experimental protocols conformed to NIH guidelines and were approved by the University of Kansas Medical Center Animal Care and Use Committee. Thirty-three female Sprague-Dawley rats (60 days old, ~ 200 g, Harlan Breeding Laboratories) were anesthetized by intraperitoneal injection of 60 mg/kg ketamine, 8 mg/kg xylazine, and 0.4 mg/kg atropine. To minimize the influence of reproductive hormones and also to simulate postmenopausal conditions which are associated with reduced cardioprotective effects (Kannel, et al., 1976, Grace, et al., 2004), ovaries were removed by flank incisions (Zoubina, et al., 2001). Seven days after ovariectomy, rats were anesthetized as above, respired mechanically, and the left anterior descending coronary artery was ligated with silk suture as described previously (Selye, et al., 1960, Hasan, et al., 2006). In sham surgeries, a suture was passed around the artery but left untied. Following coronary artery ligation (CAL) or sham-ligation, rats received tail vein injections on days 1, 3, and 5 of liposomes containing clodronate (Popovich, et al., 1999) or phosphate-buffered saline, or normal saline (1ml/100 g body weight). Clodronate was a gift from Roche Diagnostics GmbH, Mannheim, Germany (Van Rooijen, et al., 1994).

Seven days after coronary artery ligation, rats were reanesthetized as above and hearts were harvested. The efficacy of the ligation in producing

ventricular infarction was confirmed in preliminary observations by immersion in 1% triphenyl tetrazolium chloride (TTC) in phosphate buffer for 30 minutes at 40°C followed by fixation in formal saline (Lie, et al., 1975). Other hearts were harvested, embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) and snap frozen in isopentane cooled in liquid nitrogen and stored at -80°C.

Infarct Assessment

Because of anatomical variation in coronary arterial vasculature, ischemic damage was variable and was more limited in some hearts than in others. To ensure that analyses were conducted on specimens with comparable damage in all groups, infarcts that did not span the entire thickness of ventricular wall or only partially affected the left ventricle were excluded from further study. The numbers of infarctions excluded in each group was 2 of 7 (28%) in the saline liposome group, 1 of 7 (14%) in the saline injected group, and 4 of 11 (36%) in the clodronate liposome treatment group.

Hearts were cryosectioned serially at 10 µm thickness perpendicular to the apical-basal axis. To establish the physical dimensions of infarcts, one set of 6 sections taken at 1.7 mm intervals through the entire ventricle was stained with Masson's Trichrome according to a standard protocol (Diagnostic BioSystems, Pleasanton, CA). Sections were fixed for 10 minutes in 4%

paraformaldehyde and 20 minutes at 50 °C in Bouin's solution (Electron Microscopy Sciences, Hatfield, PA). Images of infarcts were captured using a Nikon SMZ 1500 microscope with a 1x objective at 0.75 optical zoom, and the area of the infarction was measured (AnalySis, Soft Imaging System, Lakewood, CO), multiplied by the distance between sampled sections, and these values summed to provide an estimate of the infarct size for each heart.

Sympathetic Innervation

To assess sympathetic innervation density, we analyzed four sections adjacent to the Masson's trichrome-stained set; the first section for analysis was taken 1.5 to 2 mm apical to the ligation, which ensured that we did not include tissue with local inflammation due to the ligation. Sections were fixed at 4°C in methanol, blocked with normal goat serum, and immunostained overnight for the selective sympathetic axon marker dopamine β -hydroxylase (DBH, 1:600, rabbit IgG, Immunostar, Hudson, WI), followed by a 2 h incubation in goat anti-rabbit IgG conjugated to Alexa 555 (1:500, Invitrogen, Carlsbad, CA). For each section, 6 fields evenly distributed within the left lateral posterior aspect of the infarct were captured with a 40x objective using a Nikon Eclipse TE300 (indicated by boxes in Fig 1.A and C); preliminary observations showed sympathetic innervation associated with the infarcted tissue was preferentially distributed within this region. In sham hearts, images were taken in an identical fashion from comparable areas. Nerve density was

calculated by superimposing a stereological grid (8 μ m line interval) over the captured image, counting grid intersections over nerves and dividing by the total number of intersections over the sampled tissue (Zoubina, et al., 1998, Zoubina, et al., 2001). Vascular innervation was excluded from quantification. To confirm that the changes observed with the quantification of DBH were not caused by changes of the level of that enzyme alone, adjacent sections to the ones stained for DBH were fixed in 4% paraformaldehyde for 5 minutes, blocked with normal goat serum, and immunostained overnight for the intermediate filament marker peripherin (1:1000, chicken IgG, Chemicon, Temecula, CA) followed by a 2 h incubation with goat anti-chicken IgG conjugated to cy2 (1:200, GeneTex, San Antonio, TX). Another set of adjacent sections was fixed in 4% paraformaldehyde for 5 minutes, blocked with normal goat serum, and immunostained overnight for the selective sympathetic axon marker tyrosine hydroxylase (TH, 1:300, rabbit IgG, Chemicon, Temecula, CA) followed by a 2 h incubation in goat anti-rabbit IgG conjugated to cy3 (1:400, Jackson, West Grove, PA).

Inflammatory Cells

A second set of sections adjacent to the DBH-immunostained series was stained for CD68 (1:200, mouse monoclonal, MAB 1435, Chemicon, Temecula, CA), a marker for tissue macrophages and blood monocytes (Micklem, et al., 1989). A third set was stained for α -smooth muscle actin

(1/200, mouse monoclonal 1A4, Sigma, St. Louis, MO), which identifies myofibroblasts (Skalli, et al., 1986, Hasan, et al., 2000). A fourth set of adjacent sections was immunostained for the T-cell antigen receptor TCR α/β (1:100, mouse monoclonal, clone R73, Serotec, Oxford, UK). All inflammatory cell markers were co-stained with rabbit IgG recognizing rat NGF (1:100, M-20, Santa Cruz, Santa Cruz, CA). Secondary antibodies were Alexa 488-conjugated goat anti-mouse (1:1000 Invitrogen, Carlsbad, CA) and Alexa 555-conjugated goat anti-rabbit (1:500 Invitrogen, Carlsbad, CA). Antibody specificities were confirmed using antigen preadsorption with a 10 fold excess of the peptide and primary antibody omissions.

Macrophage, myofibroblasts and T-cells were quantified by capturing 4 images with a 40x objective on a Nikon Eclipse C1 Si confocal microscope from each stained section in the same areas as those used for innervation assessment. To quantify macrophages, numbers of CD68-immunoreactive (ir) cells per field were counted and expressed as the average number of cells per mm² (Metamorph, Molecular Devices, Downingtown, PA). In addition, average macrophage size was estimated by measuring total section area occupied by CD68-ir by threshold detection and dividing it by the total number of CD68-ir macrophages. Because myofibroblasts form sheet-like aggregates within the infarct, counting individual cells is not feasible. Therefore, myofibroblasts were quantified by measuring the area per field occupied by α -SMA-ir cells, taking care to exclude α -SMA-ir vascular smooth muscle; the

percentage of field area was assessed by counting stereological grid intersections overlying myofibroblasts, dividing this by total number of intersects within that field, and expressing this as area per mm² (AnalySis, Soft Imaging System, Lakewood, CO). T-cells associated with the infarct were assessed by counting TCR α/β -ir cells per each of the 4 fields and expressed as the average numbers of cells per mm².

NGF and Cytokine content

The content of NGF protein in infarct tissue was quantified by western blots. Infarcted hearts from 3 saline liposome and 4 clodronate liposome rats were snap frozen and the border region with adjacent intact myocardium was dissected out and homogenized in Ripa buffer (50mM TrisHCl pH 7.4, 150mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1mM EDTA) with 10% protease inhibitor (Sigma, St. Louis, MO) and 1% phosphatase inhibitor (Sigma, St. Louis, MO). Fifty μ g of protein was loaded on a gel and western blot was performed according to protocol (Invitrogen, Carlsbad, CA). The membrane was stained overnight with rabbit anti-NGF (1:500, Santa Cruz, Santa Cruz, CA) in 5% goat serum and 2hr in goat anti-rabbit HRP (1:5000, Sigma, St. Louis, MO). After revealing the bands with SuperSignal West Pico Stable Peroxide Solution (Thermo Sc., Rockford, IL), their intensities were analyzed using a Chemi Doc XRS and quantification was performed using Quantity One software (BioRad, Hercules, CA). Total protein stained with

India ink was used to verify that equal amount of proteins were loaded on the gel and transferred to the membrane. The total density assessed by India ink was constant between samples and was used to normalize the density of the NGF bands.

The contribution of each cell type to the NGF content within the infarct was assessed in samples co-stained for the cell-selective markers and for NGF. For both CD68-ir and TCR α/β -ir cells, numbers of cells co-expressing both proteins were counted in each of the 4 sample regions and normalized to 1 mm². In addition, for both markers the total field area occupied by NGF-ir/CD68-ir macrophages and NGF-ir/ TCR α/β -ir T-cells was measured by threshold detection (Metamorph, Molecular Devices, Downingtown, PA) and the average area for the 4 fields expressed as $\mu\text{m}^2/\text{mm}^2$. The contribution of myofibroblasts to NGF expression within the infarct was assessed by superimposing a stereological grid (30 μm line interval, AnalySis, Soft Imaging System, Lakewood, CO) over captured images and counting intersections overlying regions of NGF/ α -SMA-ir colocalization (i.e. yellow in merged images), dividing by the total number of sample area intersections, and normalizing to 1mm².

The effect of clodronate liposomes on the infarct content of mRNA encoding the inflammatory cytokines interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α) and macrophage chemotactic protein-1 (MCP-1) was evaluated in the samples obtained from the same specimens used for NGF

western blot analysis. Dissected regions were homogenized in ice cold trizol and total RNA (1µg) reverse transcribed using the iScript kit (Biorad, Hercules, CA). Amplification of cDNA was conducted using specific primers for IFN- γ , TNF- α , MCP-1 and GAPDH. Conditions for the PCR were 94°C for 40 s, 60°C, 57°C, 53.7 °C and 60°C for 30s, and 72°C for 1 min for 30, 32, 31 and 28 cycles respectively for IFN- γ , TNF- α , MCP-1 and GAPDH, with a final extension at 72°C for 5min. The primers were 5'-ATGGATGCTATGGAAGGAAAG-3' (sense) and 5'-TATTGGCACACTCTCTACCC-5' (antisense) for IFN- γ ; 5'-CTTATCTACTCCCAGGTTCTC-3' (sense) and 5'-ACTTCAGCATCTCGTGTG-3' (antisense) for TNF- α ; 5'-ACTCATTCACTGGCAAGATG-3' (sense) and 5'-GGTCAAGTTCACATTCAAAGG-3' (antisense) for MCP-1; 5'-CTCTACCCACGGCAAGTTC-3' (sense) and 5'-CTCAGCACCCAGCATCACC-3' (antisense) for GAPDH. Five microliters of PCR product was analyzed by electrophoresis (1.5% agarose gel with ethidium bromide). Bands were visualized under UV transillumination using a Chemi Doc XRS and quantified as above.

Statistics

Data are expressed as mean \pm SEM. Data were analyzed using one way ANOVA followed by a Student-Newman-Keuls test or Student T-test (SigmaStat 3.11, Systat Software, Inc., San Jose, CA). Statistical significance was accepted at $P \leq 0.05$. No statistically significant differences were evident in any of the measurements obtained from groups receiving CAL followed by saline or by saline-containing liposomes, and these groups were therefore pooled into a single 'saline' group for subsequent analyses.

RESULTS

Infarct characterization

No post-operative deaths occurred in the sham-ligated subjects (N=4 in each sham group), while mortality was 21% (3/14) in the group receiving CAL plus saline and 27% (3/11) in the group receiving CAL plus clodronate liposomes. Characterization of the infarct by TTC and light microscopic analysis showed that left coronary artery ligation consistently produced an extensive area of necrosis that was confined to the left ventricle with minimal septal or right ventricular involvement (Fig. 1). Following exclusion of hearts that did not meet the transmural and size criteria, infarcts occupied $38.7 \pm 4.5\%$ of the left ventricle in the saline CAL group and $42.3 \pm 10.1\%$ of the left ventricle in the clodronate liposome CAL group.

Innervation and inflammatory cell composition of uninjured hearts

Ventricular tissue from rats receiving a sham ligation displayed morphological features similar to those we have reported previously for control animals (Hasan, et al., 2006). DBH-ir nerves were sparse (Fig. 2A) and present mainly in association with ventricular cardiomyocytes and vasculature. Quantitative analysis showed that DBH-ir sympathetic innervation occurred with a density of $0.84 \pm 0.07\%$ throughout the 6 ventricular sample regions. In rats with a sham ligation, treatment with clodronate liposomes had no effect on DBH-ir sympathetic innervation density relative to saline controls (Fig. 2A, D).

In sections apical to the untied ligature, essentially no CD68-ir macrophages were observed (Fig, 2E, H). We occasionally encountered α -SMA-ir cells resembling myofibroblasts but their incidence was low (Fig. 2I, L). Similarly, TCR α/β -ir T cells were encountered infrequently (Fig. 2M, P).

Effect of infarction on innervation and inflammatory cell composition

Seven days following CAL, DBH-ir sympathetic axon density at the border region of the infarct was increased 2-fold relative to that of control ventricular tissue (Fig. 2B, D). Consistent with previous findings (Hasan, et al., 2006), this was associated with a dramatic increase in macrophage density to about 1,200 cells/mm² (Fig. 2.F, H). Area occupied by α -SMA-ir myofibroblasts area was increased approximately 40-fold to about 130,000 $\mu\text{m}^2/\text{mm}^2$ (Fig. 2J, L).

T-cells also increased from negligible numbers in controls to about 39 cells/mm² (Fig. 2N, P).

Effect of clodronate liposomes on innervation and inflammatory cell composition following CAL

In rats receiving CAL followed by intravenous injections of clodronate liposomes, DBH-ir sympathetic innervation density was reduced by 66% relative to untreated CAL ventricles, and was comparable to that of unligated ventricles (Fig. 2C, D). To confirm that this reduction was not simply due to DBH depletion within structurally intact sympathetic axons, we immunostained adjacent sections for the intermediate filament protein peripherin, which labels all intact unmyelinated axons, and TH as an alternative noradrenergic marker. Staining patterns for DBH-ir axons in saline-injected rats with coronary artery ligation were comparable to those of TH and peripherin (Fig. 3A, B, C). Similarly, the reduction in numbers of DBH-ir fibers in rats with coronary artery ligation and clodronate liposome treatment was comparable to that observed for TH and peripherin (Fig. 3D, E, F).

Clodronate liposome treatment also reduced numbers of macrophages by 69% (Fig. 2G, H) and macrophage size by 21% ($41.5 \pm 2.2 \mu\text{m}^2$ with saline treatment and $32.7 \pm 2.3 \mu\text{m}^2$ with clodronate treatment, $p=0.015$), although they remained significantly more abundant than in control ventricles.

Consistent with previous findings, macrophage depletion was associated with

reduced IFN- γ gene expression (Fig. 4A) in the infarct (Salkowski, et al., 1995, Torres, et al., 1999), while TNF- α and MCP-1 were unchanged (McClellan, et al., 2003, Summan, et al., 2006) (Fig. 4B and C).

The wound area occupied by myofibroblasts was also significantly reduced by 22% relative to untreated CAL tissue but remained elevated relative to unligated ventricles (Fig. 2K, L). T-cell numbers were not affected by clodronate liposome treatment (Fig. 2O, P).

Effect of clodronate liposomes on infarct NGF expression

Previous studies have implicated NGF in post-infarct sympathetic sprouting (Zhou, et al., 2004, Chen, et al., 2007) and we have shown in culture that NGF neutralization eliminates sprouting induced by the infarcted myocardium (Hasan, et al., 2006). Accordingly, we assessed the extent to which clodronate liposome treatment altered infarct NGF.

In the infarct border region of saline treated rats, mature NGF protein was 3.34×10^{-3} INT*mm². After treatment with clodronate liposomes, NGF was reduced by 67% to 1.10×10^{-3} INT*mm² (Figure 5).

To determine the contribution of the predominant NGF-expressing cell types in the vicinity of the infarct (Hasan, et al., 2000, Moalem, et al., 2000, Caroleo, et al., 2001, Hasan, et al., 2006), we analyzed the NGF expression of macrophages, myofibroblasts and T-cells. In ventricles with sham ligations, no macrophages were observed below the untied ligature and therefore did

not contribute to NGF expression in the control ventricle (Fig. 6A, B, C). Following CAL, all CD68-ir macrophages expressed NGF-ir (Fig. 6D, E, F, G). After clodronate liposome treatment, CD68-ir macrophages that remained continued to express NGF-ir at intensities comparable to untreated CAL (Fig. 6D, I, J, K). To assess how macrophage depletion affected NGF tissue content, we computed section area occupied by NGF-ir macrophages. While macrophages did not contribute to NGF-ir in control tissues, section area occupied by NGF-ir macrophages was $\sim 50,000 \mu\text{m}^2/\text{mm}^2$ in the saline CAL group, and this was reduced to $\sim 13,000 \mu\text{m}^2/\text{mm}^2$ by clodronate liposome treatment (Fig. 6H).

In ventricles of rats with sham ligations, NGF-ir was observed in only 17 ± 3 % of myofibroblast α -SMA-ir cytoplasmic area (Fig. 7A, B, C, D) and this was unchanged by clodronate liposome injections (Fig. 7D). Section area occupied by NGF-ir and α SMA-ir colocalization was about $1,300 \mu\text{m}^2/\text{mm}^2$. Because of the limited numbers of myofibroblasts, the contribution of myofibroblasts to NGF expression in sham-ligated ventricles was negligible (Fig. 7H). Following CAL, the percentage of α -SMA-ir cytoplasm occupied by NGF-ir increased to 37 ± 2 % (Fig. 7D ,E , F, G), and total NGF-ir+ α SMA-ir area increased 97-fold (Fig. 7H). Clodronate liposome-treatment did not alter the percentage of α -SMA-ir cytoplasm with NGF-ir (Fig. 7D, I, J, K), but the total area occupied by these colocalized markers decreased in concert with the reduction in myofibroblast content (Fig. 7 H).

In ventricles with sham ligations, 8 ± 8 % of TCR α/β -ir T-cells showed NGF-ir (Fig. 8A, B, C, D), and these occupied $32 \pm 19 \mu\text{m}^2/\text{mm}^2$ (Fig. 8H). In ventricles of sham-ligated rats receiving clodronate liposomes, the percentage of T-cells with NGF-ir was greater than in saline-injected controls (Fig. 8D) and area occupied was $517 \pm 207 \mu\text{m}^2/\text{mm}^2$ (Fig. 8H). CAL increased the percentage of T-cells expressing NGF and their area relative to saline injected sham controls (Fig. 8D, E, F, G, H). In addition, in CAL rats receiving clodronate liposomes, the number of TCR α/β -ir T-cells expressing NGF-ir was increased, as was the area occupied by NGF-ir within these cells (Fig. 8H, I, J, K).

DISCUSSION

Prolonged myocardial ischemia leads to extensive cardiomyocyte necrosis, which results in both a reduction in cardiac performance and a thinning of the myocardial wall. Accordingly, it is important that cellular debris be removed and that tissue repair be initiated quickly in order to maintain cardiovascular homeostasis. In association with this damage, locally produced chemokines and cytokines initiate an inflammatory response that attract and activate circulating macrophages. These cells perform an important role in removing debris left by the necrotic cells (Frangogiannis, 2006). Additionally, they are essential for tissue repair (Fischer, et al., 2007, Westermann, et al., 2007, Frantz, et al., 2008, Miura, et al., 2008). Growth

factors released by these cells promote proliferation of endothelial cells, fibroblasts and myofibroblasts, which are important in revascularization, forming extracellular matrices, and providing tensile strength to the scar. Proinflammatory cytokines are present in experimental models of myocardial infarction (Frangogiannis, et al., 1998a, Frangogiannis, et al., 1998b, Dewald, et al., 2004) and are produced by many cells types (Gessani, et al., 1998, Deng, et al., 2000, Frucht, et al., 2001, Akasaka, et al., 2006, Spiekstra, et al., 2007). These cytokines, activate macrophages (Green, et al., 1990) and promote T-cell activation and proliferation (Mizutani, et al., 1989). NGF also plays important roles in differentiation and proliferation of inflammatory cells (Mizutani, et al., 1989, Barouch, et al., 2001, Micera, et al., 2003). In addition to its role in inflammatory cell differentiation, NGF is known to promote aggressive sprouting of sympathetic nerves. By activating the trkA receptor present on sympathetic axons, NGF promotes regeneration and outgrowth. Hence, NGF is important in both the inflammatory response and in the remodeling of innervation following infarction.

Previous studies have shown that, within the cardiac ischemic wound, multiple cell types can synthesize NGF. Both NGF mRNA and protein have been detected in post-infarction macrophages and myofibroblasts, in T-cells and in surviving cardiac ventricular myocytes as well as vascular smooth muscle (Zhou, et al., 2004, Hasan, et al., 2006, El-Helou, et al., 2007). Therefore, multiple cell types may contribute to local NGF production. The

extent to which these various cell types contribute to sympathetic axon remodeling after infarction is not clear. In the present study, we focused on the role of macrophages. Macrophages have been implicated in neuronal sprouting after wounds in the central nervous system (Batchelor, et al., 2002). In addition to synthesizing NGF, macrophages release a variety of other factors that may be important in orchestrating recruitment, proliferation and differentiation of other wound cell types.

To selectively deplete macrophages, we used liposomes containing clodronate (Van Rooijen, et al., 1994). Clodronate liposomes do not cross blood vessel walls but are phagocytosed by circulating monocytes where the accumulated clodronate leads to irreversible toxicity and apoptosis (van Rooijen, et al., 1996). Sustained treatment is reported to effectively reduce tissue macrophage populations (Popovich, et al., 1999, Galeazzi, et al., 2000), and our regimen reduced CD68-ir macrophages by 69% in infarcted hearts, comparable to that reported previously (van Amerongen, et al., 2007). In addition, we observed that the size of surviving macrophages was modestly reduced. Consistent with this reduction, mRNA encoding IFN- γ , which is expressed mainly by macrophages, T-cells and NK cells (Gessani, et al., 1998, Munder, et al., 1998, Frucht, et al., 2001) was reduced by 31%, and this reduction is consistent with findings by others (Salkowski, et al., 1995, Torres, et al., 1999). However, some other cytokines did not show significant changes. Neither TNF- α , a cytokine secreted by multiple cell types including

macrophages, neutrophils, mast cells and cardiomyocytes (Frangogiannis, et al., 1998a, Irwin, et al., 1999) nor MCP-1, which is expressed by macrophages, endothelial cells, fibroblasts, neutrophils and cardiomyocytes (Onai, et al., 2004), were significantly decreased by clodronate liposomes. While it is unclear how cytokine content is maintained in the face of macrophage depletion, our findings are consistent with others where these proteins were found to be unchanged (McClellan, et al., 2003, Summan, et al., 2006). These observations suggest that the ability of clodronate liposomes to prevent sympathetic hyperinnervation is probably not due to a generalized suppression of inflammatory proteins within the infarcted tissue.

While clodronate liposome treatment depleted wound macrophages, effects on other cells were limited. Clodronate liposome treatment did not affect T-cell numbers, although it did cause a modest reduction in wound area occupied by α SMA-ir myofibroblasts. This effect appears to be confined to the wound myofibroblasts, as treatment did not alter the small numbers of myofibroblasts present in non-infarcted hearts. Clodronate liposomes are known to be highly selective for phagocytic macrophages (Van Rooijen, et al., 1994), so it seems unlikely that this is a direct effect on myofibroblasts, which are not known to be phagocytic. Rather, it is likely to have occurred secondary to the depletion in macrophages. Myofibroblasts transdifferentiate from several cell types including fibroblasts and pericytes (Schmitt-Graff, et al., 1994, Gabbiani, 1996, Hasan, et al., 2000). Development of the

myofibroblast phenotype requires the presence of various secreted proteins including TGF- β (Desmouliere, et al., 1993, Dubinett, et al., 1993) which can be released by macrophages (Assoian, et al., 1987). In addition, macrophages can express α SMA (Hasan, et al., 2000) and may themselves be precursors to myofibroblasts. Depletion of macrophages by clodronate liposomes may thus account for some of the reduction of α SMA-ir myofibroblasts as well. Accordingly, clodronate-induced depletion appears to have a small but significant effect in reducing myofibroblast contributions to the ischemic wound as a result of an altered wound milieu, reduced precursor cell numbers, or both.

Clodronate treatment produced a marked reduction in the numbers of sympathetic nerves in the vicinity of the infarct. Sympathetic hyperinnervation is well documented in the infarct border zone (Vracko, et al., 1991, Hasan, et al., 2006) . While sympathetic nerves may play a role in tissue remodeling after injury (Kim, et al., 1998, Smith, et al., 2002, Souza, et al., 2005), a link between sympathetic hyperinnervation and post-infarct arrhythmias is believed to underlie sudden cardiac death (Cao, et al., 2000b). While the present study provides strong evidence that sympathetic hyperinnervation requires increased numbers of macrophages, additional studies with larger numbers of rats and a more protracted time course will required to assess any impact of clodronate liposome treatment on post-infarct survival. Similarly, the extent to which other factors reported to modulate infarct sympathetic

hyperinnervation, which include estrogen (Lee, et al., 2007b), statin treatment (Lee, et al., 2007a), and glucocorticoids (El-Helou, et al., 2007), affect macrophage dynamics and NGF expression remain to be elucidated.

The present findings show that, in the absence of normal numbers of macrophages, sympathetic hyperinnervation fails to develop at the site of the ischemic injury. This is not likely to be due to an effect of clodronate liposomes on sympathetic innervation as sympathetic nerve density was unaffected in sham-ligation subjects. Because clodronate liposomes directly affect macrophage numbers, this represents a probable cause for the reduced numbers of sympathetic nerves. Moreover, macrophages are robust sources of NGF, and this neurotrophin is required for post-infarct sympathetic sprouting (Hasan, et al., 2006). However, macrophages secrete other factors that may modulate phenotype of other cell types within the wound, including their NGF expression. For example, treatments with clodronate liposomes were found to reduce cytokines produced by macrophages such as IFN- γ (Salkowski, et al., 1995, Torres, et al., 1999), which may indirectly reduce sympathetic outgrowth by modulating NGF but is not known to directly elicit sympathetic sprouting. Accordingly, we assessed changes in NGF expression.

Clodronate liposome treatment decreased the expression of NGF by 67% in the border region compared to saline liposome treatment suggesting that the decrease of this neurotrophin could account for the suppression of

sympathetic hyperinnervation following clodronate liposome treatment. To determine the contribution of the different inflammatory cell types to this decrease, we analyzed the cellular distribution of NGF.

In sham operated hearts, NGF-ir was present at low levels in cardiomyocytes and in a few CD68-ir myofibroblasts and TCR α/β -ir T-cells. Following infarction, there was a marked increase in NGF-ir in the infarct border region, and most was colocalized with CD68-ir macrophages and α SMA-ir myofibroblasts; TCR α/β -ir T-cells showed intense NGF immunofluorescence but were relatively few in number so that their contribution to overall NGF expression was quite small (~1% of the tissue area showing NGF-ir).

Clodronate liposome treatment elicited a marked reduction in overall NGF immunostaining in the infarcted tissue. Coincident with the reductions in macrophage numbers (and to a lesser extent size), the total area occupied by colocalized CD68 and NGF immunofluorescence was reduced some 76% relative to saline treatment. Due to the reduction of α SMA-ir myofibroblast area, NGF expression by this cell population was also reduced after clodronate liposome treatment. Although α SMA-ir myofibroblast area showed a modest reduction after clodronate liposome treatment, the reduction in NGF content appeared to be more marked, suggesting that the reduction in macrophages may have also suppressed NGF expression in the myofibroblasts. Interestingly, while NGF immunoreactivity presented by both

macrophages and myofibroblasts was reduced, expression by T-cells was increased, now accounting for about 10% of NGF-ir cellular area. Overall however, macrophage depletion by clodronate liposomes substantially reduced NGF in the region of the wound.

While some regions of the post-infarct heart show hyperinnervation, other ventricular regions have a pronounced loss of sympathetic nerves (Li, et al., 2004), suggesting that the wound environment contains both attractive and repulsive factors for sympathetic axons which could account for the inhomogeneity of sympathetic innervation throughout the infarct. It is known that cardiac tissue expresses Sema3A, which acts to suppress cardiac innervation (Ieda, et al., 2007). It may therefore be the case that the abundance of NGF in the infarct region induces sympathetic sprouting by overcoming repulsive effects of semaphorins or other unidentified proteins.

ACKNOWLEDGMENTS

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FIGURES

Figure 1.

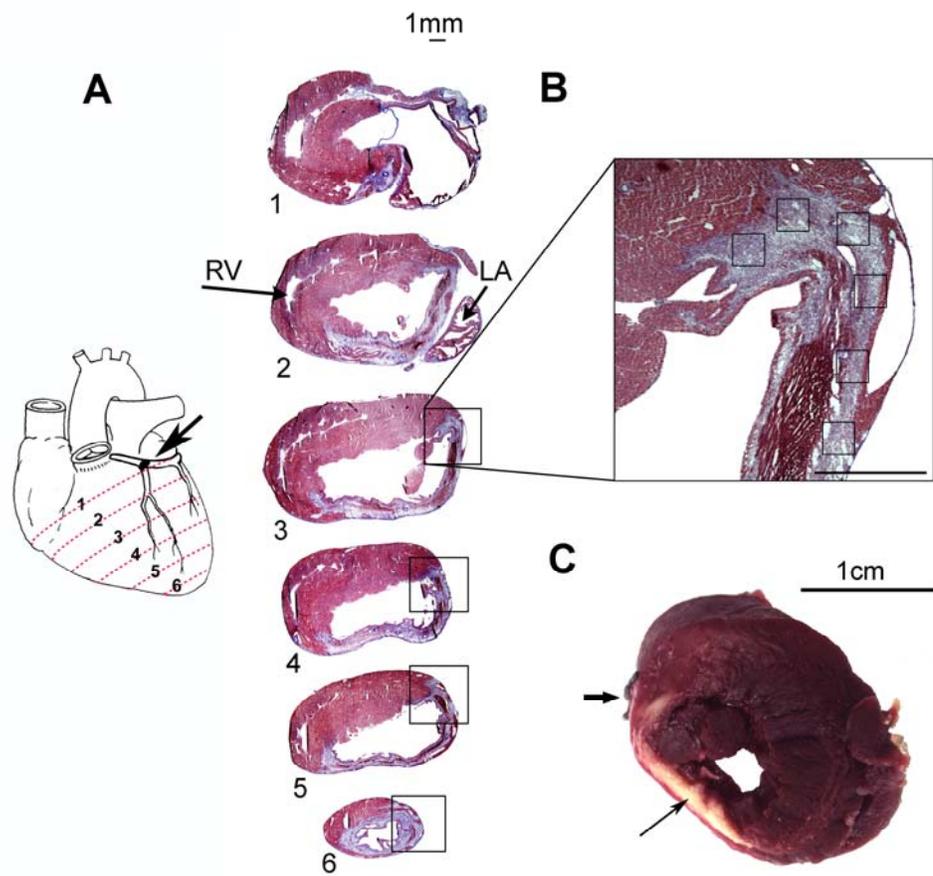


Figure 1. Infarct assessment. A: Six sections taken at ~1.7 mm intervals through the entire ventricle stained with Masson's trichrome. Collagen is lightly stained and cardiomyocytes are darker. Arrow in the schematic diagram shows where the left coronary artery was ligated, and dotted lines show the levels from which each of the representative sections was taken. RV: right ventricle, LV: left ventricle. LA: left atrium. Boxes show the regions sampled for the analysis sympathetic innervation and inflammatory cells. B: Higher magnification of area shown in section 3. Boxes in B show fields within the left lateral posterior aspect of the infarct sampled for quantification. Scale = 1mm. C: Approximately 2mm section through a CAL heart stained with TTC. Large arrow shows the suture. Thin arrow shows the infarct, which fails to stain with TTC.

Figure 2.

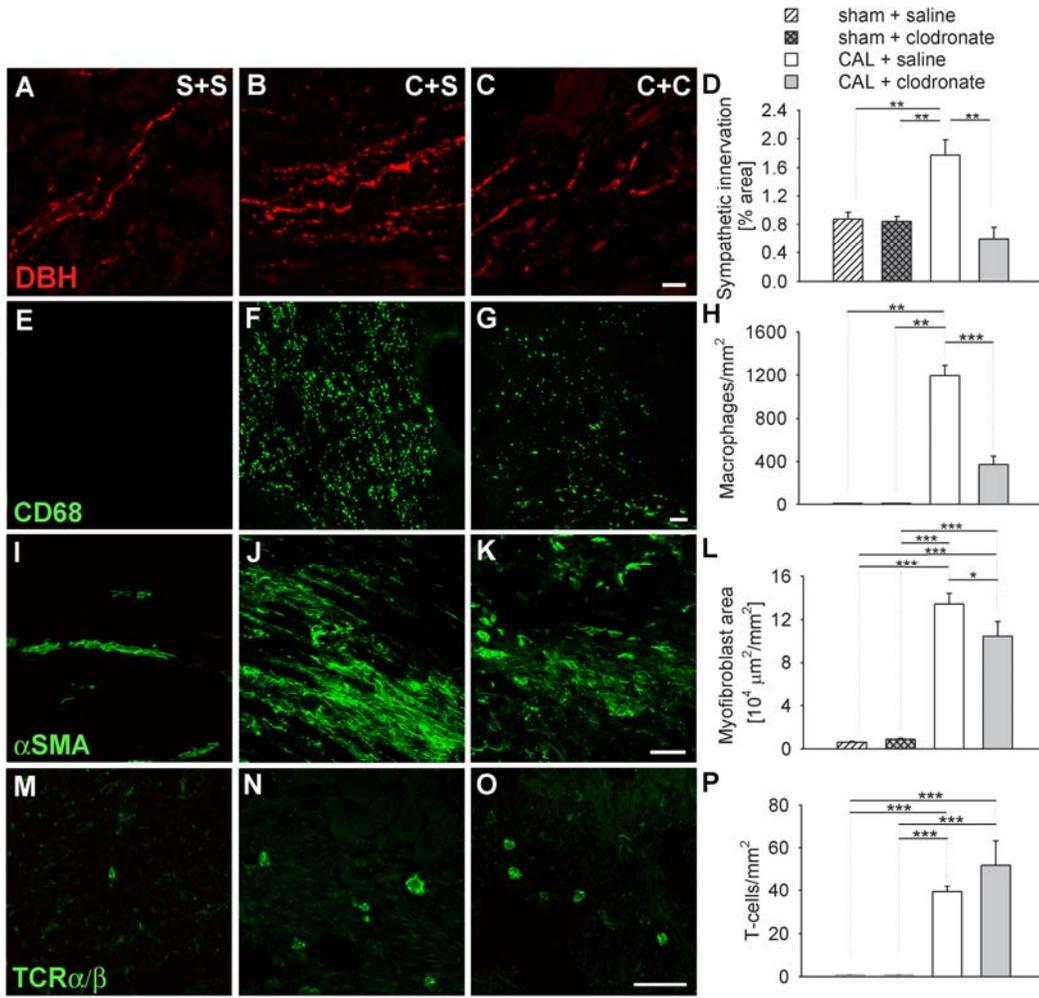


Figure 2. Innervation and inflammatory cell composition of uninjured and injured hearts. Immunostaining of ventricular tissue sections for dopamine β -hydroxylase (DBH) as a sympathetic nerve marker (A,B,C), CD68 as a macrophage marker (E,F,G), α -Smooth muscle actin (α SMA) as a myofibroblast marker, and TCR α/β as a T cell marker (M,N,O). Sections were obtained from rats receiving sham ligation and saline injections (S+S; A,E,I,M), coronary artery ligation and saline injections (C+S; B,F,J,N), or coronary artery ligations plus clodronate liposomes (C+C;C,G,K,O). Scale bar = 10 μ m in C and 50 μ m in G, K, and O. D. Quantitative analysis of sympathetic innervation density as determined by the percentage of section sample area occupied by DBH-ir nerves. H. Quantitative analysis of tissue macrophages expressed as the number of CD68-ir cells per mm^2 . L. Quantitative analysis of tissue myofibroblasts as determined by the section sample area occupied by α -SMA-ir. P. Quantitation of tissue T-cells as determined by the number of TcR α/β cells per mm^2 . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3.

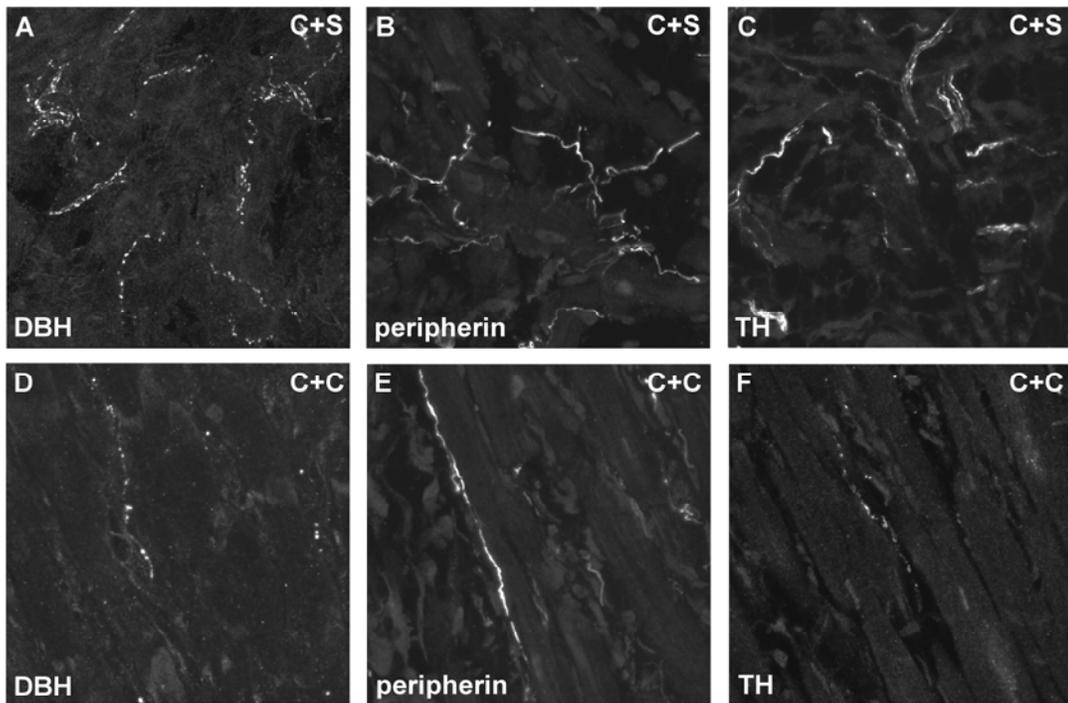


Figure 3. Changes in sympathetic innervation after clodronate liposome treatment are reflected by multiple markers. Adjacent sections from hearts receiving coronary artery ligation plus saline (C+S) or ligation plus clodronate liposomes (C+C) were immunostained for DBH which characterizes noradrenergic neurons (A,D), the intermediate filament protein peripheral which stains intact unmyelinated axons (B, E), and tyrosine hydroxylase which is a marker for catecholaminergic axons (C, F). Levels of innervation density were comparable under different treatments with all markers. Scale bar = 10 μ m.

Figure 4.

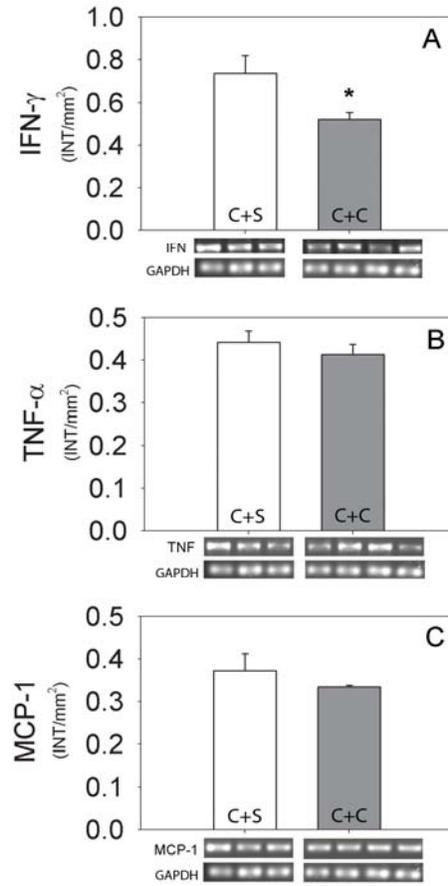


Figure 4. Effect of clodronate liposomes on IFN- γ , TNF- α and MCP-1 mRNA in the infarction border region. Infarct tissue was assessed by RT-PCR for Interferon- γ (IFN), tumor necrosis factor (TNF- α) and macrophage chemotactic protein-1 in infarct tissue 7 days after CAL in rats receiving saline-containing liposomes (C+S) or clodronate-containing liposomes (C+C). RT-PCR product was analyzed densitometrically (Intensity/mm²) and normalized to GAPDH. * $P < 0.05$.

Figure 5.

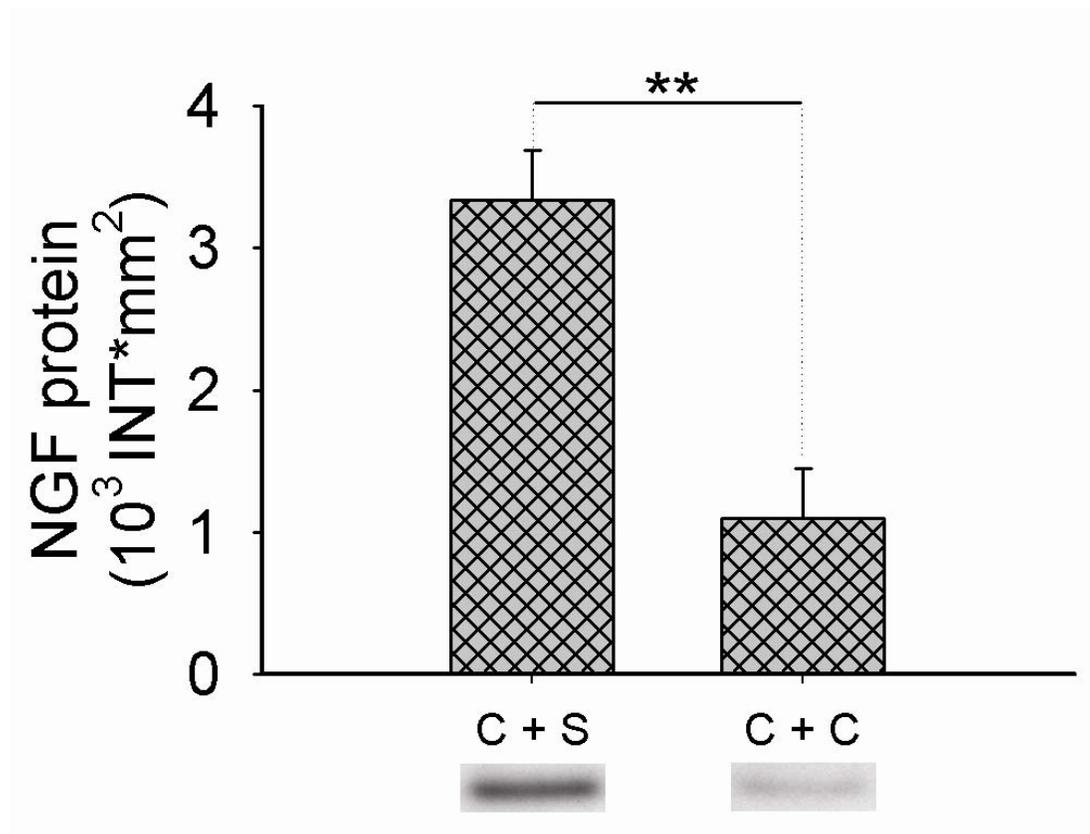


Figure 5. Effect of clodronate liposomes on NGF in the infarction border region. Tissue was assessed by western blot for NGF 7 days after CAL in rats receiving saline-containing liposomes (C+S) or clodronate-containing liposomes (C+C). Product was analyzed densitometrically and normalized to total protein. ** $P < 0.01$.

Figure 6.

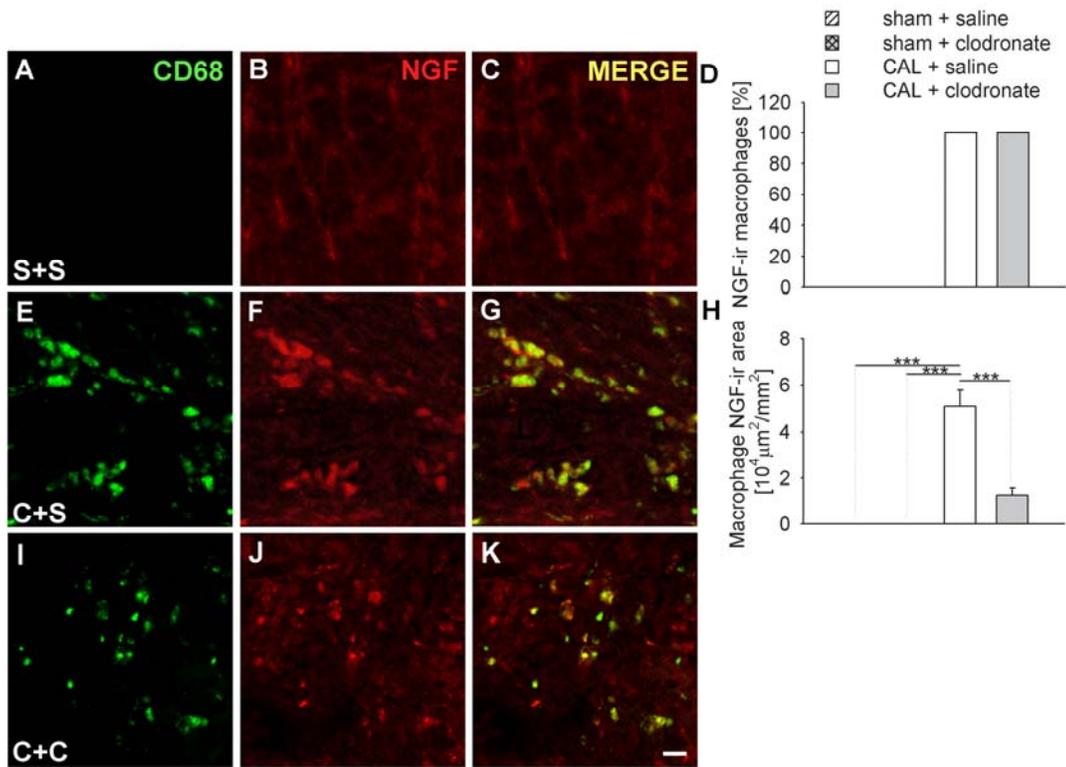


Figure 6. Effect of clodronate liposomes on macrophage NGF expression. Immunostaining for CD68 as a macrophage marker (A, E,I), and costained for NGF (B,F,J), merged images (C,G,K). Sections were obtained from rats receiving sham ligation and saline injections (S+S,A,B,C), coronary artery ligation and saline injection (C+S,E,F,G), or coronary artery ligation plus clodronate liposomes (C+C,I,J,K). Scale bar = 10 μ m. Quantitative analysis of the percentage (D) and the area (H) of macrophages expressing NGF-ir as determined by the area of CD68-ir coexpressing NGF-ir. *** P <0.001.

Figure 7.

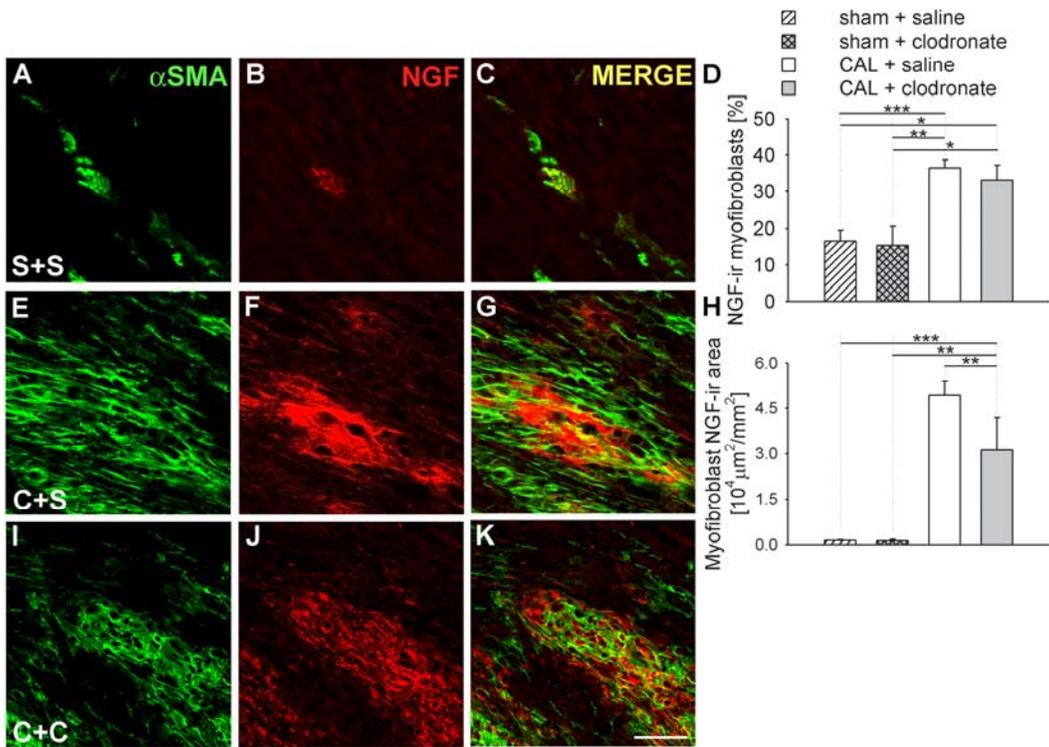


Figure 7. Effect of clodronate liposomes on NGF expression by myofibroblasts. Immunostaining for α -SMA as a myofibroblast marker (A,E,I), and costained for NGF (B,F,J), and merged images (C,G,K). Sections were obtained from rats receiving sham ligation and saline injections (S+S,A,B,C), coronary artery ligation and saline injection (C+S,E,F,G), or coronary artery ligation plus clodronate liposomes (C+C,I,J,K). Scale bar = 50 μ m. Quantitative analysis of the percentage (D) and the area (H) of myofibroblasts expressing NGF-ir as determined by the area of α -SMA-ir coexpressing NGF-ir. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 8.

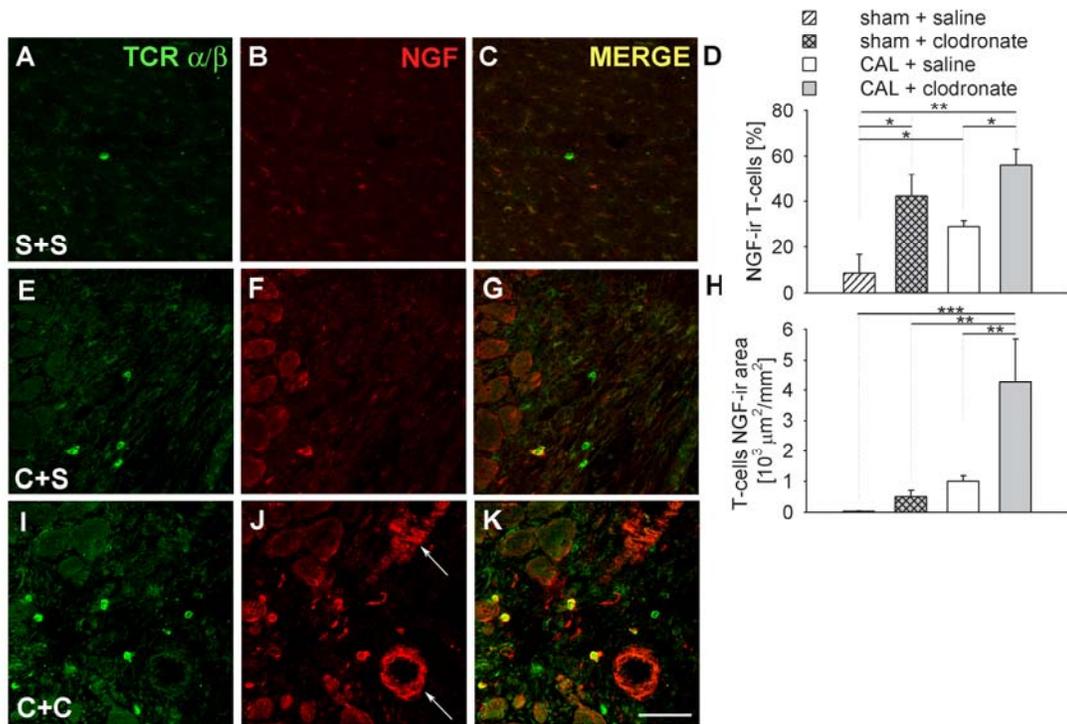


Figure 8. Effect of clodronate liposomes on NGF expression by T-cells. Immunostaining for TCR α/β as a T-cell marker (A,E,I), and costained for NGF (B,F,J), merged images (C,G,K). Sections were obtained from rats receiving sham ligation and saline injections (S+S,A,B,C), coronary artery ligation and saline injection (C+S,E,F,G), or coronary artery ligation plus clodronate liposomes (C+C,I,J,K). Arrows shows blood vessels expressing NGF. Scale bar = 50 μm . Quantitative analysis of the percentage (D) and the area (H) of T-cells expressing NGF-ir as determined by the area of TCR α/β -ir coexpressing NGF-ir. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

**CHAPTER 4. CHRONIC β_1 ADRENOCEPTOR BLOCKADE INCREASES
CARDIAC VENTRICULAR SYMPATHETIC INNERVATION DENSITY IN
RATS.**

ABSTRACT

Rationale: β -adrenoceptor antagonists are useful in treating cardiovascular disorders, but discontinuation is associated with sympathetic hyperactivity that can lead to severe complications.

Objective: We evaluated whether β -blockers alters sympathetic axon outgrowth, ventricular sympathetic innervation density, and ventricular function.

Methods and Results: Sprague-Dawley rats with or without coronary artery ligation received adrenergic receptor blockers or saline infusions for a week. Infarct sympathetic hyperinnervation was unaffected by the non-selective β -blocker propranolol, but this treatment increased innervation of myocardium with intact perfusion in ligated and control rats. The β_1 adrenoceptor antagonist metoprolol also increased sympathetic axon density but the α -blocker phentolamine did not. Two days following propranolol withdrawal, left ventricular responsivity to direct β receptor activation was similar to untreated rats, but end systolic pressure and Max dP/dt in response to norepinephrine mobilization by tyramine were greater, consistent with enhanced sympathetic neuroeffector transmission. To assess mechanisms of hyperinnervation, we cultured neonatal sympathetic neurons with propranolol or metoprolol; both increased axonal outgrowth. RT-PCR revealed β_1 -adrenoceptor mRNA in

sympathetic cultures, implying that β -blockers could act directly on neuronal receptors. To determine if sprouting is due to β -blockade of growth-inhibiting signals by intrinsically synthesized norepinephrine, we prevented synthesis with α -methyl p-tyrosine. This enhanced outgrowth, while concurrent dobutamine administration reduced sprouting, confirming norepinephrine-mediated inhibition of outgrowth via β_1 adrenoceptors.

Conclusions: Sympathetic axon outgrowth, and therefore target innervation density, is negatively regulated by β_1 autoreceptors. Sympathetic hyperinnervation occurring as a result of chronic β -adrenoceptor blockade is likely to contribute to sympathetic hyper-responsiveness following treatment cessation.

INTRODUCTION

β -adrenergic receptor (β AR) antagonists are used to manage a variety of disorders including hypertension, angina, arrhythmias, myocardial infarction and heart failure (Yusuf, et al., 1985, Viskin, et al., 1995). These agents block norepinephrine (NE) and epinephrine interactions with β -adrenergic G protein-coupled receptors, thus preventing G_s protein activation, formation of the second messenger cAMP, and activation of protein kinase A (Lefkowitz, 2004). Beta-blockers decrease cardiac inotropy, chronotropy and dromotropy, thus reducing blood pressure, conduction system excitability, and

myocardial oxygen demand. Most ventricular AR effects are attributed to β_1 receptors on cardiomyocytes, which induce calcium influx via the L-type calcium channels and enhance contractile force (Wang, et al., 2004).

Although β -blockers are widely used clinically, discontinuation is associated with serious adverse effects including chest pain, tachycardia and other arrhythmias, palpitations, and occasionally myocardial infarction (Nattel, et al., 1979). Moreover, withdrawal can contribute to increased mortality; β -blocker discontinuation following vascular surgery increases mortality 6-fold relative to patients remaining on β -blockers, and 3-fold relative to those not receiving drugs (Hoeks, et al., 2007).

Adverse effects typically are attributed to exaggerated sympatho-adrenal responsiveness, generally ascribed to increased β -receptor density and sensitivity resulting from long-term receptor blockade (Heilbrunn, et al., 1989). While receptor supersensitivity can certainly contribute to increased sympatho-adrenal tone, it is not clear whether other factors also participate in hyper-responsiveness after β AR blockade. For example, propranolol treatment increases plasma catecholamine levels (Rahn, et al., 1978), which may reflect reflex compensation but could also suggest changes in sympathetic neuroeffector gain. There is precedence for suggesting that transmitter blockade can influence neuronal architecture; in developing central monoaminergic neurons, serotonin administration reduces outgrowth, presumably as a result of autoreceptor activation (Haydon, et al., 1984,

Whitaker-Azmitia, et al., 1986). If comparable processes occur in adult sympathetic neurons, then cardiac innervation density may be increased by AR blockade. Since sympathetic hyperinnervation is believed to underlie enhanced end organ responsiveness in animal models (Kondo, et al., 1996), increased cardiac innervation may therefore contribute to cardiac dysfunction after β -blocker withdrawal. In the present study, we examined effects of β AR blockade on cardiac sympathetic innervation of adult rats and neurite outgrowth in sympathetic neuronal cultures.

MATERIAL AND METHODS

In vivo experimental preparations

All experimental protocols conformed to NIH guidelines and were approved by the University of Kansas Medical Center Animal Care and Use Committee. Sprague Dawley rats aged 60 days received coronary artery ligation (CAL) (Wernli, et al., 2009) or were unoperated. Concurrently, minipumps were implanted containing saline or propranolol (release rate of 13.2 mg/kg/day) to produce β receptor blockade (Conlon, et al., 1991) or phentolamine (release rate of 1 mg/kg/day) to produce α blockade (Steinle, et al., 2002). Unoperated rats also received metoprolol (36 mg/kg/day) which blocks β_1 but not β_2 receptors (Ablad, et al., 1975, DiBona, et al., 1999). Effectiveness of blockade was confirmed in subsets of rats at 7 days following minipump implantation. Phentolamine prevented the normal increase in pupil diameter following

topical application of 1% NE in saline to the eye. Propranolol and metoprolol blocked tachycardic responses to iv administration of isoproterenol by 79 and 45%, respectively. Metoprolol's selectivity for β 1AR was confirmed by showing that the β 2AR-mediated increase in gracilis muscle blood flow in response to isoproterenol was not affected.

Cardiac sympathetic neuroeffector function after β -blocker discontinuation

Cardiac ventricular neuroeffector function was assessed 2 days following discontinuation of chronic β -AR blockade in 4 saline-infused and 4 propranolol-treated rats. A calibrated catheter (SPR 838, Millar Instruments) for measuring pressure-volume loops was introduced in the left ventricle via the right carotid artery (Pacher, et al., 2008). After administering the ganglionic blocker chlorisondamine, tyramine was administered iv in graded doses until maximum left ventricular (LV) functions were achieved. After parameters returned to baseline, graded doses of isoproterenol were administered until maximum values were achieved. Heart rate, end systolic pressure, end diastolic pressure, and maximal and minimal first derivative of pressure (Max dP/dt and Min dP/dt) were measured. Isoproterenol ED₅₀ for heart rate and Max dP/dt were calculated as indices of β receptor sensitivity.

Analysis of cardiac sympathetic innervation density and infarct volume

Following adrenergic receptor blockade for 7 days, hearts were excised, snap-frozen and cryosectioned at 10 μm thickness. Six stepped serial sections separated by $\sim 2\text{mm}$ were fixed in 4°C methanol and immunostained for dopamine- β -hydroxylase (DBH) as a marker for noradrenergic sympathetic axons. In sections from non-ligated hearts, 6 fields evenly distributed in the left lateral posterior area of the heart were captured. In CAL hearts, images of 6 fields evenly distributed in the left lateral posterior area (infarct border region) and images of 6 fields evenly distributed in the non-ischemic posterior area of the heart were taken (Figure 1A) (Wernli, et al., 2009). Nerve density was calculated as reported previously (Wernli, et al., 2009). Infarct volume was calculated by measuring infarct area stained with Masson's Trichrome (Wernli, et al., 2009) in each section and multiplying this by the distance between sections.

Cardiomyocyte culture and protein synthesis

To investigate whether ARs modulate cardiac nerve growth factor (NGF) synthesis, hearts were harvested from pups (postnatal day 0-2), disassociated, and cardiomyocytes plated in DMEM/F12. After 3 days, culture medium was replaced with medium containing the non-selective α -agonist phenylephrine (0.1-10 $\mu\text{mol/L}$) or the non-selective β -agonist isoproterenol (0.1-10 $\mu\text{mol/L}$). Media was collected after 24 hours, proteins precipitated and

resolved using SDS-PAGE, transferred to polyvinylidene difluoride membranes and probed in triplicate with an antibody to NGF. Cultured cardiomyocytes were fixed in buffered formalin and stained for actin cytoskeleton (Alexa Fluor594-Phalloidin), NGF or the pro-protein convertase, furin.

Sympathetic Neuronal Cultures

To assess effects of β -AR blockade on sympathetic axon outgrowth, cultures were conducted on superior cervical ganglia (SCG) neurons obtained from newborn rats. Neurons were dissociated, suspended in neurobasal medium, and plated ($\sim 4 \times 10^4$ per well) on glass coverslips in control medium or medium containing propranolol (10^{-10} mol/L to 10^{-8} mol/L) or metoprolol (10^{-10} mol/L to 10^{-6} mol/L). Other cultures were conducted with 10^{-8} mol/L dobutamine, with or without 5 μ mol/L α -methyl-p-tyrosine (AMPT). Treatments were conducted in triplicate, and each experiment repeated at least twice.

After 48h, cultures were fixed in formalin and immunostained for peripherin to reveal all axons and soma. Neurite outgrowth was measured in six images distributed equally within each well and expressed as area per neuron (Chakrabarty, et al., 2008).

RT-PCR for β 1 receptor mRNA

β 1AR gene expression was assessed in SCG cultures by RT-PCR. Heart tissue or SCG neurons cultured for 48h were homogenized in ice cold trizol and total RNA (1 μ g) was reverse transcribed. β 1-AR cDNA was amplified using specific primers and PCR product analyzed by electrophoresis.

Statistical Analyses

Data were analyzed using student's T-test or one way ANOVA for multiple comparisons followed by Newman-Keuls test. Data are expressed as mean \pm SEM. Statistical significance was accepted at $P \leq 0.05$.

RESULTS

Effect of AR blockade on sympathetic innervation following CAL

Clinically, β -blockers are administered following myocardial infarction to reduce mortality in the post-infarct period. Because the infarct border region is characterized by sympathetic axon proliferation that is linked to ventricular fibrillation and sudden cardiac death (Cao, et al., 2000b), we examined whether AR blockade alters infarct sympathetic hyperinnervation in rats following CAL. To exclude the possibility that AR blockade influences innervation indirectly by affecting infarct size, we measured infarct volumes. These were comparable in all groups (saline = 118 ± 5 mm³; propranolol = 124 ± 13 mm³; phentolamine = 129 ± 7 mm³).

Infarcted myocardia showed increased innervation density similar to that reported previously using analogous experimental approaches (Hasan, et al., 2006, Wernli, et al., 2009). Quantitative analysis of border region sympathetic innervation in saline-infused rats confirmed that innervation density was substantially increased relative to non-ischemic myocardium remote to the infarct (Figure 1). Relative to the saline-infused CAL rats, neither propranolol nor phentolamine altered innervation density within the infarct border region.

Examination of innervation in the posterior ventricular region remote to the infarct in CAL rats showed that propranolol affected sympathetic axon distribution in non-ischemic myocardium. Relative to saline-infused rats, sympathetic axon density in the remote region was increased by 41% ($P<0.05$), whereas innervation density in hearts receiving the α AR blocker phentolamine was similar to that to those receiving saline (Figure 1B).

Effect of AR blockade on sympathetic innervation density in uninjured hearts

To determine if β AR antagonism alters ventricular innervation density in hearts not subject to ischemic damage, we infused control rats with saline, propranolol, metoprolol or phentolamine for 7 days. In control rats receiving saline, quantitative analysis of DBH-immunoreactive (-ir) sympathetic axons showed that innervation occurred at a density similar to that reported

previously (Wernli, et al., 2009) (Figure 2A). DBH-ir fibers appeared more abundant in rats receiving propranolol for 7 days (Figure 2B) and quantitative analysis revealed a 48% increase in ventricular sympathetic axon density (Figure 2E).

Propranolol may increase innervation density by blocking β_1 or β_2 ARs. To determine if selective blockade of β_1 ARs induces outgrowth, metoprolol was given for 7 days by minipump. DBH-ir fiber density after metoprolol treatment was also increased (Figure 2C), and quantitative analysis showed a 44% increase relative to saline controls (Figure 2E). The density of DBH-ir fibers after phentolamine treatment was similar to that of controls (Figure 2D), showing that α -blockade did not modify cardiac sympathetic innervation.

Ventricular sympathetic neuroeffector function following β -blocker discontinuation

We assessed ventricular function at 48 h after propranolol discontinuation to determine if sympathetic hyperinnervation leads to increased sympathetic neuroeffector properties. We used the β AR agonist isoproterenol to assess whether chronic propranolol infusion for 7 days had a direct effect on cardiac function. Determination of the isoproterenol ED_{50} for heart rate and Max dP/dt showed that these were comparable in saline- and propranolol infused rats (0.06 ± 0.03 vs. 0.05 ± 0.02 mg/kg for heart rate, and 0.05 ± 0.01 and 0.06 ± 0.01 mg/kg for MaxdP/dt, in saline or propranolol-

treated rats, respectively), confirming prior reports in humans and rats that β AR sensitivity is normal at this time (Nattel, et al., 1979, Hedberg, et al., 1980).

Left ventricular function in response to a maximally effective dose of isoproterenol was comparable in both saline- and propranolol-infused rats 2 days after discontinuation, indicating that chronic propranolol did not affect ventricular performance under these conditions. Maximal displacement of NE stores by tyramine increased cardiac function to a greater extent in propranolol treated rats. Relative to saline-infused controls, propranolol treated rats had 57% greater end systolic pressure, Max dP/dt was increased by 56%, and Min dP/dt by 92% (Table 1).

AR and cardiomyocyte NGF synthesis

To determine if β -blockers increase sympathetic cardiac innervation by modulating NGF synthesis, we cultured cardiomyocytes in the presence or absence of AR agonists and measured NGF protein produced and secreted *in vitro*. Cardiomyocytes in culture showed spontaneous contractions and mature cytoskeletal features, as visualized with phalloidin (Figure 3A). These cells were immunoreactive for NGF protein (Figure 3B), and the endopeptidase furin (Figure 3C), which converts pro-NGF to mature NGF, thus determining neuritogenic properties of the secreted neurotrophin (Lee, et al., 2001). Western blot analysis of the medium revealed a strong band at 40

kDa corresponding to pro-NGF, with weaker bands of lower molecular weights. Addition of either the α -agonist phenylephrine or the β -agonist isoproterenol in concentrations from 10^{-10} to 10^{-8} mol/L failed to alter the amount or isoform profile of secreted protein (Figure 3D).

AR and sympathetic neuritogenesis in vitro

We used neuronal cultures to assess whether β AR blockade promotes sympathetic neuritogenesis by acting directly on postganglionic sympathetic neurons. After 48 h in culture, SCG neurons had elaborated many neurites (Figure 4A). Addition of 10^{-10} mol/L propranolol to the culture medium appeared to increase neurite area (Figure 4B), whereas outgrowth appeared more comparable to controls in cultures with propranolol at 10^{-8} mol/L (Figure 4C). Quantitative analysis confirmed a significant increase in neurite area per neuron of 17 % (Figure 4D).

To assess whether a β 1-AR antagonist is also effective in increasing neurite outgrowth, we cultured SCG neurons in the presence of metoprolol. At a concentration of 10^{-8} mol/L, neurite outgrowth appeared greater than in control cultures (Figure 5A, B), while outgrowth again appeared comparable to controls at the higher concentration of 10^{-6} mol/L (Figure 5C). Quantitation confirmed that the lower concentration of metoprolol increased neurite outgrowth by 29% compared to control (Figure 5D).

Beta-1 AR gene expression in SCG neurons

To confirm that sympathetic neurons express mRNA encoding the β 1 AR and are therefore potentially capable of responding to β 1AR blockade, we performed RT-PCR analysis of SCG neurons after 48h in culture. The sensitivity of our primers was confirmed using ventricular myocardium as a control, which showed a strong band at the predicted product size (Figure 6). RT-PCR of SCG neurons also yielded PCR product of the correct size, albeit of less intensity than that of cardiac muscle (Figure 6).

Norepinephrine synthesis and sympathetic axon outgrowth

We investigated the mechanism by which β -AR antagonists act on sympathetic neurons to enhance axonal outgrowth. Because β -AR antagonists increase axonal outgrowth, we queried as to whether a β 1AR agonist would inhibit outgrowth. We cultured SCG neurons alone (Figure 7A) or in the presence of the selective β 1-agonist dobutamine (Figure 7B). Quantitative analysis showed that dobutamine was ineffective in decreasing axonal outgrowth over a wide range of concentrations (Figure 7E, a representative concentration of 10^{-8} mol/L is shown here).

Because SCG neurons in culture express proteins for catecholamine biosynthesis and storage, NE is likely to be synthesized and released in these preparations, raising the possibility that β 1-AR activation is ongoing and may be already maximal. To determine if NE synthesized in culture tonically

inhibits axonal outgrowth, we blocked NE synthesis with AMPT, a competitive inhibitor of tyrosine hydroxylase. Neurite outgrowth appeared to be greater in cultures containing AMPT (Figure 7C), and quantitation confirmed a 35% increase relative to controls (Figure 7E). To confirm that AMPT acted to increase neurite outgrowth by eliminating tonic β 1AR activation, we now added dobutamine to these cultures. This β 1 agonist suppressed the AMPT-mediated increase in axon outgrowth to a level comparable to that of controls (Figure 7D, E).

DISCUSSION

β AR antagonists and post-infarct sympathetic hyperinnervation

Beta-blocker administration is a mainstay in managing patients with reduced left ventricular function after ischemic injury. Multiple studies have shown that administration of β blockers that selectively block β 1-ARs, or combine this effect with antagonism of other ARs, are important in achieving long-term improvement and in preventing post-myocardial infarction sudden death (Hunt, 2005). β blockers exhibit a number of actions that may contribute to improved outcome including reducing cardiac excitability and myocardial oxygen consumption, and reducing plasma angiotensin II levels via β 1AR-associated changes in cAMP formation and channel ion flux (Ichihara, et al., 1995, Lipsky, et al., 2008). In addition to these direct effects, β AR blockade is also associated with a number of indirect effects. For example, reduced

blood pressure that can occur with β blocker administration can lead to reflexive increases in sympathetic tone (Floras, et al., 1988), and chronic receptor inactivation may result in increased receptor numbers and enhanced signaling mechanisms (Haeusler, 1990).

In keeping with its ability to reduce the risk of post-infarct sudden cardiac death, it has been postulated that β AR blockade might improve outcomes by reducing sympathetic hyperinnervation in the infarct border zone (Jiang, et al., 2007). Because β ARs can upregulate NGF synthesis in some cell types (Carswell, et al., 1992) and NGF is required for post-infarct sympathetic sprouting, β AR blockade might reduce cardiac excitability by limiting the proliferation of excitatory nerves by reducing NGF. However, our findings here (and in contrast to an earlier report (Jiang, et al., 2007)) showed no reduction in sympathetic innervation associated with the infarct. Benefits of β AR blocker therapy in preventing sudden cardiac death after myocardial infarction are therefore likely to derive from actions aside from suppressing sympathetic axon sprouting at the infarct border.

β AR blockers increase ventricular sympathetic innervation density in non-ischemic myocardium

Despite the lack of effect on infarct innervation, healthy myocardium remote to the injury showed significantly greater numbers of DBH-ir axons following 7 days of propranolol infusion than observed in saline or

phentolamine-infused rats. Because we have previously shown using pan-neuronal markers that changes in numbers of structurally intact ventricular sympathetic fibers are accurately reflected in changes in numbers of DBH-ir or TH-ir fibers (Hasan, et al., 2006, Wernli, et al., 2009), this is consistent with an increase in sympathetic axon density.

This unexpected increase in sympathetic innervation could reflect an anomalous interaction between the damaged heart and propranolol. To exclude this possibility, we examined the effects of propranolol on non-infarcted hearts and found that sympathetic axon density was similarly increased, thus implying that this is attributable directly to the β blocker.

Propranolol is a non-selective β antagonist and, while still widely used, has been largely supplanted in treatment of post-ischemia sequelae by more selective β_1 antagonists. To determine if these newer agents also increase sympathetic innervation, we used metoprolol, a selective β_1 antagonist. Metoprolol was as effective as propranolol in increasing ventricular sympathetic innervation density, which suggests that blockade of β_1 receptors alone is sufficient to attain this effect.

Increased ventricular sympathetic innervation has functional consequences

The finding of increased ventricular innervation density following β blocker discontinuation could have important implications if the greater

numbers of axons results in increased ventricular sympathetic neurotransmission. In smooth muscle, sympathetic hyperinnervation has been associated with hypertrophy and hyper-reactivity (Scott, et al., 1983, Fan, et al., 1995), and increased ventricular innervation could lead to comparable changes. Assessment of ventricular function 48 h after discontinuation, when β ARs show normal sensitivity (Nattel, et al., 1979, Hedberg, et al., 1980), supports the idea that sympathetic hyperinnervation does contribute to some aspects of enhanced ventricular function. Thus, chronic propranolol infusion did not itself alter cardiac properties, as determined by direct β AR activation by isoproterenol. In contrast, in response to release of sympathetic NE stores by tyramine, both end systolic pressure and maximum dP/dt were increased by propranolol relative to saline-infused rats. While we cannot entirely rule out contributions of alterations in afterload that may be occurring (particularly if arteriolar sympathetic innervation is also increased), maximum dP/dt values are generally considered to be largely independent of changes in afterload (Rhodes, et al., 1993). These findings therefore support the idea that increased sympathetic innervation occurring with β blocker administration may contribute to heightened sympathetic activation following discontinuation.

β blockers act directly on sympathetic neurons to promote axon outgrowth

Our findings show that β blockers increase ventricular sympathetic innervation density, implying that they provide signals that encourage axonal sprouting. NGF, the prototypical neurotogenic protein, potently promotes sympathetic neurogenesis, and βAR-mediated changes in NGF could account for increased innervation. However, we were unable to modulate cardiomyocyte NGF synthesis by AR receptor activation, suggesting a minimal role of target-derived NGF in this process.

Consistent with an action independent of the target, we found that both propranolol and metoprolol, at physiological concentrations (Abrahamsson, et al., 1990, Takahashi, et al., 1993), promoted axon growth in sympathetic neuronal culture. Interestingly, a 100-fold increase in concentration of either agent resulted in loss of neurotogenic activity. This may not be surprising, as both agents at high concentrations have membrane stabilizing properties (Goodman, et al., 2006), and membrane stabilization by drugs with local anesthetic properties inhibit axon outgrowth (Ibarretxe, et al., 2007). It therefore appears that the increased ventricular sympathetic innervation density we observed *in vivo* can be attributed in whole or large part to a direct effect of the βAR antagonist on the neuron itself.

β1 autoceptors inhibit sympathetic neurite outgrowth

Because both propranolol and metoprolol promote sympathetic axon outgrowth, it is reasonable to speculate that this action occurs via β1ARs on sympathetic neurons. Prior reports have identified β1 receptors on postganglionic sympathetic neurons (Butler, et al., 1990, Watson-Wright, et al., 1991), and our RT-PCR studies confirm the presence of β1AR mRNA in our neuronal cultures. Thus modulation of the activity of these β1 receptors could provide a mechanism whereby neurite outgrowth is regulated.

While no previous studies have identified autoreceptor regulation of sympathetic outgrowth, there are several examples of neurotransmitter modulation of outgrowth in other systems. Studies on the *Helisoma* snail show that neurotransmitters such as dopamine and serotonin can suppress neurite outgrowth (Haydon, et al., 1984, McCobb, et al., 1988). A role for autoreceptors in this process may be implied, as serotonin inhibits outgrowth of serotonergic axons from rat raphe neurons (Whitaker-Azmitia, et al., 1986), and glutamate at high doses reduces axonal and dendritic outgrowth by immobilizing growth cones of glutaminergic pyramidal neurons (Mattson, et al., 1988). Accordingly, precedents exist for proposing that sympathetic neurotransmitter autoreceptors may play a role in axon outgrowth.

If β1AR do play a role in regulating sympathetic outgrowth and therefore target innervation density, then a β1 agonist should serve to inhibit outgrowth. Our initial attempts to demonstrate this in culture using dobutamine were

unsuccessful, but it is well known that sympathetic neurons synthesize NE in culture (Landis, 1978), raising the possibility that sufficient quantities of ligand are produced in culture to maximally activate β 1ARs so that additional ligand is ineffective.

To test the hypothesis that locally synthesized NE tonically inhibits axon outgrowth in culture, we inhibited catecholamine biosynthesis with the tyrosine hydroxylase inhibitor AMPT. Inhibition of NE synthesis increased axon outgrowth to an extent similar to that seen with β AR blockade, indicating that NE synthesized in culture does indeed act to inhibit outgrowth. Consistent with our hypothesis that β 1ARs inhibit outgrowth, addition of dobutamine to these culture where NE synthesis is blocked resulted in suppressed outgrowth typical of cultures in which NE is normally synthesized.

Conclusion

Our findings provide evidence for a mechanism whereby β 1 autoreceptors regulate the growth of sympathetic axons in vitro and in vivo. Autoreceptor blockade removes this inhibition, enabling axons to sprout to greater extents and thus increasing ventricular sympathetic innervation density. This increased innervation is functionally relevant, as release of NE stores from sympathetic terminals elicits greater ventricular contractile responses after blocker discontinuation. These findings suggest that rebound sympathetic hyperexcitability following cessation of β blocker therapy may, in

addition to altered receptor sensitivity, be attributable to sympathetic structural plasticity leading to increased target neuroeffector input.

ACKNOWLEDGMENTS

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Sources of Funding

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TABLE

Table 1.

Isoproterenol	Saline	Propranolol
Heart rate, bpm	458 ± 25	499 ± 9
End systolic pressure, mm Hg	82 ± 10	99 ± 14
End diastolic pressure, mmHg	5.3 ± 0.8	3.8 ± 0.4
Peak +dPdt, mm Hg/s	7379 ± 1246	9807 ± 361
Peak -dPdt, mm Hg/s	4258 ± 807	5922 ± 1519

Tyramine	Saline	Propranolol
Heart rate, bpm	443 ± 33	480 ± 18
End systolic pressure, mm Hg	78 ± 3	121 ± 19*
End diastolic pressure, mmHg	6.3 ± 0.8	4.3 ± 0.4
Peak +dPdt, mm Hg/s	6888 ± 735	10775 ± 916*
Peak -dPdt, mm Hg/s	4175 ± 498	8053 ± 1402*

Table 1. Cardiovascular function in response to isoproterenol or tyramine. Cardiovascular responses obtained 48 h after discontinuation of a 7 day infusion of saline or propranolol in response to a maximally effective dose of the direct β agonist, isoproterenol, or displacement of sympathetic norepinephrine stores by a maximal dose of tyramine. * $P < 0.05$ compared to saline infusion.

FIGURES

Figure 1.

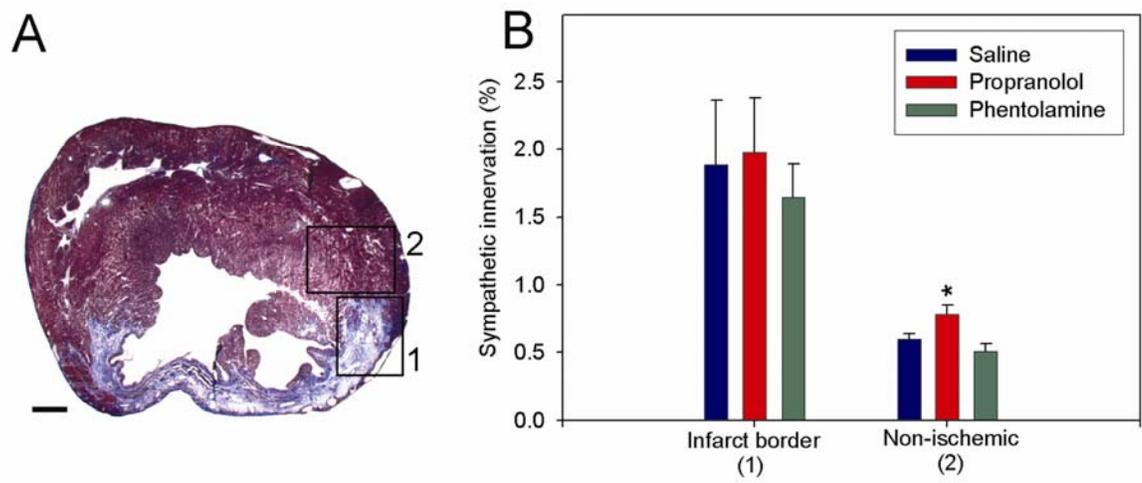


Figure 1. Adrenergic receptor blockade increases ventricular sympathetic innervation in non-ischemic myocardium. A: The left coronary artery was ligated, creating an infarcted region as shown in this Masson's Trichrome stained section through the ventricles at 7 days post-ligation. Two regions were selected for analysis of innervation density: region 1 comprising the infarct border region, and region 2, containing spared (non-ischemic) myocardium. Scale bar=1mm. B: Quantitative analyses of dopamine β -hydroxylase-immunoreactive innervation in regions 1 and 2 expressed as percentage area occupied by stained axons. The infarct border showed hyperinnervation unaffected by administration of saline or adrenergic blockers by minipump for 7 days. Non-ischemic myocardial innervation density was increased by propranolol relative to saline or phentolamine infusion. * $P < 0.05$ vs saline and phentolamine.

Figure 2.

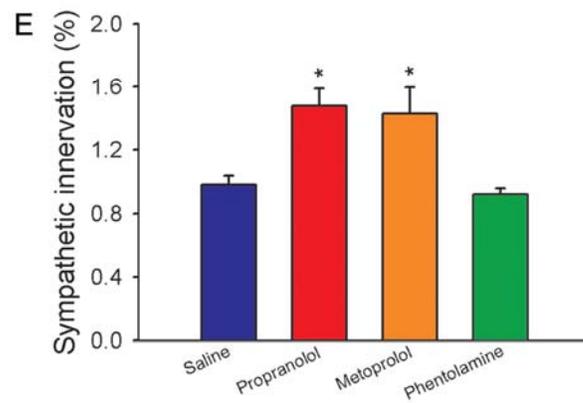
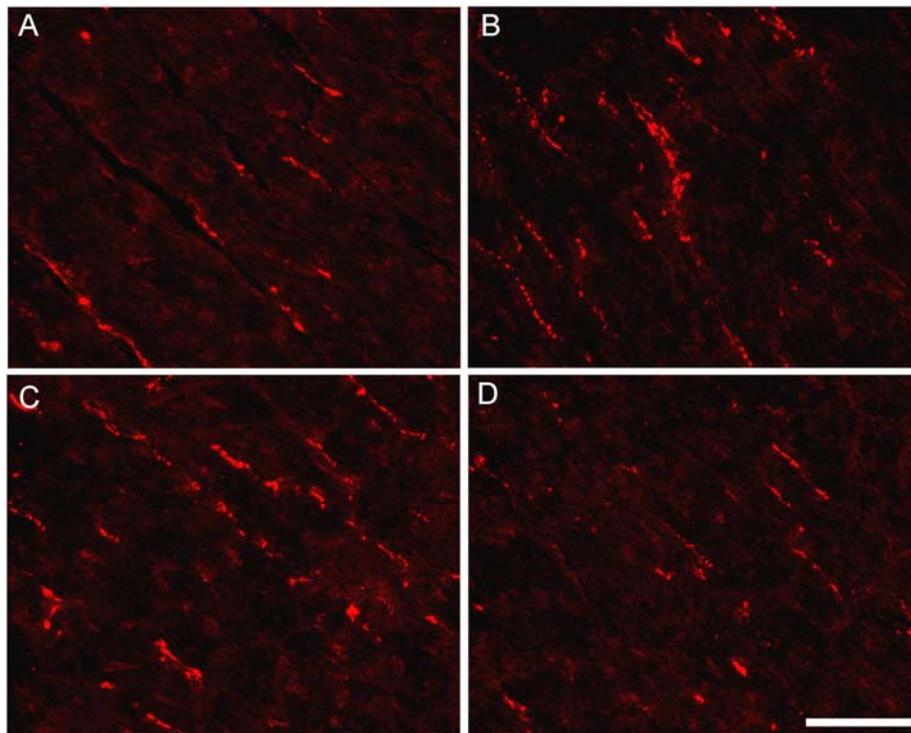


Figure 2. β adrenergic receptor blockers increase ventricular sympathetic innervation in uninjured hearts. A: Sections of ventricular myocardium in myocardium of saline-infused rats show Dopamine β -hydroxylase immunoreactive axons. Innervation density appears to be increased following a 7 day infusion of propranolol (B) or metoprolol (C), but not phentolamine (C). E: Quantitative analysis confirms increased myocardial innervation density with propranolol and metoprolol. * $P < 0.05$ for propranolol and metoprolol relative to saline or phentolamine.

Figure 3.

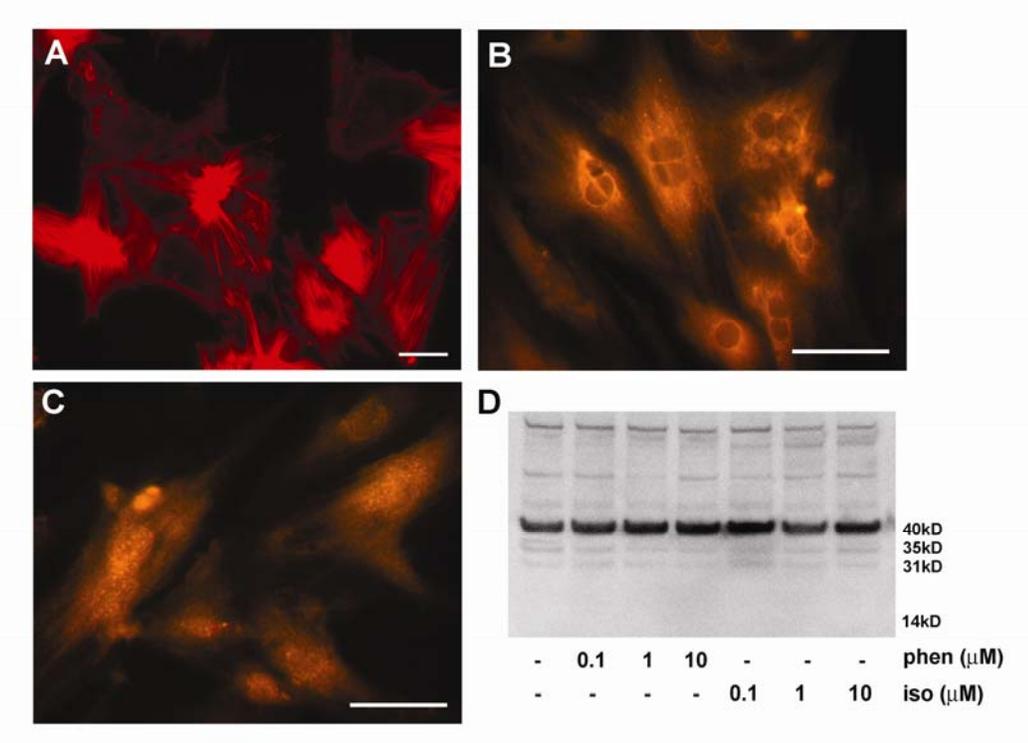


Figure 3. Adrenergic receptors do not modulate cardiomyocyte NGF secretion. Dissociated cardiomyocytes were cultured for 3 days prior to treatment with phentolamine or phenylephrine. Cardiomyocytes in culture show typical actin cytoskeleton after staining with phalloidin (A). Cardiomyocytes express NGF protein (B) and contain the pro-neurotrophin convertase, furin (C). Scale bar in B = 50 μ m. D: Following culture for 48 hours, western blot analysis of the culture medium revealed immunoreactive NGF, mainly as pro-NGF with a molecular mass of about 40 kDa. Treatment of the cultures with 0-10 μ M concentrations of the α -agonist phenylephrine (phen) or the β -agonist isoproterenol (iso) did not alter NGF content within the medium.

Figure 4.

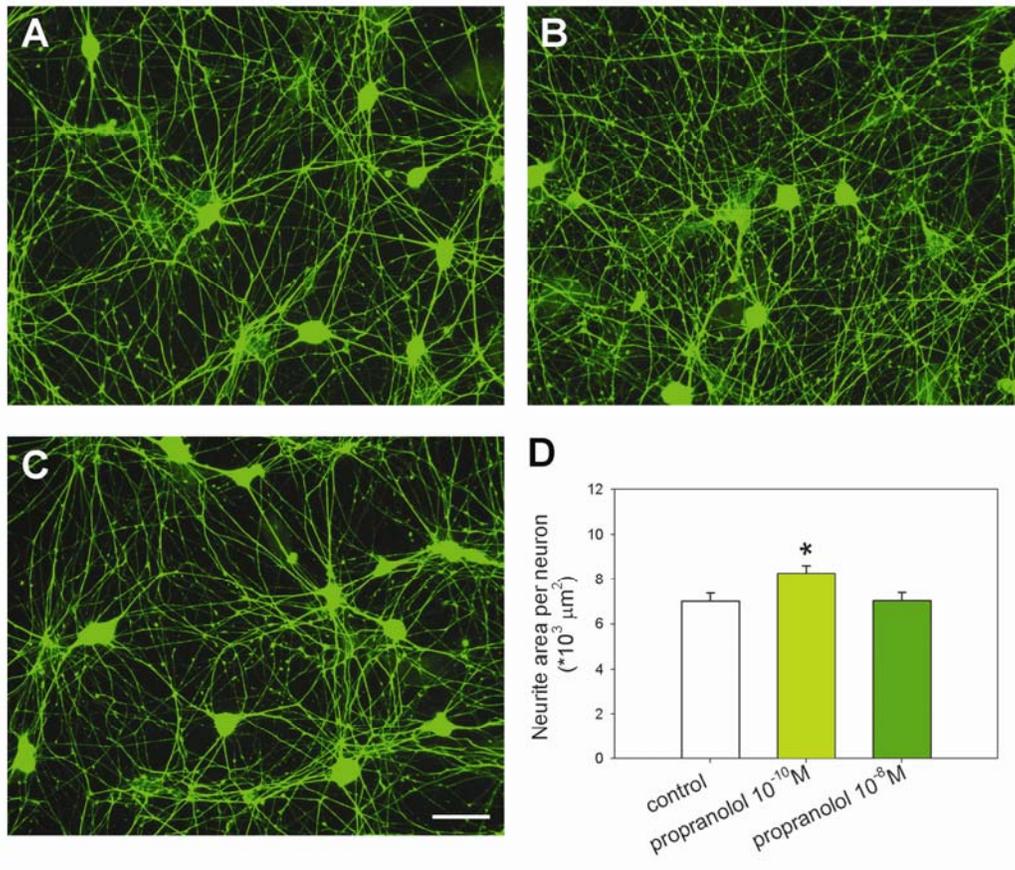


Figure 4. Propranolol increases sympathetic neurite outgrowth. Relative to superior cervical ganglion neurons cultured without treatment (A), propranolol at a concentration of 10^{-10} mol/L appeared to increase neurite outgrowth (B). At supraphysiological doses of propranolol (10^{-8} mol/L, C) this effect was not observed. Scale bar = 50 μ m. D: Quantitative analysis of peripherin-immunoreactive neurite area confirmed a dose-dependent effect of propranolol on neuritogenesis. * $P < 0.05$ compared to control.

Figure 5.

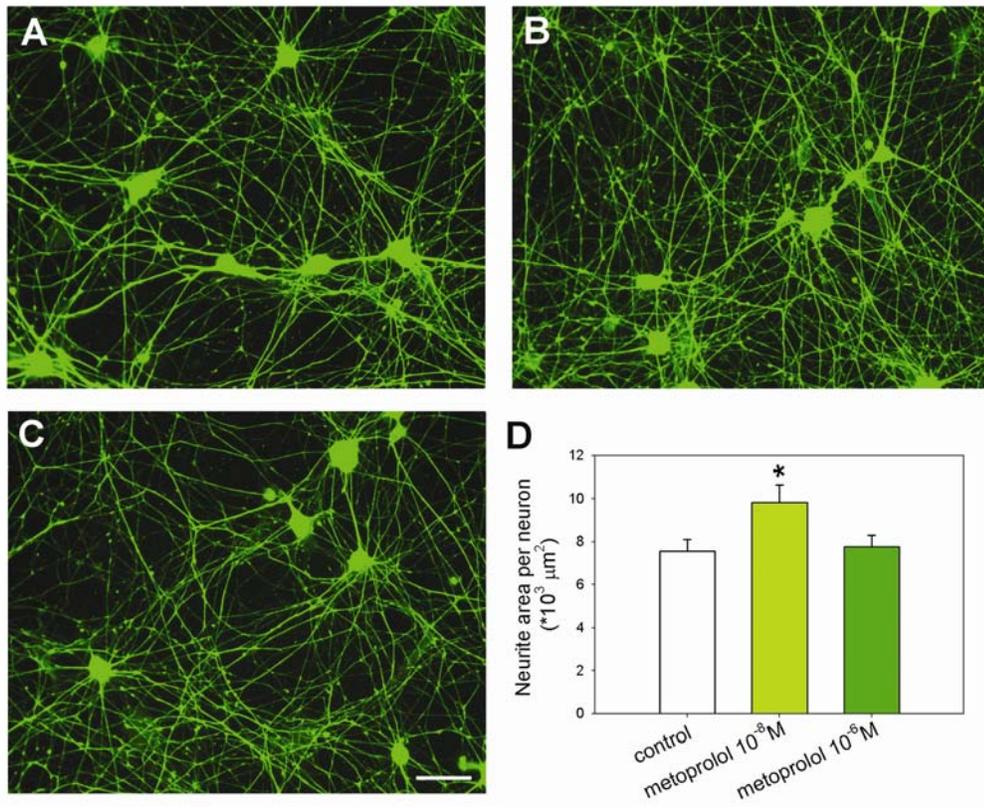


Figure 5. Metoprolol increases sympathetic neurite outgrowth. As compared to control cultures (A), cultures treated for 48h in the presence of 10^{-8} mol/L metoprolol (B) showed increased neurite outgrowth, while supra-physiological concentrations of 10^{-6} mol/L (C) were associated with outgrowth similar to controls. Scale bar = 50 μ m. D: quantitative analysis confirmed a pro-neuritogenic effect of physiological concentrations of metoprolol. * $P < 0.05$ compared to control.

Figure 6.

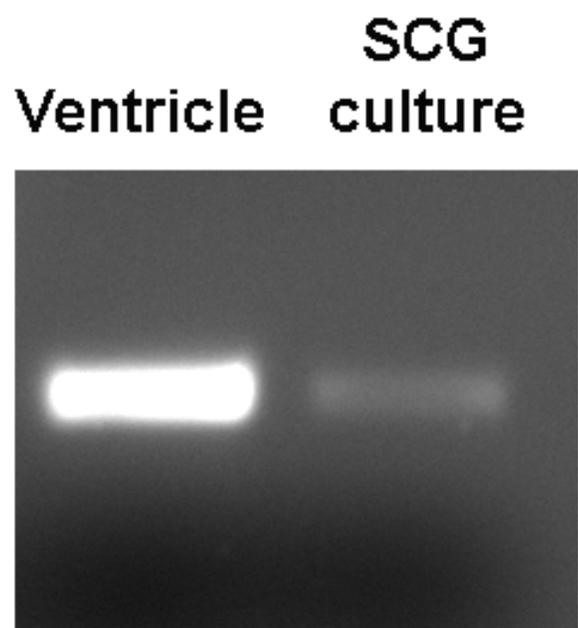


Figure 6. Sympathetic neurons contain genes encoding β 1 receptors. mRNA extracted from purified sympathetic neuronal cultures encode sequences corresponding to the β 1 adrenoceptor, as revealed by RT-PCR. Ventricular myocardium was used as a positive control.

Figure 7.

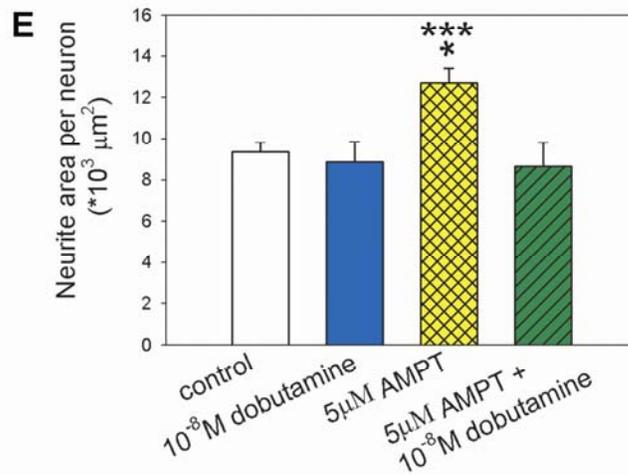
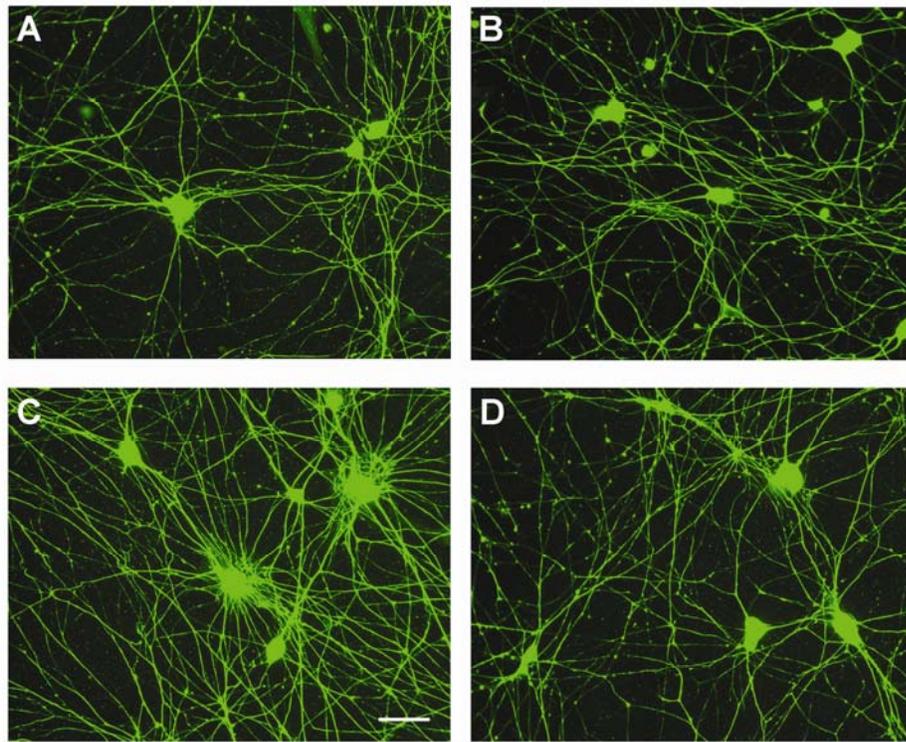


Figure 7. Sympathetic neurite outgrowth in culture is regulated by locally synthesized norepinephrine acting on β_1 autoreceptors. In comparison to control cultures (A), dobutamine at a concentration of 10^{-8} mol/L failed to affect outgrowth (B) and this was confirmed over a wide range of concentrations. When norepinephrine synthesis was blocked by α -methyl para-tyrosine (AMPT), neurite outgrowth appeared to be enhanced (C). Addition of dobutamine to cultures in which norepinephrine synthesis was blocked diminished outgrowth (D). Scale bar = 50 μ m. E: Quantitative analysis confirmed the increase in neurite area in response to AMPT (***P*<0.001, compared to control) and its reduction by addition of dobutamine (* *P*<0.05).

SUPPLEMENTARY METHODS

In vivo experimental preparations

All experimental protocols conformed to NIH guidelines and were approved by the University of Kansas Medical Center Animal Care and Use Committee. Forty six female Sprague Dawley rats (Harlan Breeding Laboratories; Indianapolis, IN) were obtained at 60 days of age (~200g), and entered into experimental groups within a week of arrival. Twelve rats receiving left ventricular infarctions were anesthetized with KAX (i.p. injection of 60 mg/kg ketamine HCl Keta-Ject, Phoenix Scientific, St. Joseph, MO; 0.4 mg/kg atropine sulfate, Healthcare Corp., Deerfield, IL; 8 mg/kg xylazine (Xyla-Ject, Phoenix Scientific, St. Joseph, MO), respired mechanically, and the left anterior descending coronary artery was ligated (CAL) with a silk suture as described previously (Wernli, et al., 2009).

Adrenergic receptor blockade

Directly following coronary artery ligation or in otherwise unoperated rats, osmotic minipumps (Durect Corporation, Cupertino, CA) were inserted subcutaneously between the scapulae after anesthesia with KAX. Rats were implanted with 2ML1 minipumps containing saline (N=12 unoperated, 4 CAL) or propranolol (N=8 unoperated, N=4 CAL, released at a rate of 13.2mg/kg/day to produce β receptor blockade; Sigma Chemical Co, St. Louis, MO) (Conlon, et al., 1991) or 2001 minipumps containing

phentolamine (N=4 unoperated, N=4 CAL, released at a rate of 1mg/kg/day via to produce α blockade (Steinle, et al., 2002); Sigma Chemical Co, St. Louis, MO). In addition, six unoperated rats were implanted with 2ML1 minipumps and received metoprolol (released at a dose 36mg/kg/day which blocks β 1 but not β 2 receptors (Ablad, et al., 1975, DiBona, et al., 1999); Sigma Chemical Co, St. Louis, MO). To ensure that changes in reproductive hormones during the estrous cycle did not influence sympathetic innervation, 4 unoperated saline control rats were anesthetized as above and ovariectomized through bilateral incisions (Zoubina, et al., 2003) a week prior to minipump insertion. No differences in cardiac innervation between intact and ovariectomized controls were observed and data from all saline controls were subsequently pooled.

Confirmation of AR-blockade

The effectiveness of adrenergic receptor blockade was assessed 7 days following minipump implantations in rats anesthetized with urethane (1.5 g/kg and given additional doses as needed, Sigma Chemical Co, St. Louis, MO). Because the pupillary dilator muscle mediates its action by α 1 receptor, we confirmed α -AR blockade by topically applying a drop of 1% NE in saline to the eye and by measuring the pupil diameter.

To confirm β -AR blockade, a femoral vein was cannulated, heart rate was recorded with a lead II electrocardiogram and a single dose of 0.2 μ g/kg

isoproterenol was given. We also confirmed that the dose of metoprolol was selective for β_1 receptors (Ablad, et al., 1975, DiBona, et al., 1999) by recording β_2 -AR-mediated blood flow from the gracilis muscle using an OxyFlow laser Doppler flowmetry system (Oxford Optronix Ltd, Oxford, UK).

Cardiac sympathetic neuroeffector function after β -blocker discontinuation

The effect of chronic β -AR blockade on cardiac ventricular neuroeffector function was assessed 2 days following discontinuation of drug administration. Seven days after onset of propranolol or saline infusion, pumps were removed and, 2 days later, rats were anesthetized with urethane, the jugular vein was cannulated, and the ganglionic blocker chlorisondamine (Ecolid, Ciba Pharmaceuticals, Summit, New Jersey, 2.5 mg/kg) was administered intravenously. Body temperature was maintained at 37°C with a heating lamp. A calibrated catheter for measuring ventricular function (SPR 838, Millar Instruments, Huston, TX) was introduced in the left ventricle via the right carotid artery (Pacher, et al., 2008). After heart rate had stabilized (~15 minutes later), tyramine (Sigma Chemical Co, St. Louis, MO) was administered iv in graded doses ranging from 2 to 30 $\mu\text{g}/\text{kg}$ until maximum left ventricular (LV) functions were achieved (65 $\mu\text{g}/\text{kg}$ tyramine). After heart rate returned to baseline (~10 min), isoproterenol (Sigma Chemical Co, St. Louis, MO) was administered in graded doses ranging from 0.01 to 0.15 $\mu\text{g}/\text{kg}$ until

maximum left ventricular functions were attained (0.33 $\mu\text{g}/\text{kg}$). To assess LV blood volume, a cuvette calibration was performed with warm heparinized blood at the end of the experiments (Pacher, et al., 2008). LV functions were calculated using LabChart software (AD Instruments, Colorado Springs, CO) and exporting 5 sec selections after the last dose of tyramine and isoproterenol to PVAN Software (Millar Instruments, Huston, TX). Parameters measured were heart rate, end systolic pressure, end diastolic pressure, maximal and minimal first derivative of pressure (Max dP/dt and Min dP/dt) and arteriolar elastance. In addition, the isoproterenol ED₅₀ of heart rate and Max dP/dt were calculated as indexes of β receptor sensitivity.

Analysis of cardiac sympathetic innervation density and infarct volume

Following adrenergic receptor blockade for 7 days, hearts were excised through a ventral midline incision, snap-frozen in Tissue Freezing Medium (Electron Microscopy Sciences, Hatfield, PA), and stored at -80°C. Sections were cut at 10 μm thickness perpendicular to the apical-basal axis and thaw-mounted onto slides. Sections were fixed at 4°C in methanol for 10 min, blocked with normal goat serum supplemented with 1% bovine serum albumin and incubated overnight with antibodies against dopamine- β - hydroxylase (DBH: 1:600, rabbit IgG, Immunostar, Hudson, WI) followed by application of goat anti-rabbit IgG conjugated to Alexa 555 (1:500, Invitrogen, Carlsbad, CA) for 2 h. This antibody was previously found to be robust and

provide staining consistent with other sympathetic markers (Wernli, et al., 2009). Six stepped serial sections, each separated by ~2mm, were selected for analysis. In sections from non-ligated hearts, images of 6 fields evenly distributed in the left lateral posterior area of the heart were taken with a 40x objective using a Nikon Eclipse TE300 microscope. In CAL hearts, images of 6 fields evenly distributed in the left lateral posterior area (infarct border region) and images of 6 fields evenly distributed in the non-ischemic posterior area of the heart were taken (Wernli, et al., 2009). Nerve density was calculated as reported previously (Wernli, et al., 2009) by superimposing a stereological grid (8 μ m line interval) over the captured image, counting grid intersections transecting nerves and dividing by the total number of intersections within the sampled tissue. Infarct volume was calculated from stepped serial sections stained with Masson's trichrome (Wernli, et al., 2009). Infarct volume in CAL hearts was calculated by measuring infarct area in each section and multiplying it by the distance between sections.

Cardiomyocyte Culture and protein synthesis

To investigate whether AR activation modulates cardiomyocyte nerve growth factor (NGF) synthesis, hearts were harvested from pups and dissociated (Worthington Biochemical Co., Lakewood, New Jersey). Cardiomyocytes were plated in DMEM/F12 (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma Chemical Co, St Louis,

MO) and 0.02 μ g/ml cytosine arabinoside (Sigma Chemical Co, St Louis, MO) to reduce fibroblast outgrowth. After 3 days, the culture medium was replaced by medium containing the non-selective α -agonist phenylephrine (0.1-10 μ mol/L; Research Biochemicals International, Natick, MA) or the non-selective β -agonist isoproterenol (0.1-10 μ mol/L; Sigma Chemical Co, St Louis, MO). Triplicates of each treatment media were collected after 24 hours and proteins were precipitated by incubating the sample with 2% Na deoxycholate (1% v/v sample; Sigma Chemical Co, St Louis, MO) followed by incubation in trichloroacetic acid (TCA; 10% v/v sample; Sigma Chemical Co, St Louis, MO). After centrifugation, pellets were dried and TCA was removed with ice cold acetone. Pellets were resuspended in LDS sample buffer containing 2.5% β -mercaptoethanol (Sigma Chemical Co, St Louis, MO). Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk in TBST (10mM Tris HCl, 150mM NaCl, pH 7.4 and 0.05% Tween solution-20) and incubated overnight at 4°C in 1:1000 rabbit anti-NGF (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS followed by goat anti-rabbit 1:5000 AP-conjugated (Jackson, West Grove, PA), and detected using Immun-Star AP Substrate (BioRad, Hercules, CA). Cultured cardiomyocytes were fixed in buffered 4% formaldehyde prepared from paraformaldehyde (Sigma Chemical Co, St Louis, MO) for 1h, blocked with normal goat serum, stained with actin cytoskeleton staining with Alexa Fluor594-Phalloidin (1:400,

Molecular Probes, Carlsbad, CA) or immunostained with anti-NGF rabbit IgG (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) or with anti-furin rabbit IgG (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using goat anti-rabbit IgG conjugated to cy3 (1:200, Jackson, West Grove, PA). The selectivity of these antibodies has been previously confirmed, by omission and preabsorbtion (Hasan, et al., 2006, Wernli, et al., 2009).

Sympathetic Neuronal Cultures

To assess effects of β -AR blockade on sympathetic axon outgrowth, cultures were conducted on superior cervical ganglia (SCG) neurons obtained from Sprague-Dawley pups (postnatal day 0-2). Neurons were dissociated in collagenase IA (Sigma Chemical Co, St Louis, MO) followed by incubation in 0.25% trypsin-EDTA (Sigma Chemical Co, St Louis, MO) and suspended in neurobasal medium (Gibco, Carlsbad, CA) with 0.5mM glutamine (Sigma Chemical Co, St Louis, MO), 2% B27 (Gibco, Carsbad, CA), 0.1 mg/ml primocin (Invivogen, San Diego, CA), FrdU and uridine (Sigma Chemical Co, St Louis, MO) 20 μ M each to reduce the number of glia and 10 ng/ml NGF (Alomone Labs, Jerusalem, Israel). Neurons were plated ($\sim 4 \times 10^4$ per well) onto glass coverslip (12mm diameter, Bellco glass, Vineland, NJ) preliminary coated with poly-D-lysine (MP Biomedicals LLC, Illkrich, France) and laminin (Gibco, Carsbad, CA). After adhesion of the cells, medium alone or containing propranolol (10^{-10} mol/L to 10^{-8} mol/L; Sigma Chemical Co, St Louis, MO) or

metoprolol (10^{-10} mol/L to 10^{-6} mol/L; Sigma Chemical Co, St Louis, MO) was added, and was changed after 24h. Other cultures were conducted in the presence of 10^{-8} mol/L dobutamine (Sigma Chemical Co, St Louis, MO), with or without 5 μ mol/L alpha-methyl-p-tyrosine (α MPT; Research Biochemicals International, Natick, MA). All treatments were conducted in triplicate, and each experiment was repeated at least twice.

After 48h, cultures were fixed in buffered paraformaldehyde as before for 1h, blocked with normal goat serum, immunostained overnight for peripherin (1:250, chicken IgG, Chemicon International, Billerica, MA) followed by a 2 h incubation in goat anti-chicken cy2 (1:200, GeneTex, San Antonio, TX). Six images equally distributed within each well were taken with a 20x objective using a Nikon Eclipse TE300 microscope. Neurite outgrowth was calculated by superimposing a stereological grid (25 μ m line interval) over the captured image (Chakrabarty, et al., 2008), counting grid intersections over neurites, dividing by the total number of intersections and multiplying by total image area. Neurite area was then divided by the total number of somata in the image, and neurite outgrowth expressed as neurite area in μm^2 per neuron. To confirm the selectivity of the staining, neurons were co-stained with tyrosine hydroxylase (TH, 1:300, rabbit IgG; Chemicon International, Billerica, MA) followed by secondary antibody goat anti-rabbit cy3 (1:200, Jackson, West Grove, PA).

RT-PCR for β 1 receptor mRNA

The presence of β 1 receptors in SCG cultures was assessed by RT-PCR. Heart tissue and dissociated SCG cultured for 48h were homogenized in ice cold trizol and total RNA (1 μ g) was reverse transcribed using the iScript kit (Biorad, Hercules, CA). Amplification of cDNA was conducted using primers for β 1 receptors (5'-CAACGGCGGGACGACCACTG -3' sense and 5'-ACACCTTGGACTCGGAGGAGAGC -5' antisense). Conditions for the PCR were an initial heating step at 94°C for 2min followed by 94°C for 40 s, 59°C for 30 s, and 72°C for 1 min for 30 cycles with a final extension at 72°C for 5min. Five microliters of PCR product was analyzed by electrophoresis (1.5% agarose gel with ethidium bromide). Bands were visualized under UV transillumination using a Chemi Doc XRS (BioRad, Hercules, CA).

Statistical Analyses

Data were analyzed using Student T-test or one way ANOVA for multiple comparisons followed by a Student Newman-Keuls test (SigmaStat 3.11, Systat Software, Inc., San Jose, CA). Data are expressed as mean \pm SEM. Statistical significance was accepted at $P \leq 0.05$.

CHAPTER 5. GENERAL CONCLUSIONS

Abrupt thrombosis in a coronary artery can lead to extensive ventricular tissue injury (Fuster, et al., 2007). Myocyte necrosis attracts inflammatory cells which secrete many factors in order to repair the ventricle (Frangogiannis, 2006). Nerve growth factor (NGF) is produced by several inflammatory cells (Zhou, et al., 2004, Hasan, et al., 2006) and we found that NGF from the infarcted tissue induces neuritogenesis in an explant co-culture system (Hasan, et al., 2006). As a result, the border region of the infarct becomes densely innervated with sympathetic fibers. The hyperexcitability of that region can lead to arrhythmias and sudden cardiac death (Vracko, et al., 1990, Cao, et al., 2000a, Cao, et al., 2000b). Surviving individuals are at risk of developing congestive heart failure which is also characterized by sympathetic hyperexcitability (Ferrari, et al., 1998). As a result, β -blockers are commonly administered to reduce oxygen demand of the heart and blood pressure. Based on these findings, our first goal was to examine possible ways to downregulate sympathetic hyperinnervation following infarction by reducing inflammation. This may in turn restore some aspects of the electrical conduction in the heart. Our second goal was to study the effect of β -blockers on cardiac sympathetic innervation.

Effects of anti-inflammatory drug treatments on sympathetic hyperinnervation

Dexamethasone presents advantages as a anti-inflammatory drug that can suppresses many inflammatory cytokines and is generally well tolerated (Koj, et al., 1988). Following post-infarction treatment with dexamethasone or saline, we first ensured that infarct size was similar between groups and the efficacy of the treatment was confirmed by measuring body weight.

Dexamethasone provoked the anticipated effect by reducing body weight by ~15% (Clark, et al., 1996, Caparroz-Assef, et al., 2007). However, we found that the treatment failed to reduce sympathetic innervation, suggesting that dexamethasone may have counteracting effects on the tropic inflammatory environment and on neuronal sprouting. Dexamethasone has been shown to increase macrophage apoptosis (Schmidt, et al., 1999), but can also increase macrophage colony stimulating factor, which controls macrophage growth and survival (Lloberas, et al., 1998). In addition, while dexamethasone reduces peripheral NGF (Lindholm, et al., 1994), it can also promote neuronal recovery (Sheehan, et al., 2006). Thus, the generalized actions of dexamethasone may not provide enough selectivity as a therapeutic treatment to reduce sympathetic hyperinnervation.

We previously showed that macrophages are one of the cell types synthesizing NGF in the infarct (Hasan, et al., 2006) and for this reason, we selectively targeted their depletion. Macrophages contribute to wound repair

by phagocytosing cellular debris (van Furth, et al., 1970, Adams, et al., 1984). During this process they become activated triggering the release of inflammatory and growth factors (Adams, et al., 1984) including NGF (Hasan, et al., 2006). We administered clodronate liposomes intravenously which are selectively phagocytosed by macrophages and provoke their apoptosis (Van Rooijen, et al., 1994, van Rooijen, et al., 1996). As a result of the treatment, macrophages were depleted in the infarct and their reduction was associated with a slight decrease of myofibroblasts, suggesting that macrophages influence the recruitment or transdifferentiation of this cell type. T-cell numbers were unaffected by macrophage reduction. The depletion of macrophages induced a 67% reduction in mature NGF and immunohistochemistry analysis showed that the main inflammatory cells contributing to this decrease were macrophages and to a much lesser extent myofibroblasts.

Together, these results demonstrate that macrophages play a central role in the development of sympathetic hyperinnervation. Therefore, depleting macrophages could reduce arrhythmogenesis. However, macrophages are required for the proper healing (van Amerongen, et al., 2007) of the infarct and depleting this cell type may cause alternative side-effects. A possible method to selectively reduce their NGF production may be to target their activation. Phagocytosis or the presence of cytokines and chemokines are critical for macrophage activation (Adams, et al., 1984), which is associated

with upregulation of NGF production (Caroleo, et al., 2001). The use of macrophage gene therapy has already been the object of several studies (Andreesen, et al., 1990, Griffiths, et al., 2000) and it may be possible to introduce macrophages that produce low levels of NGF upon activation in the heart to reduce sympathetic hyperinnervation without impairing wound healing.

Adrenergic receptors activation and beta-blockade

As a result of sympathetic hyperinnervation, norepinephrine is often present in excessive amounts in the infarcted heart. However, besides the high level of innervation, sympathetic hyperactivity (Dae, et al., 1995) and reduced norepinephrine reuptake by the norepinephrine transporter (Li, et al., 2004, Parrish, et al., 2008) are factors that contribute to norepinephrine spillover. Moreover, after infarction, axons at the base of the heart have elevated levels of tyrosine hydroxylase (Inoue, et al., 1988) the rate-limiting enzyme in the production of norepinephrine, responsible for catalyzing the conversion of L-tyrosine to dihydroxyphenylalanine (DOPA), and could contribute to excessive norepinephrine production.

Plasma norepinephrine levels are also elevated in congestive heart failure (Chidsey, et al., 1962), a frequent syndrome resulting from myocardial infarction. Although congestive heart failure is used as an umbrella term for many different complex syndromes, one common feature is the potential for

fluid retention and low blood pressure (Ferrari, et al., 1998). Generally reduced cardiac output and raised venous pressure have opposite effects on baroreceptor firing as an attempt to restore blood pressure, but the vasodilator response overwhelms the vasoconstriction response causing fluid retention (Ferrari, et al., 1998). Furthermore, the presence of norepinephrine spillover can lead to adaptive changes in congestive heart failure by reducing the ratio of β_1/β_2 in the heart (Pritchett, et al., 2002). In addition, norepinephrine release is also elevated in spontaneously hypertensive rats (Tabei, et al., 1995, Luff, et al., 2005) and this symptom can lead to congestive heart failure in the absence of a previous myocardial infarction (Lavie, et al., 2002).

In order to counteract some of these maladaptive changes caused by high norepinephrine levels, β -blockers are a frequent treatment for myocardial infarction, congestive heart failure and hypertension. A number of clinical trials have demonstrated that β -blockers can reduce mortality and decrease the risk of reinfarction and adverse effects like arrhythmias and chest pain (Viskin, et al., 1995).

Therefore, we studied whether adrenergic receptor activation affects sympathetic axonal sprouting in the heart. Although border region was hyperinnervated in all groups, neither the α -blocker phentolamine, nor the β -blocker propranolol provoked any further change of innervations in that region. However, propranolol enhanced sympathetic innervation by nearly

50% in un-ischemic regions of the heart and this was also the case in uninjured hearts. This response was selective for β_1 receptors since metoprolol induces the same change. On the other hand, phentolamine did not alter sympathetic innervation in the un-ischemic ventricular region or in unoperated hearts.

During the treatments, β -blockade was confirmed by the decrease in heart rate with isoproterenol administration and α -blockade was demonstrated by the lack of pupil dilation after norepinephrine topical administration. The blockade of β receptors prevented the appearance of any adverse effect caused by sympathetic hyperinnervation. However, changes became visible two-days following treatment withdrawal. Propranolol-induced hyperinnervation increased left ventricular functions after the release of endogenous norepinephrine stores by tyramine compared to controls. Meanwhile, propranolol withdrawal lacked any effect on receptor sensitization since cardiac response to isoproterenol was similar to control. These results could explain some aspects of the dramatic findings showing that β -blocker discontinuation induced 24% mortality rate in patients with peripheral vascular disease, who are often at risk of coronary artery disease, compared to a 5% mortality rate in non-users (Hoeks, et al., 2007).

Alternative treatments

Because of the controversial results on sympathetic innervation obtained after β -blocker treatment, alternative treatments for the management of myocardial infarction may become increasingly used to prevent adverse effects. Immediately following myocardial infarction, guidelines recommend the administration of nitroglycerin to promote vasodilatation, morphine to release pain, aspirin for its anti-thrombotic effect and a β -blocker to reduce myocardial oxygen demand caused by hyperexcitability (Frigerio, et al., 2005). As a continuous treatment following infarction and to treat congestive heart failure, it is advised to administer a diuretic, a β -blocker, an angiotensin II receptor blocker (ARB) or an angiotensin converting enzyme (ACE) inhibitor (Hunt, et al., 2005).

ACE inhibitors downregulate the renin-angiotensin system (RAS) (Fauci, et al., 2008). The release of renin is induced by a decrease in renal blood flow and by sympathetic activation of the juxtaglomerular cells of the kidney. Renin cleaves angiotensinogen into angiotensin I in the plasma. Angiotensin I is then cleaved into angiotensin II by ACE in the pulmonary circulation. Angiotensin II causes primarily arteriolar vasoconstriction, which contributes to increasing blood pressure. After infarction and during congestive heart failure the RAS is upregulated, therefore ACE inhibitors are often administered to downregulate that system (Landmesser, et al., 2009). Because angiotensin II has been shown to induce neuritogenesis in PC12

cells and NG108-15 cells (Stroth, et al., 1998, Cote, et al., 1999, Gendron, et al., 2002), reducing angiotensin II via ACE inhibitor may also reduce border region sympathetic hyperinnervation. Therefore ACE inhibitors may not only attenuate maladaptive left ventricular remodeling (Landmesser, et al., 2009), they may also reduce hyperinnervation leading to decreased arrhythmias. Thus they are an important treatment, not only to reduce blood pressure but also to reduce cardiac sympathetic hyperactivity.

Variability between β -blockers

The main β -blockers recommended are sustained-release metoprolol (ie. in a matrix tablet made of hydrophilic polymers), bisoprolol, which block β_1 adrenergic receptors, or carvedilol, which blocks α_1 , β_1 and β_2 adrenergic receptors (Hunt, 2005). Although one trial showed that carvedilol reduced mortality compared to metoprolol tartrate, the dose of metoprolol tartrate was not the conventional dose given by physicians (Hjalmarson, et al., 2000). Thus, carvedilol may reduce the risk of mortality due to its effects on multiple receptors, hence sympathetic hyperinnervation may also be reduced, but this needs to be further evaluated.

Moreover, β receptors are known to have single nucleotide polymorphisms (SNP) (Ahles, et al., 2009), which appear to modify drug efficacy. The most common SNPs are Ser49Gly located in the extracellular N terminus of the receptor, and Gly389Arg located in the C terminus (Tesson, et

al., 1999). This second variant is associated with higher adenylyl cyclase activity upon isoproterenol activation, consistent with its role on the C terminus, which conveys the response intracellularly. Due to this variability in genetic background and response, increasing research is being done to achieve individualized drug therapies.

Sympathetic neuronal outgrowth and its regulation by $\beta 1$ autoreceptors

To identify the molecular mechanisms leading to β -blocker-induced hyperinnervation, we first determined if adrenergic-blockers mediate their effect by modulating cardiac NGF. We induced chemical sympathectomy with guanethidine (Nielsen, 1977a, Nielsen, 1977b). Although this treatment enhanced the production of some proNGF isoforms, total protein was unchanged. Moreover, adrenergic agonists did not influence NGF synthesis by cardiomyocytes. These results suggest that cardiac sympathetic sprouting induced by β -blockers is independent of target organ NGF synthesis.

Besides NGF, other factors could modulate sympathetic innervation. In fact, sympathetic outgrowth is mediated by the convergence of many extracellular factors on receptors that trigger intracellular neurotrophic cascades. Besides neurotrophins, other trophic factors include adhesion molecules (Nagtegaal, et al., 1998), fibroblast growth factors (Delgado-Rivera, et al., 2009) and insulin-like growth factors (Homma, et al., 2007). Neurotransmitters have also been identified as modulator of neuronal

plasticity in the central nervous system. For example, increasing doses of glutamate reduced axonal and dendritic outgrowth of pyramidal neurons by immobilizing growth cones (Mattson, et al., 1988). Likewise, dopamine suppressed outgrowth of neurons from the snail *Helisoma* (McCobb, et al., 1988). Serotonin had a similar effect on *Helisoma* neurons (Haydon, et al., 1984). Moreover, outgrowth of serotonergic neurons from the rat raphe nucleus was reduced by serotonin, suggesting the regulation of outgrowth by an autocrine mechanism (Whitaker-Azmitia, et al., 1986).

Accordingly, we studied the effect of adrenergic receptor activation on sympathetic neuronal sprouting. We found that deactivating β adrenergic receptors with propranolol, metoprolol, and by decreasing norepinephrine content with AMPT, all induced outgrowth in a bell-shaped curve pattern. Moreover, maximal outgrowth by AMPT could be blocked with the selective β_1 agonist dobutamine. These results suggest that sympathetic outgrowth is mediated in an autocrine fashion via β_1 autoreceptors.

Post-ganglionic sympathetic neurons are known to express β_1 adrenoceptors (Butler, et al., 1990, Watson-Wright, et al., 1991) and we confirmed that sympathetic neuronal cultures express β_1 receptors, however it is still unclear whether these receptors respond to epinephrine or to intraganglionic norepinephrine. Nonetheless, this suggests the presence of a highly complex and plastic system modulating target sympathetic innervation via central or peripheral feedbacks. The rapid plasticity may provide an

adaptive mechanism for survival to stress. Hence high stress levels may reduce target organ sympathetic innervation, while low stress levels may increase it.

The intracellular mechanisms leading to this neuronal plasticity remain unknown. However, neurotransmitter-mediated plasticity has been extensively studied and documented in the context of memory (Kandel, et al., 1982). The addition of serotonin to *Aplysia* sensory neurons induced a 3.5-fold increase of cAMP (Bernier, et al., 1982). Because β -receptors are coupled to G_s protein leading to the activation of cAMP (Lefkowitz, 2004), a similar reaction could result in peripheral sympathetic neurons. In cardiomyocytes (Wang, et al., 2004) and in *Aplysia* sensory neurons (Klein, et al., 1978), cAMP enhances the influx of Ca^{2+} . This influx can be enhanced by serotonin, which decreases the probability of a specific K^+ channel to open, which broadens the action potentials by 10 or 20 percent leading to greater Ca^{2+} influx (Klein, et al., 1980). Because a permissive range of intracellular Ca^{2+} levels appears required for maximal sprouting (Kater, et al., 1991), it is possible that Ca^{2+} mediates sympathetic neuronal outgrowth via AR, thus explaining the bell-shape curve effect (Fig. 1).

Additionally, the hippocampus is one of the CNS structures with the highest inputs of adrenergic (norepinephrine) terminals (Schroeter, et al., 2000), suggesting the important implication of the noradrenergic system in memory. In particular, the administration of β_1 receptor agonist to $DbH^{-/-}$ mice

was sufficient to promote context-specific enhancement of freezing, suggesting that these receptors are important for memory retrieval (Murchison, et al., 2004).

Together, this suggests that an autocrine feedback similar to the one observed with peripheral sympathetic neurons may apply to hippocampal neurons of the CNS as well, hence 1) stress or hypertension which increase norepinephrine release may reduce neuronal plasticity and prevent the formation of neuronal synapses and 2) β -blockers may promote outgrowth followed by abnormal rewiring that could become apparent following withdrawal. Both could affect memory and in particular memory retrieval which is mediated by β_1 adrenergic receptor (Ouyang, et al., 2005).

The future of β -blockers

In many cases β -blockers are required, but due to resulting sympathetic hyperinnervation, their use should be restricted. Starting a β -blocker treatment should be a long term strategy and patients should be aware that interrupting the treatment may result in severe adverse effects which may not outweigh their benefit. Patients unable to comply with the treatment regimen should also avoid taking β -blockers.

As it is already advised, in case patients need to withdraw from β -blockers, the drug dosage should be reduced progressively. In addition, ACE inhibitors may need to be increased to counteract the effect of sympathetic

hyperactivity and possibly by reducing sympathetic neuronal density (Stroth, et al., 1998, Cote, et al., 1999, Gendron, et al., 2002).

Research on β receptor polymorphisms may result in increased selectivity of the treatment and a decrease in adverse effects. In addition, blocking norepinephrine release from the axons is another alternative treatment to reduce hyperexcitability. Because the release of norepinephrine is inhibited by a feedback via α_2 adrenergic receptors, α_2 agonists such as Clonidine may become increasingly administered to replace β -blockers.

Conclusion

Collectively, these results identify macrophage NGF production as a central contributor to sympathetic hyperinnervation at the infarct border region. Therefore, macrophage NGF could be a potential therapeutic target to reduce post-infarction adverse effects. Secondly, our findings show that β -blockers induce cardiac sympathetic outgrowth which results in enhanced ventricular neuroeffector functions. This sprouting is mediated by an autocrine neuronal feedback via β_1 adrenergic receptors. Rapid plasticity could thus result as a mean for rapid physiological adaptative responses. Beta-blockers, which inhibit this feedback, should be administered carefully and may be replaced by other therapies in the future.

FIGURE

Figure 1.

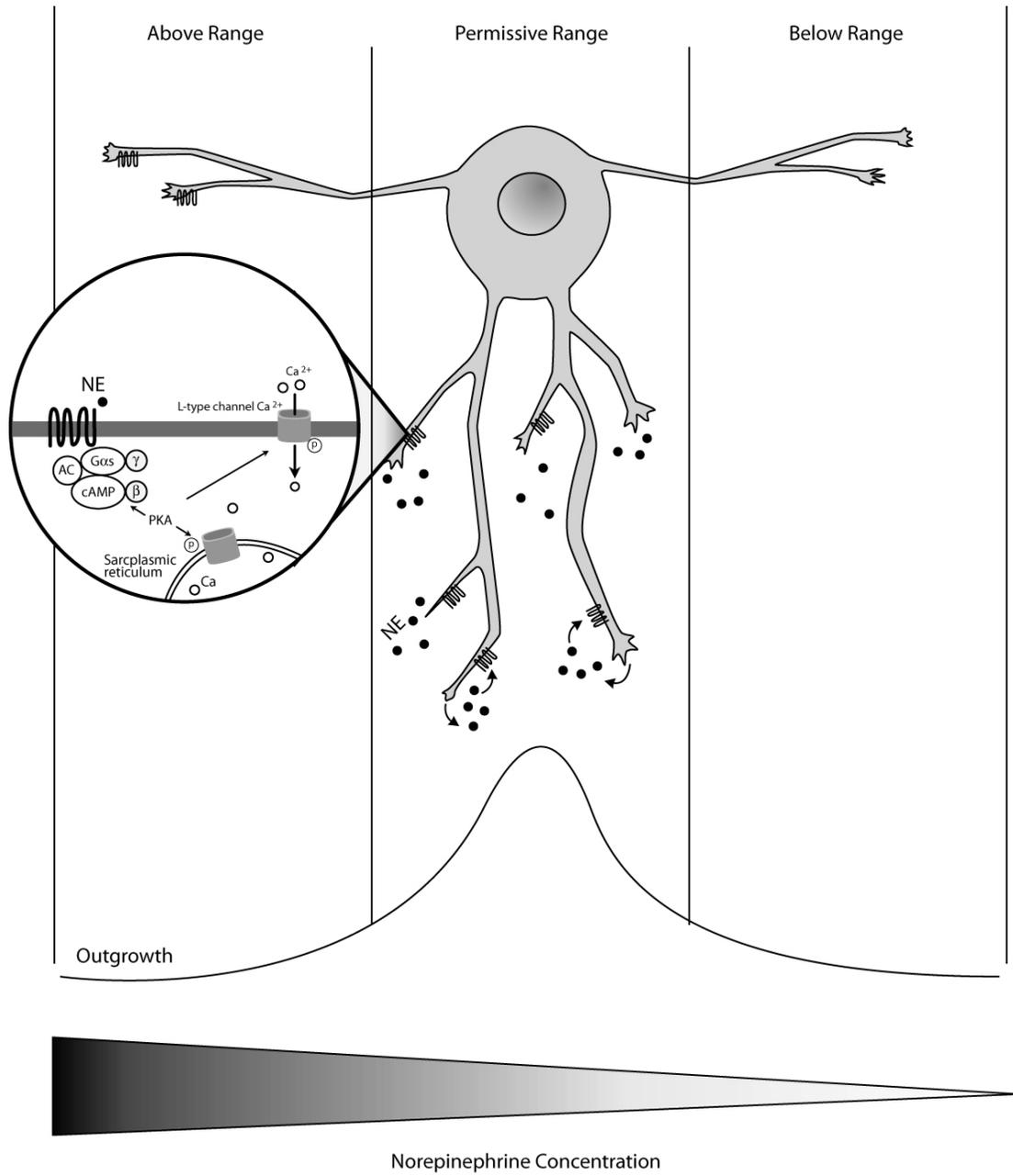


Figure 1. Schematic showing the possible intracellular pathways leading to sympathetic axon outgrowth induced by norepinephrine activation of $\beta 1$ adrenoceptors. A permissive range of norepinephrine binding to $\beta 1$ adrenergic receptor may activate the G_s protein causing the activation of adenylate cyclase (AC) promoting the dissociation of the α subunit from the $\beta\gamma$ subunits. This induces the formation of the second messenger cAMP followed by the activation of protein kinase A (PKA). This cascade may result in the release of calcium from intracellular storage and the influx of calcium via the L-type calcium channel. Since calcium levels are critical for growth cone extension, this mechanism may promote axonal outgrowth.

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