A Novel Role of Sympathetic Activity in Regulating Mitral Valve Prolapse

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**Background:** Increased sympathetic activity, commonly reported in mitral valve prolapse, indicates that the sympathetic nervous system might play an important role in regulating mitral interstitial cells. Hence, the aim of this study is to determine the level and pattern of adrenergic receptors expressed in human mitral valve leaflets and to investigate the effect of norepinephrine on physiologic behaviors of mitral interstitial cells.

**Methods and Results:** Immunohistochemistry displayed significantly increased expressions of β1, β2, and α1 adrenergic receptors in mitral valve prolapse. Norepinephrine was found to activate the phenotype of interstitial cells with increased α-SMA expression (2.26 fold). In synthesis, norepinephrine downregulated levels of mRNA for type I to type III collagen in ratio, but increased the elastin gene transcription and glycosaminoglycan levels in valve interstitial cells greatly. In view of the extracellular matrix remodel, sympathetic effects presented catabolic metabolism displaying significantly increased expressions of total, secretory and active MMP-2 protein (matrix metalloproteinase-2), as well as MMP-9 protein. Diminished MMP inhibitor expression, TIMP2, also could reflect this effect in the norepinephrine medium.

**Conclusions:** A novel role for the sympathetic effect in influencing physiologic behaviors in mitral interstitial cells was identified. It is indicated that sympathetic activity could promote myxomatous degeneration in mitral valve prolapse, propagating the disease severity, which might identify potential therapeutic targets. (Circ J 2014; 78: 1486–1493)

**Key Words:** Adrenergic receptors; Mitral interstitial cells; Mitral valve prolapse; Sympathetic activity
USA) and collagenase (Sigma-Aldrich, USA) were used to digest the tissue. After digestion, the cell pellet was collected and plated into a tissue culture flask for 1 h. After preplating, the unattached cells were removed and fresh DEMEM (Dulbecco’s Modified Eagle’s Medium) (Invitrogen, USA) with 10% HIFBS and plated into a tissue culture flask for 1 h. After preplating, the digest the tissue. After digestion, the cell pellet was collected USA) and collagenase (Sigma-Aldrich, USA) were used to

**Immunohistochemistry**

Sections of tissue were incubated separately for 1 h with antibodies against β1-ARs (ABBIOTEC), β2-ARs, and α1-ARs (Abcam). Negative controls consisted of 3% BSA (bovine serum albumin) in PBS (phosphate buffered saline). An anti-rabbit HRP/DAB (horseradish peroxidase/diaminobenzidine) detection system was used to visualize the expression according to the protocol. Sections were counterstained with hematoxylin and viewed under a microscope. The intensity of positive staining in tissue sections was analyzed by integrated optical density (IOD) using Image-Pro Plus 6.0. Briefly, three 40× TIF format images from 3 individuals in each group were analyzed in a blinded manner. All of the images were taken using the same microscope and camera sets. Image-Pro Plus software was used to calculate the average IOD per stained area (μm²) (IOD/area) for positive staining, namely protein expression. The results of 9 images were averaged for the final value of each group.

**Real-Time Quantitative PCR**

Total RNA was extracted using Trizol reagent (Takara), and cDNA was assessed by using a Promega RT-PCR kit. Quantification of the relative mRNA abundance was performed using the ABI One Step Sequence Detection System (Applied Biosystems). Specific primers were designed based upon respective cDNA sequences or from previous literature (Table). As an endogenous reference, the globally expressed housekeeping gene, GAPDH (glyceraldehyde 3-phosphate dehydrogenase), was used. Samples were performed in triplicate, and the differences of threshold cycles between target genes and GAPDH were calculated. The relative mRNA abundance in the treatment groups were calculated using the 2^-ΔΔCt method, using the control group as the calibrator according to the user manual.

**Western Blotting**

Total protein homogenates (40μg) were denatured, fractionated by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked (3% wt/vol non-fat dried milk in PBS containing 0.05% Tween-20) and then probed using primary antibodies against α-SMA (Santa Cruz), MMP2, MMP9, TIMP2 (R&D Systems) and TGF-β1(Santa Cruz). Visualization of the protein bands was accomplished using enhanced chemiluminescence (ECL) and captured on X-ray film. Data were presented as the target protein to GAPDH ratio.

**ELISA for MMP2 and Glycosaminoglycan Concentration in Culture Supernatant**

Protein concentrations in the VIC culture supernatant were measured by the human MMP2 and Glycosaminoglycan ELISA kit according to the manufacturer’s instructions (Invitrogen and EIAab). In brief, a 100μl of Standard, Blank, or Sample was added to each well for 2h at 37°C. One hundred microliters of Detection Reagent A working solution was incubated in each well for 1h at 37°C. After the last complete wash, 100μl of Detection Reagent B working solution was added to each well for 1h at 37°C before 90μl of Substrate Solution was added to each well within 30min at 37°C and then 50μl of Stop Solution was added to each well also. To determine the optical density of each well all at once, a microplate reader was set to 450nm. Concentrations of MMP2 and glycosaminoglycan in the culture supernatant were determined by interpolation from the standard curve.

**Gelatin Zymography**

From each group, 20μl of culture supernatant was applied to gelatin zymography. Gelatin-degrading activity was examined by electrophoresis on 10% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed in 2.5% Triton X-100 to remove SDS (sodium dodecyl sulfate), incubated overnight at 37°C and stained with 0.1% Coomassie Brilliant Blue R (Sigma Aldrich). Enzyme activities were quantified by densitometric analysis using Quantity One software (Bio-Rad Laboratories).

**Statistics**

Data were presented as mean±standard deviation (SD). Differences between groups were tested using one-way ANOVA, with...
post-hoc Dunnet C test. A two-sided probability level of $P<0.05$ was considered statistically significant. All analyses were done with SPSS for Windows 13.0.

### Results

#### Expression of Adrenergic Receptors in Human Mitral Valves

The mitral valve could be influenced mainly by the autonomic nervous system through neurotransmitters: norepinephrine and acetylcholine in the endocrine and paracrine systems, respectively. Consequently, binding the specific receptor triggers a cascade of reactions that alter physiological function within the cell. Therefore, expressions of adrenergic receptors in human mitral valves can reflect the regulatory state regarding sympathetic effects. In the immunohistochemistry analysis, weak staining of the cell was visualized to demonstrate low expressions of $\beta_1$-ARs, $\beta_2$-ARs, and $\alpha_1$-ARs in control mitral valve leaflets. In the mitral valve prolapse, the immunohistochemistry was intense, thus allowing us to see the deposits of strong staining for these adrenergic receptors (Figure 1).

#### Phenotype Transition in Mitral Valve Interstitial Cells

In gross cell appearance, valve interstitial cells in monolayer cultures showed elongated (spindle-shaped) morphology and formed an orthogonal pattern of overgrowth at post-confluence that resembled fibroblasts. In addition to the sympathetic neurotransmitter, norepinephrine, cells took on the features of myofibroblasts presenting increased contraction, prominent stress fibers, and other contractile appearances.

Moreover, in a molecular biology investigation, $\alpha$-smooth muscle actin ($\alpha$-SMA), the marker for activated valve interstitial cells, was evaluated in vitro. The morphological changes were accompanied by an increase in the expression of $\alpha$-SMA protein, which was 2.26-times upregulated by norepinephrine (10$^3$nmol/L) in comparison to the control ($P<0.05$). And when VICS were co-incubated with the adrenergic receptor antagonists, phenolamine, propranolol or prazosin, respectively, the enhancement of the SMA protein by NE was significantly di-
Dose response plots of collagen type I and III production are shown in Figure 3. When challenged with NE in gradient concentrations, the interstitial cell synthesized decreasing collagen type I mRNA in a concentration-dependent manner (Figure 3A), reaching the most inhibitory effect at 1 mmol/L (up to 18.3% of the control). Furthermore, pretreatment of interstitial cells with prazosin (Praz), phentolamine (Phent), and propranolol (Propr) (C and D) Collagen typeIII mRNA response to norepinephrine (NE) alone, and pretreated with prazosin (Praz), phentolamine (Phent), and propranolol (Propr). E The ratio of collagen type I and III mRNA response to norepinephrine (NE). (F) Elastin mRNA response to norepinephrine (NE). (G) Glycosaminoglycan (GAG) in the cultured supernatant and the response to norepinephrine (NE). *P<0.05 vs. Control. †P<0.05 vs. NE.

Dose response plots of procollagen III in interstitial cells diminished (all P<0.05). Of these, the antagonist with the most prominent inhibitory effect was phentolamine, a non-selective α-AR blocker (59.32% of the NE-induced group) Figure 2.

**Synthesis of Extracellular Matrix in Mitral Valve Interstitial Cells**

Valve interstitial cells produce, secrete, and degrade most of the surrounding extracellular matrix, which consists primarily of collagen, elastin, and glycosaminoglycans to maintain a physiological matrix and to remodel the valve tissue according to external signals.

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Above all, the secretion of glycosaminoglycans (GAG) in the valve interstitial cell is closely associated with parallel collagen fibrils, and the cross-linking and maturation of elastic fibers, which contributes to the mechanical function of heart valves. The administration of NE significantly upregulated the GAG secretion (3.05-fold) by interstitial cells in cultural supernatant, which was quantified by ELISA. Figure 3G illustrates the response.

Matrix Metalloproteinase Family

Integrity of the heart valve in anatomy and function is required for the maintenance of the extracellular matrix, in which disruption caused by specific matrix metalloproteinases (MMPs), called remodeling, would lead to valves disease. Among the MMPs, MMP2, MMP9, and TIMP2 were reported to play critical roles in the transformation process.

Our next initiative was to identify the response of elastin, which contributes partly to the morphogenesis of heart valves under sympathetic stimulation. We found that interstitial cells exhibited augmented elastin mRNA abundance in a dose-dependent manner when NE was administered, as assessed by quantitative real-time PCR (an increase up to 31.46%; Figure 3F). And adrenergic antagonists such as prazosin, propranolol or phentolamine did not have this same effect.

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As illustrated in Figure 4A, cells in the presence of norepi-
neurine (1×10^2 to 2×10^9 mmol/L) secreted increasingly MMP2 levels in the supernatant compared to control cells, as evaluated by ELISA. And the peak activity (3.58-fold) was observed at 100 mmol/L, which was regarded as the maximum challenging concentration as below used. To determine whether a similar process was operative in active MMP2, cultures were incubated for 48h in serum-free medium with a maximizing effective concentration of NE. That is 100 mmol/L of NE. In line with the secretary protein, Western blot analysis using total protein from cultured interstitial cells with NE (100 mmol/L) showed an increase in the level of total MMP2 expression. Pretreatment with propranolol, phenolamine or prazosin inhibited the inducing effect of norepinephrine on total MMP2 levels (all P<0.05 vs. NE). Pretreatment with propranolol maximized the inhibition (22.95%) but still exhibited a higher level of MMP2 relative to control cells (Figure 4B). In fact, MMPs are initially secreted as an inactive proenzyme or zymogen, which should be converted to the active form when gelatinolytic activity is performed. To determine whether a similar process was operative in active MMP2, cultures were incubated for 48h in serum-free medium with a maximizing effective concentration of NE. In a zymography assay, cell culture supernatant, in addition to NE, produced significantly clearer bands in a 72-kDa marker compared to the control, indicating the enhancing potential by NE in active MMP2 (1.7-fold). The bands, which corresponded to pretreatment with propranolol, phenolamine or prazosin, were stained more than that of those from the NE group, indicating the inhibitory capacity of these adrenergic receptor antagonists (all P<0.05 vs. NE). Phenolamine caused the most inhibition (43.21% decrease; Figure 4D).

To determine whether a similar process was working in MMP9, the total and active protein forms were assessed as well. We found elevated levels in total MMP9 expression challenged by NE (61.67% increase). However, pretreatment with propranolol, phenolamine or prazosin could not modify elevated MMP9 levels (all P>0.05 vs. NE; Figure 4C). Consistently, exposure to NE and adrenergic receptors antagonists (propranolol, phenolamine or prazosin) had no effect on the activity of MMP9 in the supernate (Figure 4D).

In addition, there exists an endogenous class of proteins termed the tissue inhibitor of metalloproteinases (TIMPs), of which TIMP2 has been characterized in inhibiting the activity of all active MMPs and regulating the activation of proMMP-2. In the Western blot assay, the expression of TIMP2 apparently decreased in the cells treated with norepinephrine compared to that in control cells (a 33.63% decrease). Furthermore, impairment in decreasing the number of TIMP2 induced by NE was observed only when cells were pre-incubated with propranolol. The treatment with phenolamine or prazosin did not alter the effect of norepinephrine on TIMP2 levels (Figure 4E).

**TGF-β/Smads Response to NE in Interstitial Cells**

In terms of cell signals, TGF-β/Smad-dependent pathways were investigated in an attempt to unveil the molecular mechanism of the cells response to NE, physiologically. NE administration diminished TGF-β protein levels in VIC when compared to that in control cells (29.57%, P<0.05 vs. control). Impairment in decreasing TGF-β protein by NE was only observed when pre-incubation occurred with propranolol (Figure 5), whereas it did not alter the Smad2, 3 and Smad7 levels in mRNA.

**Discussion**

It has previously been reported that human heart valves have a distinct pattern of autonomic innervations, and the dysfunction of nerve terminals in the valve leaflet could be correlated with the mitral valve prolapse.** Neurotransmitters released by the SNS might be responsible for the dysfunction of nerve terminals in the valve leaflet because they act with ARs. Indeed, the enhanced expressions of ARs were found in the degenerative mitral valves, thus providing a clue when regulating progression of the mitral valve prolapse. In mitral valve interstitial cells, we found that the AR agonist, norepinephrine, caused an increase in the expression of the α-SMA protein, the marker for active phenotype, and endowed features of myofibroblasts in cells. In other words, valve interstitial cells became activated by the sympathetic nervous system throughout the cell life to maintain day-to-day physiological activities, regulate valve repair and participate in remodeling. In line with other species and the organic system, sympathetic effects were responsible for the progression of hepatic fibrogenesis, and contributed to vascular wall remodeling.** Above all, phenotype transition responsiveness to the sympathetic nervous system eventually would regulate cell cytokine secretion, extracellular matrix (ECM) synthesis as well as degradation, and eventually affect processes that are important in heart valve disease progression. That is why reoperation after the repair of degenerative mitral regurgitation has occurred at a linear rate of up to 3.0% per year, because valve-related failure characterized by a new prolapse region and leaflet thickening due to improper autonomic state, is persistent. The extracellular matrix, which mainly includes collagens, elastins, and glycosaminoglycans synthesized by interstitial cells, could respond to external signals to influence the valve property. The process was operative via the phenotype transition of cells.
in autonomic regulation. In this study, challenged with NE, the interstitial cells synthesized caused a decrease in collagen type I, elevated levels of collagen III, and a descended ratio of collagen I to III. Heart valve quality is closely associated with the amount and ratio of type I to type III collagens. In particular, type I collagen, the mature type, is responsible for tensile strength. In contrast, type III collagen fibers are regarded as the immature collagen with a thinner diameter.12 A decreased ratio of type I to III collagen induced by NE could change the geometrical arrangement, diameter of collagen fibrils and the amount of cross-linking, consequently impairing mechanical stability of heart valves in the end. Elastins, another principal component in mitral valves, had augmented expression during sympathetic stimulation. Aiming to maintain the homeostasis in the complex compensatory process, elastase would activate partial degradation of normal elastic fibers because of abnormal elastic aggregation. Then, in turn, immaturity or incomplete cross-linking elastins were synthesized, which finally leads to mechanical deformation. By coincidence, this finding was amazingly in agreement with the results of a study that showed that the elastin content of floppy valves was elevated from a normal value of 9.5% to 14.5% of tissue dry weight.13 At last, the administration of NE significantly upregulated the GAG level in cultural supernatant (3.05-fold). In fact, glycosaminoglycans were closely associated with parallel collagen fibrils, the cross-linking and maturation of the elastic fibers, eventually contributing to mechanical function of heart valves. Concretely, accumulation of these materials disrupted the normal layered structure of the leaflet and extended into the compartment of collagens and elastics, resulting in the mechanical weakness of heart valves. Furthermore, collagen fibrils, including non-spiraling fibrils, were arranged loosely and irregularly because of the presence of abnormally large amounts of proteoglycans, which played an important role in the assembly of collagen fibrils.14 In addition, GAG were bound to elastin with hydrophobic molecules, which was associated with normal cross-linking, and it also considerably influenced the mechanical properties of elastic fibers.15 Also, glycosaminoglycans maintained hydrophobic elastin molecules in solution until their aggregation onto the growing fiber and cross-linking by matrix lysil oxidase occurred.16 Hence, these excessive amounts interfered with the cross-linking and maturation of the elastic fibers.

During remodeling of the ECM, the degradation of the matrix was regulated to respond to external signals during the synthesis of the matrix. A specific pattern of MMPs and TIMPs was expressed in heart valves in charge of the remodeling process. In view of sympathetic effects, the interstitial cells cultured with NE displayed significantly increasing expressions of total, secretory and active MMP-2 protein, as well as MMP-9, which conversely decreased the TIMP2 level. Among them, MMP-2 is capable of degrading all of the matrix molecules, mainly covering elastins, and collagens I and III within the leaflet tissue.17 Moreover, transgenic heart valves with MMP-2 had massive increases in acidic glycosaminoglycans, paralleling the severity of valvular mechanical alterations.18 Hence, myxomatous degeneration was assumed be directly driven by the extracellular matrix-degrading enzyme, matrix metalloproteinase-2.19 Extrapolating from norepinephrine-inducing MMPs expression in metastasis, the autonomic nervous system probably provided a key pathway for immune system dysregulation in the remodeling. A result of MMP2 upregulation was achieved through dysregulation of the production of cytokines, such as interferon-γ (IFN-γ), interleukin-1 (IL-1), IL-2, tumor-necrosis factor (TNF) and significantly activated NF-KB (nuclear factor kappa-light-chain-enhancer of activated B cells).20,21 In addition, the intracellular domain of a particular type of matrix was in touch with neuronal nitric oxide synthase, which, along with MMP2 and MMP9, was upregulated by NE administration. Therefore, it was thought that the activation of MMP2 and MMP9 by NE could be mediated by NO (nitric oxide).22 Currently, the detailed mechanism in heart valves about MMPs regulation by NE remains unknown and requires further investigation.

Are the sympathetic changes in the valve causative or secondary effect? Based on studies conducted to date, including this one, which have focused on mitral valve prolapsed, this question has not been answered and has been regarded as another topic of investigation in the genetics or embryogenesis field. This question might be answered by the use of genetic mapping for mitral prolapse in susceptible genes involved in dysautonomic regulation.

To rule out other confusing factors associated with an improper autonomic state in mitral valve tissue, we chose heart transplantation recipients to act as controls because these patients suffered from dilated cardiomyopathy or multi–vessel coronary artery disease, which merely had no influence on valve tissue. Although they might have been exposed to high sympathetic activity systemically, the interstitial cells, in fact, were relatively normal. And in agreement with localized sympathetic activity, serum norepinephrine levels were higher in mitral valve prolapse (data not presented) compared to the control, with no significant difference in NYHA class between the groups. What is more, given that hearts receiving β–blockers before surgery might have caused misleading results to occur and thus misinterpretation of the data, β1-ARs, β2-ARs and α1-ARs were all detected. It turned out that not only β1-ARs, but also β2-ARs and α1-ARs were upregulated in prolapsed mitral valve leaflets. In actual fact, the increased state of systemic sympathetic activity in mitral valve prolapse was detected and confirmed years ago. Therefore, increased sympathetic activity in terms of valve tissue can be confirmed in this study. Overall, the enrolled control valve is feasible and scientific. In this study, we have identified a novel role for the sympathetic effect in influencing physiologic behaviors in mitral interstitial cells. We have found that dysautonomia, which leads to improper modulation of cellular synthetic metabolism in mitral valves, promotes myxomatous degeneration in mitral valve prolapsed, propagating the disease severity. It is hoped that this study will help identifying new therapeutic strategies for preventing and blocking mitral valve prolapsed, or perhaps the autonomic state will serve as one of the triggers for referral when degenerative mitral valve regurgitation is present.23

Disclosures
Conflict of Interest: The authors declare that they have no competing interests.

References


