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Aortic Valve Cyclic Stretch Causes Increased Remodeling Activity and Enhanced Serotonin Receptor Responsiveness

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Abstract

Background—Increased serotonin(5HT) receptor(5HTR) signaling has been associated with cardiac valvulopathy. Prior cell culture studies of 5HTR signaling in heart valve interstitial cells have provided mechanistic insights concerning only static conditions. We investigated the hypothesis that aortic valve biomechanics participate in the regulation of both 5HTR expression and inter-related extracellular matrix remodeling events.

Methods—The effects of cyclic-stretch on aortic valve 5HTR, expression, signaling and extracellular matrix remodeling were investigated using a tensile stretch bioreactor in studies which also compared the effects of adding 5HT and/or the 5HT-transporter inhibitor, Fluoxetine.

Results—Cyclic-stretch alone increased both proliferation and collagen in porcine aortic valve cusp samples. However, with cyclic-stretch, unlike static conditions, 5HT plus Fluoxetine caused the greatest increase in proliferation ($p < 0.0001$), and also caused significant increases in collagen ($p < 0.0001$) and glycosaminoglycans ($p < 0.0001$). DNA microarray data demonstrated upregulation of 5HTR2A and 5HTR2B (> 4.5 fold) for cyclic-stretch versus static ($p < 0.001$), while expression of the 5HT transporter was not changed significantly. Extracellular matrix genes (eg. Collagen Types I,II,III, and proteoglycans) were also upregulated by cyclic-stretch.

Conclusions—Porcine aortic valve cusp samples subjected to cyclic stretch upregulate 5HTR2A and 2B, and also initiate remodeling activity characterized by increased proliferation and collagen production. Importantly, enhanced 5HTR responsiveness, due to increased 5HTR2A and 2B expression, results in a significantly greater response in remodeling endpoints (proliferation, collagen and GAG production) to 5HT in the presence of 5HT transporter blockade.

Keywords

Heart valve; Molecular biology; Pharmacology (serotonergic receptors)

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Introduction

Increased serum 5HT levels have been associated with a valvulopathy in a number of clinical settings including Carcinoid tumors (1–2), use of the appetite suppressant Fenfluramine-Phenteramine (3–8), and the administration of agents used to treat Parkinson's disease, such as Pergolide (9). Research related to the pathogenesis of 5HT valvulopathy has indicated in both *in vitro* studies (10–16) and animal models (17–23) that excessive 5HT levels may cause both increased heart valve interstitial cell proliferation and upregulation of extracellular matrix production. These prior studies highlight the fact that 5HT may have an important role in both heart valve physiology and pathophysiology. Thus far neither *in vitro* nor *in vivo* 5HT investigations concerning cardiac valves have explored the interrelationships of 5HTT inhibition and 5HTR signaling with the biomechanics of cardiac valve leaflets. In the present studies we examined the hypothesis that aortic valve biomechanics are mechanistically involved in the regulation of both 5HTR expression and inter-related extracellular matrix remodeling.

We investigated the following questions: 1) Does dynamic cyclic-stretch alone, simulating physiologic aortic valve leaflet motion, increase proliferation and leaflet remodeling (i.e. collagen and glycosaminoglycans) in porcine aortic valve cusp samples (PAV)? 2) Does the administration of either 5HT or Fluoxetine (Fluox), a 5HT-transporter (5HTT) inhibitor, alone or together have an impact on these endpoints? 3) If 5HTR signaling in PAV is increased through combining 5HT with Fluox, is there an even greater effect than that observed with cyclic-stretch without co-treatment? 4) Which 5HTR are predominantly involved? 5) What are the changes in gene-expression patterns associated with the interaction of cyclic-stretch and 5HTR signaling?

Materials and Methods

Materials

Chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated. Cell culture disposables were obtained from Corning Life Sciences (Lowell, MA). Fresh PAV from pigs of 12–24 months age were obtained from the Holifield Farms Abattoir (Covington, GA) within 30 minutes of slaughter. The valves were then transported to the laboratory in sterile, ice-cold Dulbecco's Phosphate Buffered Saline (DPBS).

Bioreactor studies

PAV leaflet samples were isolated from the central region of each valve cusp and incubated in cell culture medium either without or within a tensile stretch bioreactor as previously reported by the Yoganathan group (24–28). PAV leaflet samples designated “cyclic-stretch” were subjected to 10% cyclic stretch at 1.167 Hz (a rate of 70 cycles per minute) in DMEM supplemented with 10% fetal bovine serum (FBS), (24–25). These samples were randomized and assigned to one of four pharmacological groups (Control, 5HT, Fluox, and 5HT+Fluox) and further randomized to the bioreactor or maintained in parallel as static cultures (24–28). Data were collected from 6 individual PAV samples in each of the eight treatment groups. Optimization studies led to the doses used in these experiments, which were 5HT concentrations of 10^{-5} M, and Fluox concentrations of 10^{-6} M. The experiments were run for either 72 hours to obtain proliferation and extracellular matrix (ECM) production endpoints, or for 24 hours in order to obtain suitable RNA for the microarray studies. Samples flash-frozen in liquid nitrogen were stored at -80°C before RNA extraction.

Remodeling endpoints

Bromodeoxyuridine (BrdU) was used to label proliferating cells during the last 24 hours of the experiments, and BrdU immunohistochemistry performed as previously described (28). A colorimetric collagen assay (Biocolor, United Kingdom) was used to quantify the total enzyme soluble collagen content in the leaflet samples as previously described (24), and Picrosirius red staining in conjunction with *in silico* image analyses was used to assay immature collagen content as previously reported (29, 24). Sulfated glycosaminoglycan content was analyzed in PAV after control and bioreactor incubations as described above, using the Blyscan™ sGAG assay kit (Bicolor) (24).

Microarray Methods and Related Statistics

Total RNA from each leaflet sample was extracted using TRIzol (Invitrogen, Carlsbad, CA) and stored at -80°C . RNA concentration and quality were determined with RNA 6000 Nano LabChips using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). RNA amplifications were performed using NuGEN's Ovation RNA Amplification System (NuGEN Technologies, Inc. San Carlos, CA) (30). Each amplified RNA (cRNA) was labeled with ENZO BioArray High Yield RNA Transcript Labeling kit (Affymetrix, Santa Clara, CA), and biotin-labeled cRNA then fragmented and hybridized to the Affymetrix Porcine Genome GeneChip. Principal component analysis used Partek Genomic Suite software (Partek, Inc., St. Louis, MO). A two-way ANOVA method was used to calculate p-values adjusted with the False Discovery Rate (FDR) method of Benjamini-Hochberg for multiple testing corrections.

Quantitative RT-PCR

TaqMan gene expression assays-on-demand and custom assays (Applied Biosystems, Foster City, CA) were used to measure the following porcine genes: 5HTT, 5HTR2A, 5HTR2B, Aggrecan, Collagen III (Alpha-1 Chain), and Biglycan. Real time PCR assays were performed and analyzed using an ABI prism 7500 Real Time PCR System (Applied Biosystems). The relative quantitation for each gene of interest expression in each group was determined by using the comparative Ct method ($2^{-\Delta\Delta C_t}$) as described in user bulletin # 2 ABI Prism 7500.

Cell Cultures--5HTR studies

Aortic valve interstitial cells were isolated from fresh porcine aortic valves, characterized and cultured in M199 (Invitrogen, Carlsbad, CA)/10% FBS (Gemini Bio Products, West Sacramento, CA) as previously published (15). Cells were treated with 5HT for five minutes before harvest and lysis for Western blots as previously described (15, 16, 31). Membranes were probed first with an antibody for human phosphorylated extracellular signal-related kinase (pERK1/2) (anti-phospho-p42/44 MAPK; Cell Signaling Technologies, Danvers, MA), then stripped and re-probed with an antibody to total ERK1/2 (ERK1/2; Santa Cruz Biotechnologies) as a loading control. Representative blots of at least triplicate studies are shown.

Statistical Method for Remodeling Endpoints

Graphical results and all quantitative data are presented as mean $\pm$ SEM, and represent $n=6$ per group. Two-way analysis of variance (ANOVA), with pharmacological groups and the stretch condition as the two factors and interaction terms, was used to test if the relationship between outcomes (proliferation, collagen, and glycosaminoglycan content) and pharmacological groups are different for static condition and cyclic-stretch. Two-tailed $p<0.05$ was considered significant. All analyses were carried out using SAS version 9.2 (SAS Institute Inc, Cary, NC).

Results

PAV Comparisons of Cyclic-Stretch versus Static

Cyclic-stretch caused great increases in proliferation in PAV under all four conditions. The increases caused by cyclic-stretch were significant for both 5HT alone ($p=0.04$) and Fluox and 5HT combined ($p<0.0001$), but not significant in the control or Fluox alone. (Figure 1). Notably, combining 5HT and Fluox in the presence of cyclic-stretch (Figure 1H) caused the greatest increase in proliferation in PAV.

Total soluble collagen production in PAV was significantly increased under cyclic-stretch conditions compared to static in control ($p=0.007$), Fluox alone ($p=0.01$), and Fluox and 5HT combined ($p<0.0001$), but not with 5HT administered alone. (Figure 2A) Under combined 5HT and Fluox, cyclic-stretch resulted in a 2.8-fold increase in total collagen production ($p<0.0001$). Synthesis of immature collagen fibers, assessed with a picrosirius red assay, was not significantly increased by cyclic-stretch alone, 5HT alone, or Fluox alone. It was significantly increased in PAV in the presence of cyclic-stretch with co-administration of 5HT and Fluox, and the increase is 6-fold higher than co-administration of 5HT and Fluox without cyclic-stretch ($p<0.0001$, Figure 2B).

The addition of 5HT resulted in reduced total soluble glycosaminoglycans (sGAG) (Figure 2C) under both static and cyclic-stretch conditions in comparison with no treatment, although only the change under cyclic-stretch was statistically significant. In agreement with both proliferation and collagen results, cyclic-stretch plus 5HT and Fluox resulted in significantly greater sGAG levels than the other conditions studied (Figure 2C). Fluox alone or cyclic-stretch alone had no significant effect on sGAG (Figure 2C). However, 5HT plus Fluox under cyclic-stretch conditions resulted in a significant increase in sGAGs ($p<0.0001$, Figure 2C).

Microarray Results

Principal component analyses of the microarray data revealed that cyclic-stretch was the dominant factor determining differences in patterns of gene expression (Fig. 3). Selected probe sets were interrogated based on published probe set annotation for swine (32–33). Of 9 HT-processing-related genes identified, only 2 out of 9 genes, 5HTR2A and 5HTR2B, were strongly increased, with an average fold change (cyclic-stretch versus static) of 4.9 and 4.7, respectively (Table 1). Other 5HTR were significantly upregulated, but at lower levels (1.1 – 1.4-fold; cyclic-stretch versus static) than 5HTR2A and 5HTR2B, and only 5HTR1F was not significantly changed (Table 1). Importantly, 5HTT gene expression levels were not significantly altered (Table 1).

Microarray data demonstrated upregulation of structural collagen genes by cyclic-stretch, as indicated by the results for Collagen Types I, II and III (Table 2). Heat shock protein 47, a marker of immature collagen production, was also upregulated with cyclic stretch (Table 2). The microarray data also demonstrated that glycosaminoglycan-related proteins, including biglycan, versican, lumican and aggrecan, were significantly upregulated (1.50– 8.2 fold) in association with cyclic-stretch (Table 2). Interestingly matrix metalloproteinases 2 and 9, both collagenases involved in extracellular matrix remodeling, were significantly down-regulated by cyclic stretch (Table 2).

Elastin, a major structural protein, was not significantly affected by cyclic-stretch alone, but was upregulated 6.5-fold by 5HT-Fluox, when compared to static conditions in the presence of the same agents ($p=0.004$). Lesser changes in gene expression patterns associated with cyclic-stretch plus 5HT-Fluox were noted with MMP3 and MMP28 (downregulated 14.7-fold; $p=0.007$ and upregulated 2.8-fold; $p=0.004$, respectively).

Selected gene expression changes noted in the microarray results were confirmed with quantitative RT-PCR (Table 3). These data verified the micro-array trends for 5HTR2A, 5HTR2B, 5HTT, Collagen III, Biglycan and Aggrecan. The complete microarray dataset for these studies has been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) repository and are accessible through Gene Expression Omnibus Series accession number GSE21663.

5HT Receptor Activity: Western Blot Results

5HT resulted in a dose dependent increase in pERK1/2 in porcine aortic valve interstitial cells (PAVIC) (Figure 4A), and Fluox alone had no effect (data not shown). Ketansarin, an inhibitor of the human 5HTR2A, strongly suppressed pERK1/2 in PAVIC, as did the 5HTR2B inhibitor, SB204741. PD98059, a MEK inhibitor, also strongly suppressed pERK1/2 and thus served as a positive control. The other 5HTR inhibitors studied, SB206553 (a 5HTR2C antagonist), WAY100635 (a 5HT1A antagonist), GR55562 (a 5HT1B/1D antagonist), and BRL15572 (a 5HT1D antagonist) had little to no effect on inhibiting pERK1/2.

Comment

The result of greatest interest in the present studies is the demonstration that PAV cyclic stretch exposure upregulates 5HTR2A and 2B, thus making the aortic valve interstitial cells more responsive to 5HT signaling. This result is the likely explanation for the enhanced responsiveness of PAV noted in these studies to the sustained presence of 5HT due to 5HTT blockade with Fluox. Prior studies by our group (15, 16, 31) and others (7) have shown increased ECM production due to 5HTR signaling in static, serum-starved culture studies using cardiac valve interstitial cells (VIC). However, previous static VIC culture studies of Fluox alone did not show a significant effect of this agent on proliferation either with or without 5HT addition (16). Furthermore, the present results demonstrated in PAV that cyclic-stretch provoked the greatest upregulation of 5HTR2A and 5HTR2B, in agreement with prior reports indicating the importance of these receptors in 5HT-valvulopathy (5–8). In addition, previous cell culture studies by our group also demonstrated that both sheep aortic (15, 31) and human and canine mitral valve (16) interstitial cells had predominantly active 5HTR2A and 5HTR2B receptors.

Taken together the present results strongly suggest that 5HT related pathways involved in cardiac valve physiology and pathophysiology need to be investigated in an experimental setting that also involves biomechanical mechanisms. At present the role of 5HT and 5HTR in cardiac valves is not understood, and this lack of mechanistic insights was in part responsible for the Fen-Phen catastrophe (3–7). At this time there is no satisfactory approach for determining the safety of a 5HTR agonist or antagonist from the point of view of possible adverse effects on cardiac valves. The present results indicate that greater insights concerning the potential for valvulopathy may be gained by utilizing a combined pharmacologic-biomechanical approach when investigating novel serotonergic drugs.

Conclusions

PAV subjected to cyclic stretch upregulate 5HTR2A and 2B, and rapidly initiate remodeling activity characterized by increased proliferation and collagen production. Importantly, increased 5HTR expression due to cyclic stretch, resulting in increased responsiveness, was manifest by a significantly greater response in PAV remodeling endpoints (proliferation, collagen and GAG production) to 5HT in the presence of 5HTT blockade. These results emphasize the importance of investigating 5HT valvulopathy hypotheses in dynamically active heart valve leaflets rather than static cell cultures, and suggest a new paradigm for

model systems to study the physiology and pathophysiology of 5HTR signaling in cardiac valves.

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Abbreviations and Acronyms

5HT	Serotonin
5HTR	Serotonin receptor
5HTT	Serotonin transporter
BrdU	Bromo-deoxyuridine
ECM	Extracellular matrix
Fluox	Fluoxetine
PAV	Porcine aortic valve
PCA	Principal component analysis
VIC	Valve interstitial cell
sGAG	Soluble glycosaminoglycans

Figure 1.

The effects of 72 hours of cyclic-stretch and 5HT (10^{-5} M), Fluox (10^{-6} M), or combined treatment of 5HT+Fluox on cell proliferation. A–H: Representative photomicrographs (original magnification 40x) of BrdU immunostaining of porcine aortic valves (PAV):(A) Static PAV, (B) Static PAV + 5HT, (C) Static PAV + Fluox, (D) Static PAV + 5HT and Fluox, (E) Cyclic stretch PAV, (F) Cyclic stretch PAV + 5HT, (G) Cyclic stretch PAV + Fluox, (H) Cyclic stretch PAV + 5HT and Fluox; (I) Graph shows quantitative results of PAV BrdU immunostaining, $n=6$ per group, mean $\pm$ SEM. Cell proliferation (bromodeoxyuridine (BrdU)-positive stained cells) was significantly affected by stretch ($p=0.0042$), while 2-way ANOVA regressions showed no significant change under static

conditions with the addition of 5HT, Fluox, or both Flux and 5HT . Cyclic-stretch increased proliferation within treatment groups versus static ($p<0.0001$), with relative fold-changes of 15, 5, 1.3, and 3.5 for control, 5HT, Fluox, and combined treatments respectively. Regression analysis showed that only 5HT and combined 5HT+Fluox achieved statistical significance, at $t_p=0.04$ and $**p<0.0001$, respectively.

Figure 2.

The effects of 72 hours of cyclic-stretch and 5HT (10^{-5} M), Fluox (10^{-6} M), or combined treatment of 5HT+Fluox on total collagen, immature synthesized collagen production, and total soluble glycosaminoglycans (sGAG) in porcine aortic valves (PAV). Graphs show results for PAV samples, $n=6$, mean $\pm$ SEM. A) Total collagen production in PAV was increased by cyclic-stretch ($p=0.0087$), and unchanged by 5HT, Fluox, or their combination unless cyclic-stress was also applied ($p<0.0001$). With cyclic-stretch, treatment of PAV with 5HT+Fluox resulted in an average 2-fold increase over the other three cyclic-stretched groups (** $p<0.0001$ vs. static); cyclic-stretch alone or Fluox alone had lesser effects (* $p=0.007$, $^{\theta}p=0.01$ respectively). B) Immature collagen production per picrosirius red staining revealed a significantly increased proportion of newly synthesized collagen fibers with application of cyclic-stretch ($p=0.0005$), and 2-way ANOVA regressions revealed that the pharmacologic agents only had effect under cyclic stretch ($p<0.0001$). The combination of 5HT+Fluox and cyclic-stretch produced the highest increase in immature collagen fibers (** $p<0.0001$). C) sGAG content was determined in PAVs, and was increased by cyclic-stretch ($p=0.017$). As in (A) and (B) above, no statistically significant effect of pharmacologic agents was found without cyclic-stretch. However, 2-way ANOVA regressions showed a positive effect of pharmacologic agents with cyclic-stretch ($p=0.002$), and specifically that 5HT+Fluox under cyclic-stretched conditions resulted in increased sGAG in comparison to unstretched 5HT+Fluox (27.3-fold increase; ** $p<0.001$).

Figure 3.

Principal component analyses (PCA) gene expression array data from PAV after static or cyclic-stretch incubation, with two-dimensional graphical representation of the principal components without or with co-treatment with 5HT(10^{-5} M)+Fluox (10^{-6} M). PCA revealed clustering of two major principal components, indicating that the comparison of static versus cyclic-stretch conditions is the dominant factor determining dysregulation at the genetic level.

Figure 4.

(A) Representative Western blot of PAVIC results showed dose-dependent responses to 5HT in ERK phosphorylation (pERK1/2). (B) Representative Western blot of PAVIC results demonstrated that both K and SB41 (see abbreviations below) inhibit 5HT-induced pERK1/2.

Abbreviations - Mitogen activated protein kinase kinase (MEK) inhibitor = PD98059 (PD); 5HTR antagonists (Human): 5HTR2A = Ketanserin (K); 5HTR2B = SB204741 (SB41); 5HTR2C = SB206553 (SB53); 5HTR1A = WAY100635 (W); 5HTR1B = GR55562 (GR); 5HTR1D = BRL5572 (BRL).

Figure 5. 5HTR responsiveness is enhanced by cyclic stretch due to increased 5HTR2A and 2B receptor expression. Cyclic stretch also results in increased proliferation and collagen production. However, when cyclic-stretch induced increased 5HTR expression is combined with maximally enhanced 5HTR signaling due to 5HT addition with 5HTT-inhibition, increased receptor responsiveness results in significantly greater increases in all remodeling endpoints.

Table 1

5HT-Related Genes and the Effects of Cyclic-Stretch vs Static Conditions on Expression Levels (Data shown are ratios of cyclic-stretch/static microarray results)

Gene	Fold change (mean)	<i>p</i> Value
5HT-Receptor 2A	4.9	0.001
5HT-Receptor 2B	5.1	<0.001
5HT-Transporter	1.1	Not significant (NS)
5HT-Receptor 1B	1.1	<0.001
5HT-Receptor 1D	1.2	0.001
5HT-Receptor 1F	1.2	NS
5HT-Receptor 2C	1.2	0.01
5HT-Receptor 4	1.4	0.02
5HT-Receptor 7	1.1	0.002

Table 2

Changes in Extracellular Matrix Protein Gene Expression Patterns Associated with Cyclic-Stretch (Data shown are ratios of cyclic-stretch/static microarray results)

Gene	Fold change (mean)	p Value
Collagen Type I, alpha 1	3.8	0.002
Collagen Type I, alpha 2	6.4	0.004
Collagen Type II, alpha 1	3.8	0.006
Collagen Type III, alpha 1	6.2	0.002
Heat shock protein 47	2.0	0.028
Matrix metalloproteinase 2	-2.0	0.001
Matrix metalloproteinase 9	-8.9	0.005
Biglycan	3.2	<0.001
Versican	1.5	0.02
Lumican	4.5	<0.001
Aggrecan	8.2	0.001

Table 3

Quantitative RT-PCR (qRT-PCR) Confirmation of Microarray Data for Selected Genes Upregulated due to Cyclic-Stretch (Data shown are ratios of cyclic-stretch/static results)

Gene	Fold change per Microarray	p	Fold change per qRT-PCR	p
5HT-Receptor 2A	4.9	0.001	2.8	0.01
5HT-Receptor 2B	5.1	<0.001	2.0	0.006
5HT-Transporter	1.1	Not significant (NS)	1.4	NS
Collagen Type III, alpha 1	6.2	0.002	6.5	0.007
Biglycan	3.2	<0.001	2.9	0.006
Aggrecan	8.2	0.001	2.0	0.02