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

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Background. **Increased serotonin (5-hydroxytryptamine [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 interrelated 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 ($p < 0.0001$). The 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.**

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Increased serum serotonin (5-hydroxytryptamine [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 serotonin transporter (5HTT) inhibition and serotonin receptor (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 (ie, 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?

Material and Methods

Material

Chemicals were obtained from Sigma (St Louis, MO) unless otherwise stated. Cell culture disposables were obtained from Corning Life Sciences (Lowell, MA). Fresh

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Abbreviations and Acronyms

5HT = serotonin
5HTR = serotonin receptor
5HTT = serotonin transporter
BrdU = bromodeoxyuridine
ECM = extracellular matrix
Fluox = fluoxetine
PAV = porcine aortic valve
PCA = principal component analysis
sGAG = soluble glycosaminoglycans
VIC = valve interstitial cell

PAV from pigs of 12 to 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.

Bioreactor Studies

The 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]. The PAV leaflet samples designated "cyclic stretch" were subjected to 10% cyclic stretch at 1.167 Hz (a rate of 70 cycles per minute) in Dulbecco's Modified Eagles Medium supplemented with 10% fetal bovine serum [24, 25]. These samples were randomized and assigned to one of four pharmacologic 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 8 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 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 was performed as previously described [28]. A colorimetric collagen assay (Biocolor, Carrickfergus, UK) 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 (sGAG) content was analyzed in PAV after control and bioreactor incubations as described above, using the BlyscanTM sGAG assay kit (Bicolor) [24].

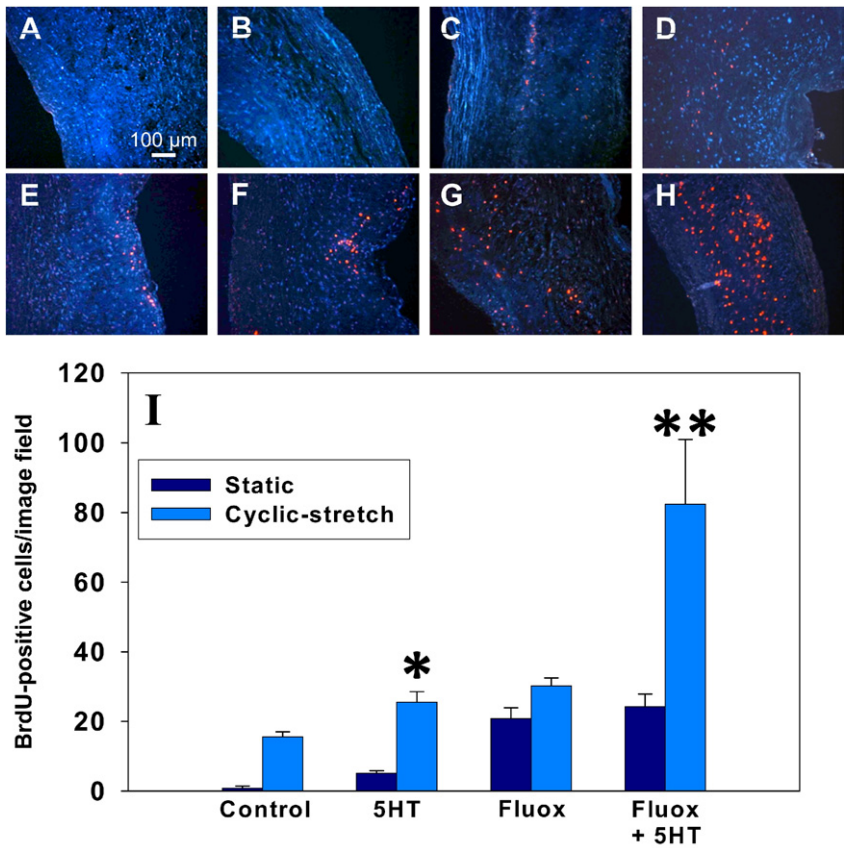

*Fig 1. The effects of 72 hours of cyclic stretch and serotonin (5HT; 10^{-5}M), fluox (10^{-6}M), or combined treatment of 5HT + fluox on cell proliferation. (A)-(H) Representative photomicrographs (original magnification 40×) of bromodeoxyuridine (BrdU) immunostaining of porcine aortic valves (PAV). (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 ± SEM. Cell proliferation (BrdU-positive stained cells) was significantly affected by stretch ($p = 0.0042$), while 2-way analysis of variance regressions showed no significant change under static conditions with the addition of 5HT, fluox, or both fluox 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 *$p = 0.04$ and **$p < 0.0001$, respectively.*