Cardiac sympathectomy promote anti-inflammatory response by activating JAK2-STAT3-mediated signaling cascade in rat myocarditis model: a novel mechanism with clinical implications

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설렘과 두려움을 안고 시작했던 학위 논문, 이제 비로소 모든 과정을 마치며 지난 시간을 되돌아 봅니다. 작지만 수많은 노력이 모여 논문이 완성되어 가는 과정은 저에게 학문의 길 뿐 아니라 인격 수양의 시간이었습니다.

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박사과정을 하면서 많은 분들의 격려와 도움 속에서 20년간의 학교생활을 드디어 마쳤다는 생각에 감사한 마음이 가득해집니다. 학교에서 보낸 제 시간들은 앞으로의 제 인생에서 많은 영향을 미치게 될 것으로 생각됩니다.

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박혜림 올림

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ABSTRACT

Cardiac sympathectomy promote anti-inflammatory response by activating JAK2-STAT3-mediated signaling cascade in rat myocarditis model: a novel mechanism with clinical implications

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Introduction: Left stellectomy has become an important therapeutic option for patients with potentially fatal arrhythmias. However, the antiarrhythmic mechanism of left stellectomy is not well known. Cholinergic anti-inflammatory pathway (CAIP) is a complex immune mechanism that regulates peripheral inflammatory responses. This study evaluated the effect of left stellectomy on CAIP using rat experimental autoimmune myocarditis (EAM) models.

Methods: EAM was produced by injecting 2 mg of porcine cardiac myosin into the footpads of rats. Left stellectomy was performed before EAM

induction. We evaluated the effect of left stellectomy on arrhythmic events, survival, inflammation and CAIP in rats without and with EAM.

Results: Left stellectomy prevented arrhythmia and improved survival in EAM rats. Left stellectomy decreased TNF-α, IL-6, and HMGB1 levels ($P<0.05$ versus EAM) in serum and heart tissues from EAM rats. Serum level of acetylcholine decreased in EAM rats, and increased after left stellectomy in EAM rats. The ratios of phosphated-STAT3/STAT3 and phosphate-JAK2/JAK2 decreased in cell lysates of spleen, liver and heart in EAM rats. However, the same ratios significantly increased after left stellectomy. NF-κB in cell lysates of spleen, liver, and heart increased in EAM rats, but decreased after left stellectomy.

Conclusions: In EAM models, left stellectomy increased survival of the rats while showing antiarrhythmic effects with reduced inflammation via activation of JAK2-STAT3-mediated signaling cascade. Our findings suggest an exciting opportunity to develop new and novel therapeutics to attenuate cardiac inflammation.

Key words: stellectomy, cholinergic, anti-inflammatory, JAK2-STAT3-mediated signaling

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I. INTRODUCTION

Left cardiac sympathetic denervation (LCSD) has become an important therapeutic option in patients with potentially fatal arrhythmias, such as long QT syndrome and catecholaminergic polymorphic ventricular tachycardia.[1-4] Left stellectomy is accompanied by a reflex increase in cardiac vagal efferent nerve activity.[5] Vagus nerve (cranial nerve X) is the main nerve of parasympathetic division of autonomic nervous system. Vagus nerve is a major constituent of inflammatory reflex, which is the neural reflex

mechanism that controls innate immune responses and inflammation during pathogen invasion and tissue injury.[6-9]

Direct electrical stimulation of cholinergic anti-inflammatory pathway (CAIP) with electrodes that generate action potentials in vagus nerve significantly inhibits cytokine production by innate immune cells in spleen, liver, gastrointestinal tract, heart, and other tissues that are innervated by vagus nerve.[10-13] Stimulation of vagus nerve significantly downregulates the production of TNF, IL-1, IL-6 and IL-8, but does not alter the production of anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGFβ). By restraining the output of potentially toxic mediators that are produced by innate immune cells, CAIP protects the organism from organ damage and death during endotoxemia, sepsis, hemorrhagic shock, colitis, arthritis, ileus, pancreatitis and other syndromes of excessive cytokine release.[7,14-20]

Inflammation can cause damage or even death. Molecular and neurophysiological studies during the past decade have revealed that immunity is coordinated by neural circuits that operate reflexively. The afferent reflex arc consists of nerves that can sense injury and infection. This activates efferent neural circuits, including CAIP, which modulate immune responses and progression of inflammatory diseases.[7] α7nAchR, a member of the acetylcholine receptor (AchR) family, is widely expressed by cells of the nervous system. In monocytes and macrophages, α7nAChR signal transduction inhibits phosphorylation of NF-κB (IκB) inhibitor, a proximal

step that regulates activation of NF-κB.[21] α7nAChR ligation also recruits JAK2 to form a heterodimeric complex that initiates signal transduction mediated by signal transducer and activator of transcription 3 (STAT3), a pathway that negatively regulates NF-κB binding to DNA and can also increase the activity of cytokine signaling 3 suppressor as part of anti-inflammatory response.[22] In agreement with the *in vivo* observations using vagus nerve stimulation, downregulation of cytokine expression by α7nAChR signaling in activated cytokine-producing immune cells *in vitro* is selective, as production of anti-inflammatory cytokines, including IL-10 and TGFβ, is not suppressed.[10,23,24] However, the relationship between left stellectomy and CAIP was not evaluated in the heart. Therefore, we hypothesized that left stellectomy could suppress heart inflammation and improve survival by affecting immune activity.

To prove this hypothesis, we evaluated the effect of left stellectomy on CAIP using rat experimental autoimmune myocarditis (EAM) models.[25] We then evaluated the effects of left stellectomy on survival, arrhythmia, and inflammation. JAK2-STAT3-mediated signaling cascade was evaluated in hearts and other organs such as spleen, liver, kidney, and lungs containing innate immune cells.

II. MATERIALS AND METHODS

Our investigation conforms to regulations of the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH Publication, 8th Edition, 2011). Protocols in this study were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine, and Cardiovascular Research Institute (Approval reference number 2011-0136), while in adherence to guidelines of the American Heart Association.

1. Experimental models

To make EAM model, male Lewis rats (250-300 g) were immunized with purified porcine cardiac myosin to induce EAM.[25] To induce myocarditis, we immunized purified cardiac myosin (M0531, Sigma Aldrich, Schnelldorf, Germany) from porcine ventricular muscle. Purified cardiac myosin was emulsified with an equal volume of Complete Freund's Adjuvant (BD biosciences, Heidelberg, Germany) supplemented with mycobacterium tuberculosis H37 Ra (Difco, Detroit, USA) at a concentration of 10 mg/ml. On days 1 and 7, rats were injected subcutaneously in the footpads with 0.2 ml of emulsion, yielding 1.0 mg of cardiac myosin per rat. Rats in the normal control group were injected only with 0.2 ml of Complete Freund's Adjuvant.

To make the left stellectomy model, rats were endotracheally intubated after induction of general anesthesia with isoflurane (2.5 vol% for

induction and 1 vol% for maintenance) in oxygen and mechanically ventilated with a rodent ventilator (Rodent ventilator 683; Harvard Apparatus, Cambridge, MA). Body temperature was kept at a constant 37°C using a heating pad. Electrodes for monitoring heart rate and rhythm were connected to the animals and electrocardiogram (ECG) data were collected for real-time or retrograde analysis. Ventilator was set at 65 cycles/min and 2.5-ml stroke volume. All nerve branches running into the left stellate ganglion were isolated and cut, while ganglion was excised, and gauze was removed. All stellectomy rats exhibited ptosis the day after surgery, which was the initial indicator of a successful left stellectomy (Figure 1A and 1B).

Rats were randomly divided into the following four groups (Figure 1C). The control rats received injections of 0.5 ml of Complete Freund's Adjuvant in the same manner (Control group, n = 12). Six-week-old male Lewis rats were immunized by subcutaneous injection of 2 mg purified cardiac myosin in each rear footpad on days 1 and 8 (Myo group, n = 12). In other rats, left stellectomy was performed before the induction of myocarditis (MyoNB group, n=10). Finally, left stellectomy was performed without myocarditis (NB group, n=7). Ambulatory Holter monitoring was performed using telemetry system (Telemetry research, Auckland, New Zealand).

2. Echocardiographic examination

Fourteen days after immunization, transthoracic echocardiography

was performed on rats that were anesthetized by intraperitoneal administration of pentobarbital sodium (0.25 mg/kg, Dainihon Chemical Co., Osaka, Japan). Echocardiographic examination was done with a 15 MHz transducer (Vivid Q, General Electric-Vingmed, Milwaukee, WI, USA). M-mode echocardiogram was evaluated along the short-axis view of left ventricle at the papillary muscles. Left ventricular end diastolic dimension (LVEDD) and left ventricular end systolic dimension (LVESD) were measured, and left ventricular ejection fraction (LVEF) were calculated from M-mode echocardiograms.[26]

3. Optical mapping

Twenty-one days after the initial immunization, rats (250 – 300 g) were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). Chests were opened via median sternotomy, and hearts were rapidly excised and immersed in cold Tyrode's solution (composition in mmol/L: 125 NaCl, 4.5 KCl, 0.25 $MgCl_2$, 24 $NaHCO_3$, 1.8 NaH_2PO_4, 1.8 $CaCl_2$, and 5.5 glucose). To maintain a pH of 7.4, ascending aorta was immediately cannulated and perfused with 37°C Tyrode's solution equilibrated with 95% O_2 and 5% CO_2. Coronary perfusion pressure was regulated between 80 and 95 mmHg. Two widely spaced bipolar electrodes were used for continuous pseudo-ECG monitoring.

For optical recording, heart contractility was inhibited by 10-17

μmol/L of blebbistatin.[27] Hearts were stained with di-4-ANEPPS (Invitrogen, California, USA), and excited with quasi-monochromatic light (520 ± 30 nm) from two green LED lamps. Emitting light was collected by a camera (MiCAM Ultima, BrainVision, Tokyo, Japan) with a pass filter that was 610 nm long. Data was gathered at 1 ms sampling intervals, acquiring simultaneously from 100 x 100 pixels with spatial resolution of 0.3 x 0.3 mm^2/pixel. Mapped area included parts of the right and left ventricular free walls.

Optical recordings were performed during steady-state and programmed stimulation. Programmed stimulation was performed with bipolar electrodes at the lateral side of left ventricle. Pacing was initiated at a cycle length (CL) of 300 ms, using stimuli of double the pacing threshold, and reduced at decrements of 10 ms until 2:1 capture. APD_{90} was measured at base and apex of left ventricle. APD dispersion was defined as the difference between maximum and minimum APDs. After the initial electrophysiological study, we attempt to induce ventricular tachycardia (VT) or ventricular fibrillation (VF) using standard pacing technique (burst pacing at CLs down to 70 ms). Both sustained (>30 s) and non-sustained VT or VF episodes were documented. Optical mapping and VT induction studies were performed in six rats in each group.

4. Western blotting and immunohistochemistry

Myocardial tissues were fixed in 10% neutral formalin, sliced and embedded in paraffin. The sections were stained with H&E and Masson's trichrome. Rat hearts were homogenized before the lysis-step. Protein concentration was determined using Pierce BCA Protein Assay (Sigma-Aldrich). Protein samples were separated by SDS–polyacrylamide gel and transferred onto nitrocellulose membrane (Bio-Rad, Richmond, CA). Membranes were blocked with 10% non-fat dry milk in 1×TBS containing 1% Tween 20 for one hour. Membrane was also incubated with indicated primary antibodies against GAPDH (1:5,000, Santa Cruz Biotechnology), HMGB1 (1:1,000, Abcam Reagents), IL-6 (1:1,000, Abcam Reagents), TNF-α (1:1,000, Abcam Reagents), phosphorylated STAT3 (1:1000, Santa Cruz Biotechnology), total STAT3 (1:1000, Santa Cruz Biotechnology), phosphorylated JAK2 (1:1000, Cell Signaling), total JAK2 (1:1000, Cell Signaling), α7AchR (1:1000, Cell Signaling), and NF-κB (1:1000, Cell Signaling) overnight at 4°C. After washing, primary antibodies were detected with HRP-conjugated anti-rabbit and anti-mouse IgG (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA) secondary antibodies, respectively. Detection was performed using an enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). Bands were scanned and their intensities were quantified with Image J software.

5. Enzyme-linked immunosorbent assay

Blood was obtained from abdominal aorta of each rat group on day 21. Enzyme-linked immunosorbent assay was performed to determine levels of HMGB1, IL-6, TNF-α, norepinephrine, epinephrine, and acetylcholine in serum. According to the manufacturer's instructions, protein levels in serum were quantified with commercial kits for HMGB1 (IBL International, Hamburg, Germany), IL-6 (R&D System, Minneapolis, MN, USA), TNF-α (R&D System, Minneapolis, MN, USA), norepinephrine (MyBioSource, San Diego, CA, USA), epinephrine (MyBioSource, San Diego, CA, USA) and acetylcholine (R&D System, Minneapolis, MN, USA).

6. Heart rate variability analysis

Heart rate variability (HRV) analysis was performed using the Kubios HRV program. HRV was determined from 5-min segments of ECG data recorded at 1 kHz. The segments chosen for analysis were based on the absence of movement and arrhythmia artifacts. Arrhythmic beats (beats not initiated in the atria, as determined by visual inspection of ECG) were manually removed Frequency-domain. HRV was also calculated for the very low frequency (VLF; 0–0.05 Hz), low frequency (LF; 0.05–0.75 Hz), and high frequency (HF; 0.75–2.5 Hz) bands.

7. Statistical analysis

Data were expressed as the mean ± SEM. ANOVA with post-hoc

test was used to compare the means of four numeric values. Pearson's chi-square tests were used to compare categorical variables. P values <0.05 were considered statistically significant.

III. RESULTS

1. Left stellectomy prevented heart failure and myocardial inflammation in EAM

Figure 1D shows gross pictures of hearts. Left stellectomy prevented cardiomegaly after myocarditis. Compared to EAM rats (Myo group), EAM rat after stellectomy (MyoNB) showed significant decreases in heart weights (1.09 ± 0.10 g vs. 1.70 ± 0.11 g, p<0.001) and ratio of heart to body weight (3.4 ± 0.4 vs. 5.6 ± 0.3, p<0.001) (Figure 1E).

Figure 1F shows histological analysis conducted 21 days after the initial immunization with cardiac myosin. Left stellectomy prevented inflammatory cell infiltration after EAM. EAM-induced fibrosis was also prevented by left stellectomy, showing significantly decreased fibrosis in MyoNB compared to Myo groups (16 ± 2%, vs. 46 ± 5%, p<0.001) (Figure 1G).

The EAM-induced elevation of serum level in inflammatory markers was attenuated by left stellectomy, showing lower serum level of TNF-α (17.8 ± 0.1 vs. 28.0 ± 0.6 pg/ml, p<0.001), IL-6 (55.4 ± 8.7 vs. 107.6 ± 35.8 pg/ml,

p<0.001), and HMGB1 (23.4 ± 1.5 vs. 54.0 ± 2.3 ng/ml, P<0.001) in MyoNB than in Myo group (Figure 1H).

HMGB1, IL-6, and TNF-α expressions of the heart tissue in Myo group were increased by 2.8 (p<0.001), 1.8 (p=0.003), and 2.2 (p=0.005) times than that of control, respectively. However, they did not increase after left stellectomy in MyoNB group (Figure 1I)

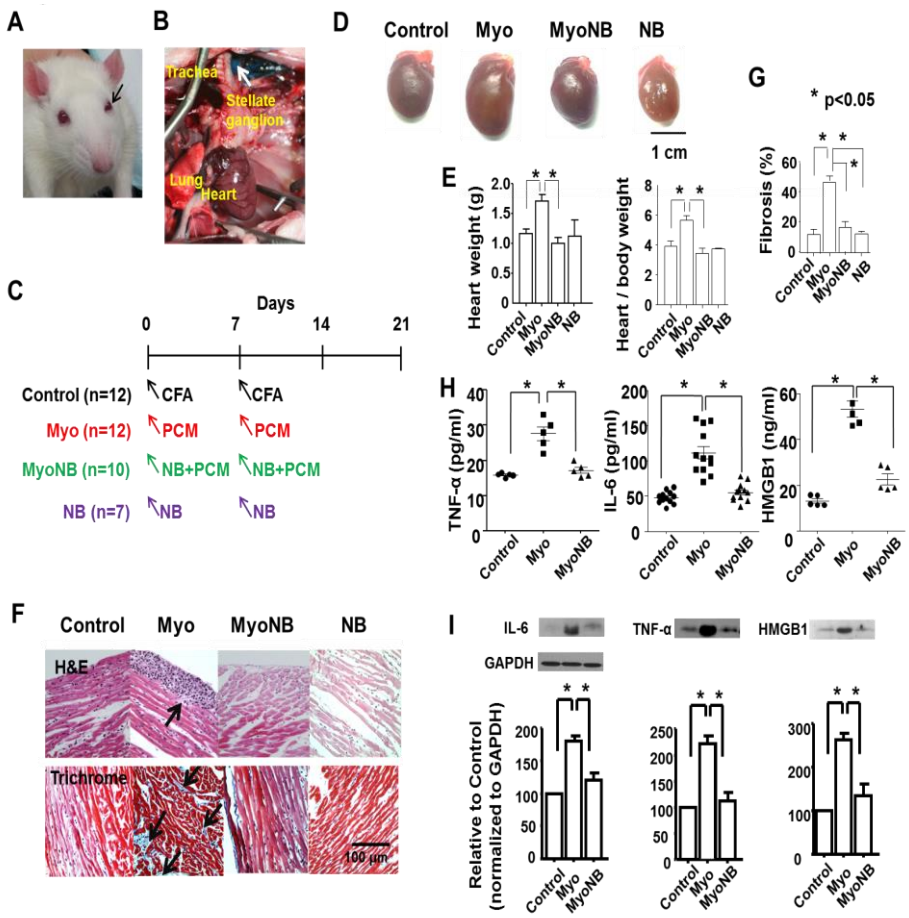

Figure 1. Left stellectomy prevents myocarditis. A, Significant ptosis of the left eye, after left stellectomy. B, Poststernotomy autopsy pictured after stellate ganglion staining with methylene blue. C, Four experimental groups. CFA, Complete Freund's Adjuvant; PCM, purified cardiac myosin; NB, left stellectomy. D, Gross pictures of hearts. F, the comparison of heart weight (*left panel*) and heart/body weight (*right panel*). F, Histological analysis on 21 days after the immunization. G, The comparison of fibrosis. H, ELISA of

serum level of TNF-α, IL-10 and HMGB1. I, Western blot of IL-6, TNF-α and HMGB1 from heart tissue. Data represent mean ± SD of four independent experiments. P-values less than 0.05 were marked with asterisks.

2. Left stellectomy improved survival rate and suppressed arrhythmias in EAM.

Left stellectomy improved survival rate after myocarditis. While four (33%) out of 12 rats died suddenly at 14 ± 4 days after myocarditis in Myo group ($p=0.03$), no (0%) rat died in MyoNB groups ($p=0.04$) (Figure 2A). Left stellectomy significantly shortened EAM-induced prolongation of QT (76.7 ± 5.2 vs. 103.3 ± 8.2 ms, $p<0.001$) and QTc intervals (178.5 ± 9.5 vs. 249.8 ± 23.8 ms, $p<0.001$) (Figure 2B). Figure 2C shows PVC (asterisk), VT (arrow), and VF recorded by ambulatory Holter monitoring. While spontaneous VT/VF were observed in seven (58%) rats in Myo group, only one rat (10%) had VT/VF episodes in MyoNB group, showing significant reduction of VT/ VF episodes after left stellectomy ($p=0.02$) (Figure 2D).

Left stellectomy prevents LV dysfunction in EAM. Figure 2E shows the representative M-mode echocardiogram of each group on day 21. MyoNB group showed significantly improved LVEF compared to Myo group (58 ± 7%, vs. 82 ± 3%, $p<0.001$). MyoNB group presented smaller LVESD compared with Myo group (3.3 ± 0.8 mm vs. 5.3 ± 0.6 mm, $p=0.003$) (Figure 2F).

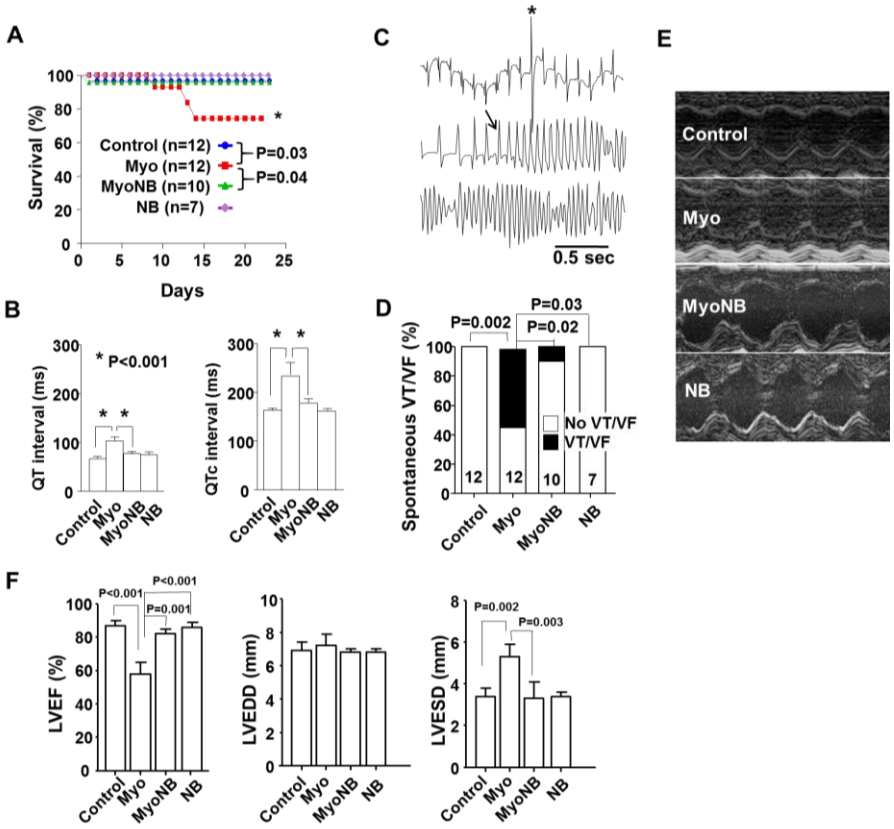

Figure 2. Left stellectomy improved survival and prevented arrhythmias and cardiac dysfunction in EAM. A, Kaplan-Meier survival curves. B, The comparison of QT and QTc intervals. C, Ambulatory Holter monitoring in Myo group. PVC was marked with asterisks at the upper panel. The initiation of VT (arrow of middle panel) and the degeneration to VF (lower panel). D, Comparison of spontaneous VT/VF among 4 groups. E, Representative M-mode echocardiogram on day 21 of 4 groups. F, Comparison of left ventricular ejection fraction (LVEF), LV end diastolic dimension (LVEDD)

and LV end systolic dimension (LVESD). Data represent mean ± SD of four independent experiments. P-values less than 0.05 were marked with asterisks.

3. Left stellectomy improved conduction time and APD dispersion in EAM

Left stellectomy improved conduction time of LV. Figure 3A shows typical action potential traces in four groups at pacing CL of 300 ms. Compared with Myo group, MyoNB showed improvements in conduction time, APD_{90} (153 ± 31 vs. 115 ± 7 ms, p=0.04) and dispersion (17 ± 5 vs. 6 ± 3 ms, p=0.02) (Figure 3B). Activation (upper panels) and APD map (lower panels) show crowding of isochronal lines in Myo group (Figure 3C).

Figure 3D (a) shows typical examples of VT/VF induction in each group. Left stellectomy significantly improved VT/VF inducibililty, showing VT/VF induction of 86% and 14% in Myo and MyoNB groups, respectively (p=0.008).

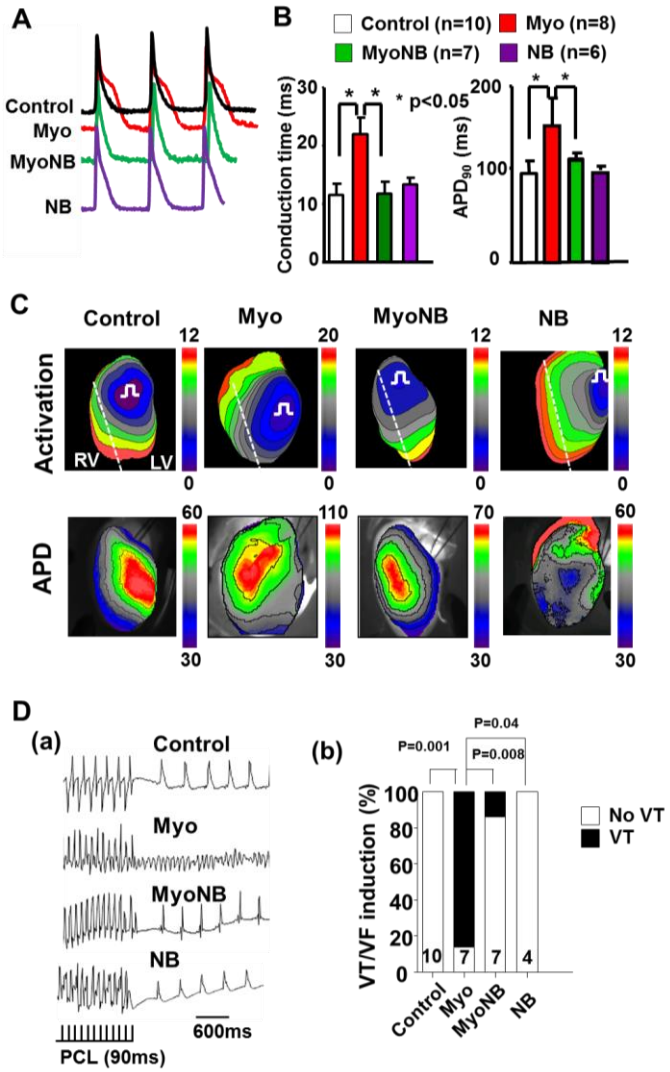

Figure 3. Left stellectomy prevented EAM-induced APD prolongation and dispersion. A, Typical action potential traces of 4 groups at the pacing CL of 300 ms. B, The comparison of conduction time, APD_{90} and APD dispersion. C, Representative activation (upper panels) and APD maps (lower

panels). The dotted line marks the interventricular septum. D. (a) Typical examples of VT induction. (b) The comparison of VT/VF induction rate. Data represent mean ± SD of four independent experiments. P-values less than 0.05 were marked with asterisks.

4. Effect of left stellectomy on cholinergic anti-inflammatory signaling

Compared to control, Myo group had significantly increased level of norepinephrinie (227±17, vs. 485±42 pg/ml, $p<0.001$) and epinephrinie (198±7, vs. 305±5 pg/ml, $p<0.001$). Left stellectomy prevented EAM-induced increases of norepinephrine (279±16 pg/ml, $p<0.001$) and epinephrine (235±12 pg/ml, $p=0.01$). Compared to control, left stellectomy also significantly increased the ratio of acetylcholine level (1.00±0.04 vs. 1.39±0.17, $p=0.03$) in MyoNB group (Figure 4C).

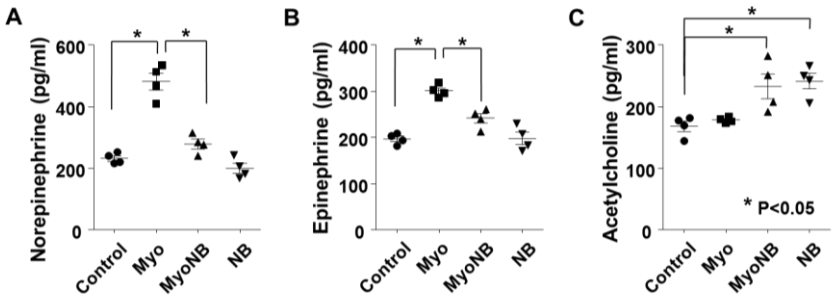

Figure 4. Effect of left stellectomy on norepinephrine, epinephrine, and acetylcholine. ELISA of norepinephrine (a), epinephrine (b) and acetylcholine (c) in blood of four groups. Data represent mean ± SD of four independent experiments. P-values less than 0.05 were marked with asterisks.

5. Effect of left stellectomy on heart rate variability

Representative power spectra from data collected from 4 groups (Figure 5A). Ratio of LF/HF shows how left stellectomy effects on autonomic regulation. It was observed that the LF/HF ratios of the Myo group. Compared to control, Myo group had significantly increased the level of LF/HF (0.40±0.17 vs 0.15±0.02, $p<0.001$). Left stellectomy prevented EAM-induced increases of LF/HF (0.18±0.04, $p<0.001$) (Figure 5B).

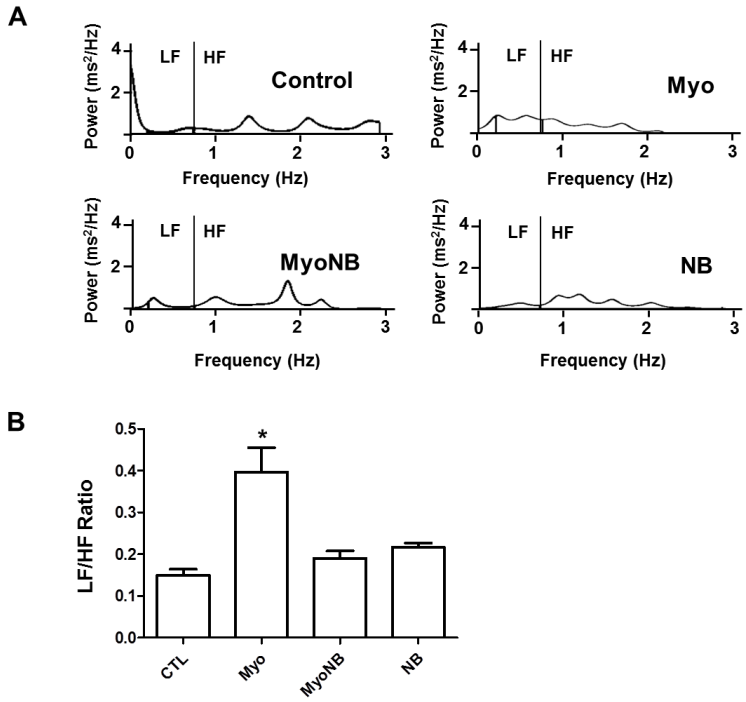

Figure 5. The findings of autonomic balance of LF and HF components of power. A. Representative power spectra. Autonomic controls in cardiac regulation and modulation for all 5-min segments. B. heart rate variability-low frequency (LF) to high frequency (HF) power ratio, an index of cardiac sympathetic activity. Results are represented as means ± SD. **P* < 0.05 CTL vs. Myo.

6. Left stellectomy activated JAK/STAT pathway in spleen, liver, heart and other organs

To determine whether JAK/STAT pathway is activated after left stellectomy, phosphorylation and total amount of STAT3 and JAK2 in cell lysates from spleen, liver, heart kidney and lung were examined (Figure 6). In cell lysate from spleen, compared with control (112±12%), Myo group showed significantly decreased p-STAT3/STAT3 (80±14%, $p=0.04$) and p-JAK2/JAK2 ratio (47±4%, $p<0.001$). However, the levels of p-STAT3/STAT3 ratio (491±10%, $p<0.001$ vs. Myo group) and p-JAK2/JAK2 ratio (177±4%, $p<0.001$ vs. Myo group) significantly increased in MyoNB group. The level of NF-κB increased in Myo group, and decreased in MyoNB group (Myo 178±10% vs. MyoNB 112±3%, $p<0.001$).

In cell lysate from liver, compared with control (105±6%), Myo group showed significantly decreased p-STAT3/STAT3 (43±6%, $p<0.001$) and p-JAK2/JAK2 ratio (63±1%, $p<0.001$). However, the level of p-STAT3/STAT3 ratio (407±7%, $p<0.001$ vs. Myo group) and p-JAK2/JAK2 ratio (90±4%, $p<0.001$ vs. Myo group) significantly increased in MyoNB group. The level of NF-κB increased in Myo group, and decreased in MyoNB group (Myo 156±8% vs. MyoNB 104±7%, $p<0.001$).

In cell lysate from heart, compared with control (100±5%), Myo group showed significantly decreased p-STAT3/STAT3 (37±7%, $p<0.001$) and p-JAK2/JAK2 ratio (65±10%, $p=0.001$). However, MyoNB group showed

significantly increased p-STAT3/STAT3 ratio (120±2%, $p<0.001$) and p-JAK2/JAK2 ratio (89±7%, $p=0.01$) compared to Myo group. The level of NF-κB increased in Myo group, and decreased in MyoNB group (Myo 132±5% vs. MyoNB 109±3%, $p<0.001$).

Left stellectomy also increased p-STAT3/STAT3 and p-JAK2/JAK2 ratio, and decreased NF-κB in kidney cell lysates. However, significant influence of left stellectomy was not observed in lung tissue lysates.

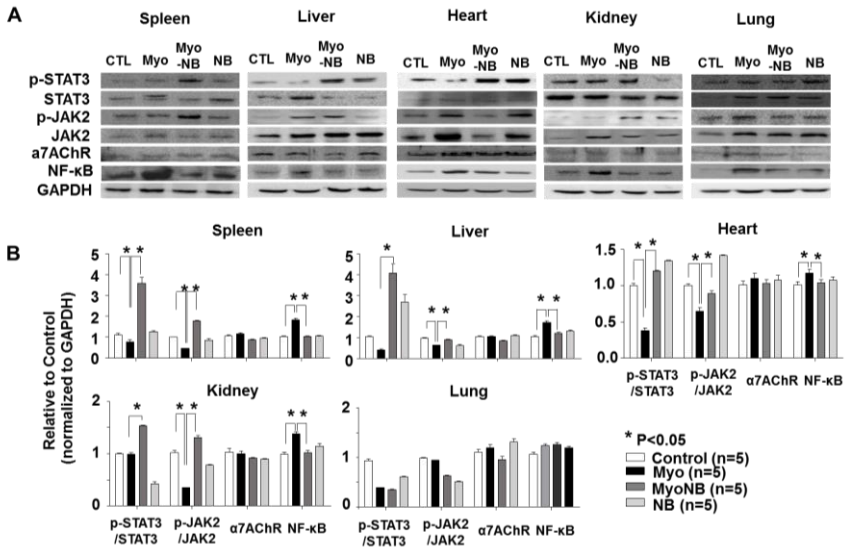

Figure 6. Left stellectomy activates STAT3 and JAK2 in spleen, kidney, liver and heart. A, Immunoblot of p-STAT3, STAT3, p-JAK2, JAK2, α7AchR, NF-κB and GAPDH in cell lysates of spleen, liver, heart, kidney and lung from four groups. B, Comparison of p-STAT3/STAT3, p-JAK2/JAK2 α7AchR and NF-κB in cell lysates of spleen, liver, heart, kidney and lung from four groups.

IV. DISCUSSION

The main findings of this study were that left stellectomy prevented arrhythmia and improved survival by attenuating inflammation in EAM. Second, left stellectomy increased acetylcholine level while decreasing norepinephrine and epinephrine. Finally, left stellectomy increased JAK2/STAT3 pathway in cell lysate from the heart, spleen, liver, and kidney. These results suggest that beneficial effects of left stellectomy might be related with CAIP activation.

Left stellectomy improved survival by attenuating inflammation in EAM

Myocarditis is a fatal inflammatory disease of the heart, with consequent loss of cardiomyocytes and fibrosis development.[28,29] Loss of cardiomyocytes could lead to ventricular remodeling, heart failure, and malignant arrhythmias. This study shows that LCSD using left stellectomy improved survival by decreasing QT prolongation and arrhythmogenicity induced by EAM. Moreover, left stellectomy prevented ventricular dysfunction after EAM.

LCSD has been shown to be a safe and effective treatment option for reducing frequency of life-threatening ventricular arrhythmias in patients with heritable channelopathies,[1-4] as well as in a small cohort of adult patients with HCM.[30] There are numerous effects of LCSD that contribute to its clinical efficacy. LCSD raises the threshold for VF in subjects with ischemia, making

it more difficult for the heart to fibrillate.[31,32] LCSD reduces arrhythmia burden after acute myocardial ischemia in anesthetized and conscious animal models[31] of sudden death. LCSD does not reduce heart rate or impair myocardial contractility, as the right-sided stellate ganglion and sympathetic chain provide adequate compensation.[32,33] However, relationship between LCSD and inflammation has not been evaluated. This study shows that left stellectomy decreased inflammation and level of pro-inflammatory cytokines in EAM.

Left stellectomy activated cholinergic anti-inflammatory pathway

In this study, left stellectomy decreased norepinephrine and epinephrine. It was consistently reported that norepinephrine level decreased after sympathetic denervation.[34,35] However, the change of acetylcholine after sympathetic denervation was not well-defined. Interestingly, left stellectomy also increased acetylcholine level. Cholinergic anti-inflammatory pathway is a brain-to-immune mechanism that regulates inflammatory responses via α7-nicotinic acetylcholine receptor subunit (α7-nAChR)-dependent, vagus nerve signaling.[36] Cholinergic stimulation (via electrical stimulation of the vagus nerve or selective cholinergic agonists) inhibits cytokine production in preclinical models of systemic inflammation, including endotoxemia, hemorrhagic shock, ischemia-reperfusion injury, and polymicrobial sepsis.[19,24,37,38] Recent studies indicate that spleen is an essential component of

the α7-nAChR dependent pathway, since neural-to-spleen signals regulate cytokine production during endotoxemia.[13,39] In addition, innervation of splenic nerve is critical for mediating the anti-inflammatory effects of vagus nerve stimulation (VNS) on LPS-induced cytokine production.[40]

Nicotine acts on macrophages via recruitment of JAK2 to α7nAChR and activation of JAK2/STAT3 pathway, thereby initiating the anti-inflammatory and SOCS3 signaling cascade.[22] We found activation of JAK2/STAT3 in cell lysates of spleen, liver, heart and kidney in response to left stellectomy, which indicates activation of STAT3 induced by acetylcholine derived from vagal efferents. Acetylcholine, which was released by vagal ends, diffused in spleen, liver, heart and kidney resident macrophages before being completely hydrolyzed by serum acetylcholinesterases and blunting the NF-κB–triggered inflammatory cascade (Figure 7).

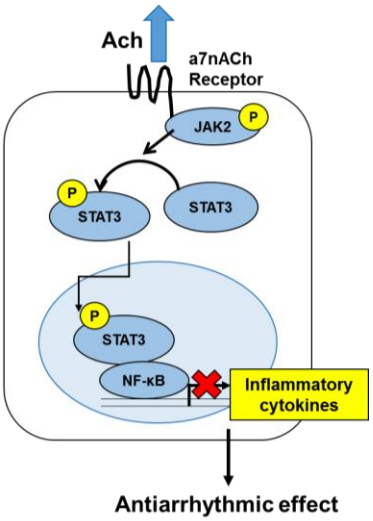

Figure 7. Mechanism of function of efferent arc. In cholinergic anti-inflammatory pathway, acetylcholine binding to nicotinic acetylcholine receptor subunit α7 (α7nAChR) leads to formation of heterodimeric protein complex with Janus kinase 1 (JAK2), which activates signal transducer and activator of transcription 2 (STAT3). Together, these signaling cascades lead to inhibition of pro-inflammatory cytokine release. NF-κB, nuclear factor κB;

We induced myocarditis by the injecting cardiac myosin. Therefore, our study cannot explain the arrhythmogenic mechanisms of myocarditis caused by viral infection or other etiologies. However, autoimmunization to myosin might be one of the final common pathways of myocarditis. Moreover, inflammation and oxidative stress are frequently observed in diseased hearts. Finally, in order to reveal which cells are involved in the process CAIP of heart, both isolation and assay of each heart cell type are needed.

V. CONCLUSION

In EAM, left stellectomy prevented arrhythmia and improved survival with suppression of inflammation. Left stellectomy increased acetylcholine level and JAK2/STAT3 pathway in cell lysate from the spleen, liver, heart, and kidney after EAM. These results suggest that beneficial effects of left stellectomy might be related with CAIP activation.

REFERENCES

1. Collura CA, Johnson JN, Moir C, Ackerman MJ. Left cardiac sympathetic denervation for the treatment of long QT syndrome and catecholaminergic polymorphic ventricular tachycardia using video-assisted thoracic surgery. *Heart Rhythm* 2009;6:752-759.
2. Moss AJ, McDonald J. Unilateral cervicothoracic sympathetic ganglionectomy for the treatment of long QT interval syndrome. *N Engl J Med* 1971;285:903-904.
3. Schwartz PJ, Priori SG, Cerrone M, Spazzolini C, Odero A, Napolitano C *et al.* Left cardiac sympathetic denervation in the management of high-risk patients affected by the long-QT syndrome. *Circulation* 2004;109:1826-1833.
4. Wilde AA, Bhuiyan ZA, Crotti L, Facchini M, De Ferrari GM, Paul T *et al.* Left cardiac sympathetic denervation for catecholaminergic polymorphic ventricular tachycardia. *N Engl J Med* 2008;358:2024-2029.
5. Cerati D, Schwartz PJ. Single cardiac vagal fiber activity, acute myocardial ischemia, and risk for sudden death. *Circ Res* 1991;69:1389-1401.
6. Tracey KJ. The inflammatory reflex. *Nature* 2002;420:853-859.
7. Tracey KJ. Reflex control of immunity. *Nat Rev Immunol* 2009;9:418-428.

8. Andersson U, Tracey KJ. Reflex principles of immunological homeostasis. *Annu Rev Immunol* 2012;30:313-335.

9. Pavlov VA, Tracey KJ. The vagus nerve and the inflammatory reflex--linking immunity and metabolism. *Nat Rev Endocrinol* 2012;8:743-754.

10. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR *et al.* Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 2000;405:458-462.

11. Bernik TR, Friedman SG, Ochani M, DiRaimo R, Ulloa L, Yang H *et al.* Pharmacological stimulation of the cholinergic antiinflammatory pathway. *J Exp Med* 2002;195:781-788.

12. Saeed RW, Varma S, Peng-Nemeroff T, Sherry B, Balakhaneh D, Huston J *et al.* Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation. *J Exp Med* 2005;201:1113-1123.

13. Huston JM, Ochani M, Rosas-Ballina M, Liao H, Ochani K, Pavlov VA *et al.* Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis. *J Exp Med* 2006;203:1623-1628.

14. Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S *et al.* Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature* 2003;421:384-388.

15. Ghia JE, Blennerhassett P, Collins SM. Impaired parasympathetic function increases susceptibility to inflammatory bowel disease in a mouse model of depression. *J Clin Invest* 2008;118:2209-2218.

16. Ghia JE, Blennerhassett P, Kumar-Ondiveeran H, Verdu EF, Collins SM. The vagus nerve: a tonic inhibitory influence associated with inflammatory bowel disease in a murine model. *Gastroenterology* 2006;131:1122-1130.

17. van Westerloo DJ, Giebelen IA, Florquin S, Bruno MJ, Larosa GJ, Ulloa L *et al.* The vagus nerve and nicotinic receptors modulate experimental pancreatitis severity in mice. *Gastroenterology* 2006;130:1822-1830.

18. van Westerloo DJ, Giebelen IA, Florquin S, Daalhuisen J, Bruno MJ, de Vos AF *et al.* The cholinergic anti-inflammatory pathway regulates the host response during septic peritonitis. *J Infect Dis* 2005;191:2138-2148.

19. Guarini S, Altavilla D, Cainazzo MM, Giuliani D, Bigiani A, Marini H *et al.* Efferent vagal fibre stimulation blunts nuclear factor-kappaB activation and protects against hypovolemic hemorrhagic shock. *Circulation* 2003;107:1189-1194.

20. Kawada T, Yamazaki T, Akiyama T, Kitagawa H, Shimizu S, Mizuno M *et al.* Vagal stimulation suppresses ischemia-induced myocardial interstitial myoglobin release. *Life Sci* 2008;83:490-495.

21. Yoshikawa H, Kurokawa M, Ozaki N, Nara K, Atou K, Takada E *et al.* Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I-kappaB phosphorylation and nuclear factor-kappaB transcriptional activity through nicotinic acetylcholine receptor alpha7. *Clin Exp Immunol* 2006;146:116-123.

22. de Jonge WJ, van der Zanden EP, The FO, Bijlsma MF, van Westerloo DJ, Bennink RJ *et al.* Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat Immunol* 2005;6:844-851.

23. Parrish WR, Rosas-Ballina M, Gallowitsch-Puerta M, Ochani M, Ochani K, Yang LH *et al.* Modulation of TNF release by choline requires alpha7 subunit nicotinic acetylcholine receptor-mediated signaling. *Mol Med* 2008;14:567-574.

24. Pavlov VA, Ochani M, Yang LH, Gallowitsch-Puerta M, Ochani K, Lin X *et al.* Selective alpha7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis. *Crit Care Med* 2007;35:1139-1144.

25. Kodama M, Matsumoto Y, Fujiwara M, Masani F, Izumi T, Shibata A. A novel experimental model of giant cell myocarditis induced in rats by immunization with cardiac myosin fraction. *Clin Immunol Immunopathol* 1990;57:250-262.

26. Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA *et al.* Recommendations for chamber quantification: a report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. *J Am Soc Echocardiogr* 2005;18:1440-1463.

27. Joung B, Tang L, Maruyama M, Han S, Chen Z, Stucky M *et al.* Intracellular calcium dynamics and acceleration of sinus rhythm by beta-adrenergic stimulation. *Circulation* 2009;119:788-796.

28. Eriksson U, Penninger JM. Autoimmune heart failure: new understandings of pathogenesis. *Int J Biochem Cell Biol* 2005;37:27-32.

29. Feldman AM, McNamara D. Myocarditis. *N Engl J Med* 2000;343:1388-1398.

30. Bourke T, Vaseghi M, Michowitz Y, Sankhla V, Shah M, Swapna N *et al.* Neuraxial modulation for refractory ventricular arrhythmias: value of thoracic epidural anesthesia and surgical left cardiac sympathetic denervation. *Circulation*;121:2255-2262.

31. Schwartz PJ, Stone HL. Left stellectomy in the prevention of ventricular fibrillation caused by acute myocardial ischemia in

conscious dogs with anterior myocardial infarction. *Circulation* 1980;62:1256-1265.

32. Schwartz PJ, Stone HL. Effects of unilateral stellectomy upon cardiac performance during exercise in dogs. *Circ Res* 1979;44:637-645.

33. Schwartz PJ. The rationale and the role of left stellectomy for the prevention of malignant arrhythmias. *Ann N Y Acad Sci* 1984;427:199-221.

34. Gao J, Kerut EK, Smart F, Katsurada A, Seth D, Navar LG *et al.* Sympathoinhibitory Effect of Radiofrequency Renal Denervation in Spontaneously Hypertensive Rats With Established Hypertension. *Am J Hypertens* 2016.

35. Brandon KW, Rand MJ. Acetylcholine and the sympathetic innervation of the spleen. *J Physiol* 1961;157:18-32.

36. Tracey KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. *J Clin Invest* 2007;117:289-296.

37. Bernik TR, Friedman SG, Ochani M, DiRaimo R, Susarla S, Czura CJ *et al.* Cholinergic antiinflammatory pathway inhibition of tumor necrosis factor during ischemia reperfusion. *J Vasc Surg* 2002;36:1231-1236.

38. Giebelen IA, van Westerloo DJ, LaRosa GJ, de Vos AF, van der Poll T. Stimulation of alpha 7 cholinergic receptors inhibits lipopolysaccharide-induced neutrophil recruitment by a tumor

necrosis factor alpha-independent mechanism. *Shock* 2007;27:443-447.

39. Huston JM, Wang H, Ochani M, Ochani K, Rosas-Ballina M, Gallowitsch-Puerta M *et al.* Splenectomy protects against sepsis lethality and reduces serum HMGB1 levels. *J Immunol* 2008;181:3535-3539.

40. Rosas-Ballina M, Ochani M, Parrish WR, Ochani K, Harris YT, Huston JM *et al.* Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia. *Proc Natl Acad Sci U S A* 2008;105:11008-11013.

< ABSTRACT(IN KOREAN)>

쥐 심근염 모델에서 심장교감신경절제술을 통한 JAK2-STAT3 신호전달 활성화에 따른 항염증 효과

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박 혜 림

연구 배경 : 교감신경절 차단술은 잠재적으로 치명적인 부정맥을 가진 환자에게 중요한 치료로 사용되어 왔다. 그러나 성상신경절 차단술의 항부정맥 메커니즘은 잘 알려져 있지 않다. 콜린성 항염증 경로는 말초의 염증 반응을 조절하는 복잡한 면역 기전이다. 이 연구는 쥐 심근염 모델을 사용하여 교감신경 절제술이 콜린성 항염증 경로에 미치는 영향을 평가하였다.

연구 방법 : 심근염 모델은 심근염 모델은 porcine cardiac myosin

(10mg/ml) 0.2 ml를 Lewis rat에 0일째 7일째 발바닥에 주사하여 만들었다 (심근염군). 교감신경차단술은 심근염 모델이 만들어지기 전 시행하였다.

연구 결과 : 교감신경차단술은 부정맥을 예방하고 심근염 쥐의 생존율을 향상시켰다. 또한 TNF-α, IL-6 및 HMGB1의 발현량을 감소시켰다. (심근염군과 비교, P<0.05). 혈청에서의 아세틸콜린은 심근염군에서 감소하였고, 신경차단술을 시행한 심근염군에서는 다시 증가함을 보였다. STAT3와 JAK2의 인산화 비율이 심근염군에서의 비장, 간 및 심장에서 줄어들었지만, 교감신경차단술을 시행한 그룹에서는 다시 회복이 되었다. NF-κB의 단백질 발현도 심근염 군에서는 유의하게 증가하였으나, 교감신경차단술을 시행한 심근염에서는 감소함을 보였다.

결론 : 심근염 모델에서의 교감신경절제술은 JAK2-STAT3 매개 신호 전달의 활성화를 통해 염증감소를 보여주었고, 항부정맥효과를 나타내며 생존율을 증가시켰다. 우리의 연구 결과를 토대로 심장 염증 반응을 조절하는데 유용한 역할을 할 수 있음을 확인하였다.

핵심되는 말: 교감신경차단술, 콜린성 항염증경로, JAK2 -STAT3 매개 신호전달, 부정맥