Shear stress activates P2X4 receptor currents via triggering Cx43 hemichannels in atrial myocytes: possible role on atrial adaptation to pressure overload

Sun-Hee Woo (shwoo@cnu.ac.kr)
Chungnam National University College of Pharmacy https://orcid.org/0000-0003-1444-3438
Joon-Chul Kim
Chungnam National University
Long Nguyen Hoang Do
Chungnam National University
Qui Anh Le
Chungnam National University
Tran Nguyet Trinh
Chungnam National University
Luong Phuong Kim
Chungnam National University

Research Article

Keywords: atrial myocytes, shear stress, Cx43 hemichannel, P2X4 receptor, transverse aortic constriction

Posted Date: April 26th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1537087/v1

Abstract

Increase of afterload induces cardiac hypertrophy leading to contractile failure and dilation. Atria to receive venus return should adapt to such mechanical changes including shear stress. However, the atrial cell responses to shear stress are poorly understood. Recent evidence suggests that atrial myocytes release ATP fast under shear stress via connexin43 (Cx43) hemichannels, which triggers action potentials and Ca^{2+} waves. We explored cellular mechanism for graded depolarization under shear stress and its implication on atrial pathogenesis during progressive increase in afterload. Using whole-cell patch clamp combined with micro fluid-jet system, we found in rat atrial myocytes that shear stress activates non-selective cation currents ($I_{S,Cs}$) that were enhanced by zero external Ca^{2+}, but eliminated by La^{3+}, high concentrations of carbenoxolone, anti-Cx43 antibodies, TAT-Gap19 or Cx43 conditional knockout (cKO), suggesting a key role of Cx43 hemichannel. Intracellular calcein fluorescence imaging to assess gap junction hemichannel activity revealed that shear stress induces a calcein efflux from atrial myocytes which was increased by zero external Ca^{2+} and suppressed by Cx43 hemichannel inhibitions (La^{3+}, Gap19 or Cx43 cKO). The $I_{S,Cs}$ was eliminated by ATP-metabolizing apyrase and suppressed by antagonists of P2 receptors, mainly P2X4 receptor (P2X4R), indicating a role of autocrine P2X4R activation. Furthermore, in a rat model of left atrial (LA) hypertrophy, induced by transverse aortic constriction (TAC) for 5-weeks, $I_{S,Cs}$ and its P2X4R current component, and P2X4R protein level were largely increased with unaltered hemichannel function. In severely dilated left atria following 20-week-TAC, Cx43 hemichannel activity and P2X4R-currents of $I_{S,Cs}$ were attenuated again with lowered protein levels for Cx43 and P2X4R. Cx43 hemichannel-P2X4R signaling could account for increase of atrial excitability under shear stress. Characteristic alterations in this response during progressive pressure overload suggest that it plays a role in atrial compensatory adaptation to hypertension.

Introduction

Cardiac muscles are subjected to mechanical stimuli during cardiac cycles, which, in turn, affects excitation and contraction [1, 2]. Atria play a role in sensing pressure at the position to which blood from other body organs returns. Increase in atrial pressure is known to release atrial natriuretic peptide (ANP) to accelerate water excretion from the kidney and balance blood-volume and -pressure [3]. Atrial enlargement is clinically associated with hypertension, heart failure and valve diseases, the major causes of atrial fibrillation [2, 4, 5]. Under these conditions mechanical stresses increase, and the responses to stretch has been well documented. Atrial myocytes are also exposed to fluid shear stress during each beat by the relative movement of laminar sheets and adjacent cells [6–8]. Pressure overload (PO) and volume overload involve significant increase in interstitial fluid shear in cardiac muscle during cardiac cycles due to detachment of myocytes from the extracellular matrix and myocyte slippage [6–9]. However, the effects of shear stress on cardiac muscle and their pathophysiological roles are poorly understood.

A line of recent evidence has suggested that shear stress increases spontaneous Ca^{2+} releases in atrial and ventricular myocytes [10, 11], triggers propagating action potentials (APs) in culture [12], and increases the beating rate of neonatal ventricular myocytes [13]. Use of single-cell approach to apply shear stress in combination with real-time two-dimensional Ca^{2+} imaging and reporter patch to detect ATP, it has been revealed that shear stress immediately releases ATP from intact atrial myocytes, thereby triggering spontaneous AP and fast Ca^{2+} release in atrial myocytes [14, 15]. The shear-induced ATP release in atrial cell is suggested to be mediated by connexin43 (Cx43) hemichannels among the several mammalian ATP release pathways [14, 15]. However, the mechanisms for graded depolarization to trigger AP under shear stress remain to be proven. In this regard, we have previously discovered in atrial myocytes that sustained monovalent cation currents via transient receptor potential melastatin 4 (TRPM4) channels are activated by shear stress-induced Ca^{2+} release [16]. Inhibition of TRPM4 or stretch-activated cation channel, however, did not suppress the shear-stress-induced Ca^{2+} waves, associated with membrane depolarization [15]. Interestingly, such Ca^{2+} signals were sensitive to the antagonists of P2X- or P2X4-receptor (P2X4R) or to enzymes metabolizing external ATP [14, 15].

Therefore, in the present study, we tested the hypothesis that shear stress activates Cx43 hemichannels to induce autocrine activation of P2X4R, thereby generating depolarizing currents at diastolic potential in atrial myocytes, and further explored its implication in atrial adaptation and pathogenesis under progressive increase in afterload. We used the whole-cell patch clamp technique to record shear-induced cation currents in atrial myocytes and used confocal imaging to measure dye flux through gap junction hemichannels under shear stress. Shear stress was applied to single cells using rapid fluid micro-jet apparatus [14, 15], and genetic and pharmacological interventions and immunoblotting were also adopted. We used a rat model of pathological left atrial (LA) hypertrophy and failure induced by transverse aortic constriction (TAC) for short- and long-duration, respectively. We demonstrated that shear stress induces Cx43 hemichannel activation to trigger cation influx through the P2X4R in resting atrial myocytes at diastolic potentials. In addition, this response was significantly enhanced during atrial compensatory hypertrophy under (PO) but decreased again with atrial contractile failure under long-term PO.

Materials And Methods

An expanded Methods section is provided in the Online Resource.

Conditional knockout (cKO) mice

A commercially available C57BL/6 $Cx43^{flox}$ mice line (Jackson Laboratory; B6.129S7-Gja1^{tm1Dlg}/J, stock number 008039; loxP sites flank exon 2 of *Gja1*) [17] was used. For *Gja1* deletion, mice were intraperitoneally injected 5 consecutive days with tamoxifen (TAM) (Cayman Chemical; 3 mg dissolved in corn oil with 10% ethanol; giving an approximate tamoxifen dose of 0.075 mg/g body weight [18]). Mice

14 days post-TAM were used for the experiments. As a control, C57BL/6J mice (Jackson Laboratory) injected with the same TAM injection protocol were used.

TAC in rats and transthoracic echocardiography

TAC was used [19] to induce LA hypertrophy and dilation in rats. Additional details are provided in the Data Supplements. Atrial dimensions were measured using 2-D guided B-mode parasternal long-axis view during simultaneous surface electrocardiograph recording (S-Sharp, S-Sharp Corporation, New Taipei City, Taiwan).

Cell isolation

Atrial myocytes were enzymatically isolated from normal male Sprague-Dawley rats (200–300 g; RaonBio, Seoul, Korea), TAC and sham rats, and from control and Cx43 cKO mice using retrograde Langendorff perfusion method as previously described [10]. This study conforms with the Guiding Principles for the Care and Use of Experimental Animals published by the Korean Food and Drug Administration and Animal and Plant Quarantine Agency in South Korea. The experimental protocols were approved by the Animal Care and Use Committees of the Chungnam National University, South Korea (No. CNU-00992).

Application of shear stress

Shear stress was applied onto single cells using micro-jet apparatus as previously described [10]. Pressurized flows of external solutions were applied onto the single myocytes through a microbarrel (internal diameter = 250 μm) the tip of which was placed at ≈150 μm from the cell and was connected to fluid reservoirs with different heights (5~40 cm). The shear stress (dyn/cm^2) was calculated as previously described [20]. We generally used ~16 dyn/cm^2 (equal to 0.16 N/m^2; 40-cm reservoir heights) which consistently generated global Ca^{2+} waves in atrial myocytes with no movement of the cells [21]. The positioning of the microbarrel was performed under microscope using a micromanipulator (Prior England 48260).

Measurement of shear-induced cation currents

Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique using an EPC7 amplifier (HEKA Elektronik, Germany) as previously described [16]. Before applying shear, conditioning pulses from -80 mV to -10 mV were continuously applied to the cells to maintain the sarcoplasmic reticulum Ca^{2+} load in the normal Tyrode's solutions composed of (in mM) 137 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$, and 10 glucose, 2 $CaCl_2$ (pH 7.4). Measurement of shear-activated current was

generally carried out in the external solutions containing (mM) 137 CsCl, 10 HEPES, 1 $MgCl_2$, 10 glucose, 2 $CaCl_2$ (pH = 7.4), with ~4 min intervals. Puffing solutions to apply shear stress contained 2 mM Ca^{2+} or zero Ca^{2+} (with 1 mM EGTA) as described in the figures and legends. Cs^+-rich pipette solutions contained (in mM) 137 CsCl, 10 HEPES, 5 MgATP, 1 $MgCl_2$ with the pH adjusted to 7.2. For K^+-rich internal solution, 137 mM CsCl was replaced with equimolar KCl (Fig. 1). In some experiments, anti-Cx43 antibodies (monoclonal, mouse, MiliporeSigma, C8093, 1:2000) and scramble for control were added to the pipette solutions. Similarly, anti-P2X4R antibodies (Rabbit polyclonal, 1:1000 dilution, epitope: intracellular, a.a. 370-388 of rat P2X4R; APR-002, Alomone labs, Jerusalem, Israel) and/or blocking peptides were added to the pipette solutions. Generation of the voltage-clamp protocol and acquisition of the data were carried out using pClamp software (v9.0, Molecular Devices, Foster City, CA, USA) via an A/D converter (Digitata 1440, Molecular Devices). The current signals were filtered (10 kHz) and analyzed using Clampfit 10.3 software and OriginPro 8.1 SR1 software (OriginLab Corporation, Northampton, MA, USA). The experiments were performed at room temperature (22–25°C).

Dye flux measurement

For dye efflux assay, cells were loaded with 2 mM Ca^{2+}-containing Tyrode's solutions (see above) supplemented with 0.1 μM calcein Red/Orange acetoxymethyl ester (Thermo Fisher Scientific-Molecular Probes, Eugene OR, USA) for 20 min, and then washed by superfusion with Tyrode's solutions for 10 min. Two-dimensional images were recorded at 0.2 Hz using a confocal laser scanning microscope (Nikon A1, Japan). Fluorescence images were analyzed with a software NIS-Elements AR (v3.2, Nikon), OriginPro 8.1 (Origin Lab Corporation) and GraphPad Prism 8. Fluorescence (F) measured immediately before the application of shear stress was considered as F_0. The F was normalized by F_0 to show a decrease (efflux) of F by shear stress.

Western blotting

To examine expression levels of P2X4R, Cx43, ANP and phosphorylated Cx43 at S368 (pS368-Cx43) in atrial muscles Western blotting was performed as previously described [14]. Approximately 15-30 μg of total proteins were run on 10% SDS-polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane, and the blots were sequentially probed with primary and secondary antibodies using standard Western blot protocol (Online Data Supplements). All blots were imaged and quantified using a Chemi-luminescence Bioimaging Instrument (CHEBI) (Cellgenetek, South Korea).

Statistics

Statistical analyses were performed with Microsoft Excel, GraphPad Prism 8, and OriginPro 8.1. The numerical results are presented as means ± standard error of the mean (S.E.M.). *n* indicates number of

cells used. Paired or unpaired Student's *t*-tests with Bonferroni correction were used for statistical comparisons depending on the experiments. Comparisons between groups were made using one-way or two-way ANOVA with post hoc or repeated measures depending on the experiments. Differences at $P < 0.05$ were considered to be statistically significant.

Results

Shear stress immediately triggers nonselective cation currents in atrial myocytes

Transient inward current was recorded in rat atrial myocytes at resting potentials during the application of shear stress (I_S) following a series of conditioning pulses (Fig. 1A). The I_S were dramatically increased and prolonged when external Ca^{2+} was removed from puffing solutions, while it was slightly reduced at 10 mM external Ca^{2+} concentrations (Fig. 1B). Next, we examined what types of ions contribute to I_S by substitutions of major cations in the external solutions with choline. Results presented in Fig. 1C and 1D show that Na^+ and K^+ are the major ions to carry I_S at -70 mV. The results in Fig. 1E and 1F obtained using high Ca^{2+}- and low Na^+-external solutions represent that Ca^{2+} also can contribute to I_S.

Because shear-activated currents were nonselective cation currents we used symmetrical CsCl solutions and zero-Ca^{2+} puffing solutions to better isolate and maximize the current for further mechanism study. Transient inward Cs^+ currents were observed under shear application ($I_{S,Cs}$), and they were consistently enhanced with zero-Ca^{2+} solutions (2–4-fold; Fig.1G). Measurements of $I_{S,Cs}$ at varying shear forces (1.4–16 dyn/cm^2) revealed that the $I_{S,Cs}$ were activated faster and more strongly as the strength of shear stress increased, reaching maximum at ~10 dyn/cm^2 (Fig. 1H, I).

Nonselective cation currents are induced by Cx43 hemichannel activity under shear stress

Our previous report suggested that ATP releases from atrial myocytes under shear stress are mediated by Cx43 hemichannels [14, 15]. Therefore, a possible role of Cx43 hemichannels in the activation of $I_{S,Cs}$ were further examined. Pretreatment of La^{3+} (2 mM) that non-selectively suppresses gap junction channels almost completely suppressed $I_{S,Cs}$ (Fig. 2A). $I_{S,Cs}$ was eliminated by the application of high concentrations (50 μM) of carbenoxolone (“CBX”) that suppresses gap junction channels, Cx and pannexin (Px) (Fig. 2B). Blockade of Px using probenecid (800 μM) did not alter $I_{S,Cs}$ (Fig. 2C), which also excludes the possibility that Cx-mediated intracellular Ca^{2+} increase secondarily activates Px [22]. Lack of the effect of external Ca^{2+} on Px [23] is also inconsistent with the property of $I_{S,Cs}$. Low external Ca^{2+} solutions can enhance the activity of calcium homeostasis modulator 1 (CALHM1). However, CALHM1 is known to insensitive to CBX [24], which excludes its contribution to $I_{S,Cs}$. TRPM4 is activated by prolonged

shear stress via intracellular Ca^{2+} releases [16]. The TRPM4 inhibition using 9-phenanthrol (9-PT) decreased $I_{S,Cs}$ by ~25% (Fig. 2D).

The role of Cx43 hemichannel in the generation of $I_{S,Cs}$ was further examined by the inhibition of Cx43 using an anti-Cx43 antibody of Cx43. $I_{S,Cs}$ was almost completely blocked by dialysis with internal solutions containing the Cx43 antibodies (400 ng/ml) for 15 min (Fig. 2E, F; "Cx43 Ab"). When the same experiments were performed in cells dialyzed with scramble as a control, $I_{S,Cs}$ was not significantly reduced up to 15-min-dialysis (Fig. 2E, F). Note that $I_{S,Cs}$ measured in control internal solutions were slightly larger than those detected in cells dialyzed with scramble-containing internal solutions (Fig. 2E, F).

Next, we used Cx43 cKO mice to further confirm the specific role of Cx43 in the activation of $I_{S,Cs}$. Cx43 expression was reduced to ~20% in Cx34 cKO mice using the present TAM injection protocol (Fig. S1). The peak magnitudes of $I_{S,Cs}$ in atrial myocytes from Cx43 cKO mice were much smaller than those measured in control mouse atrial cells (Fig. 2G). Shear-induced cation currents were similarly eliminated by Cx43-specific siRNA in HL-1 atrial cells (Fig. S2). To confirm the role of Cx43 hemichannel in the activation of $I_{S,Cs}$, we used TAT-Gap19 that inhibits Cx43 hemichannels without blocking Cx40 or Px1 hemichannel [25, 26]. External application of TAT- Gap19 suppressed $I_{S,Cs}$ in a concentration dependent manner with an almost complete inhibition at 60 μM (Fig. 2H). Taken together, these data indicate a key role of Cx43 hemichannels in the activation of $I_{S,Cs}$.

Shear stress induces a dye flux via Cx43 hemichannels in atrial myocytes

To address the activation of Cx43 hemichannels by shear stress in atrial myocytes we performed live-cell imaging for Cx-permeable fluorescent dye (calcein-red/orange). In the dye-loaded cells cellular fluorescence was immediately decayed by the application of shear stress with 2 mM Ca^{2+}-containing Tyrode's solutions (Control in Fig. 3A, B). To compare the effects of several interventions the time courses of averaged calcein fluorescence, measured in multiple cells, were normalized to control levels and presented. In addition, dye efflux rate was estimated as $\%DF/F_0$ per min to compare the peak shear effects. Results in Fig. 3B and 3C show that shear-induced dye efflux was accelerated to ~300% of the control by the removal of extracellular Ca^{2+} ("Ca^{2+}-free") and it was almost completely suppressed by the application of La^{3+}, but not by Px blockade ("probenecid").

The next series of experiments were carried out using puffing solutions containing zero extracellular Ca^{2+} as a control under repetitive applications of 1-min-long shear stress in the same myocytes to further investigate the pathway of dye efflux under shear stress. Calcein signal decay in the absence of shear stress ("no shear") was much lower than that in the presence of shear stress (Fig. 3D, E). After the myocytes were treated with 10 μM CBX, that blocks Px more selectively than Cx [23], shear-mediated dye

efflux was not significantly altered (Fig. 3D, E). This result confirms that Px does not contribute to shear-mediated calcein efflux in these myocytes.

In atrial myocytes preincubated with Gap19, there was no significant shear-dependent calcein efflux, in that the calcein signals measured without ("no shear, Gap19") and with shear stimuli ("1st and 2nd shear, Gap19") were similar (Fig. 3F, G), suggesting a role of Cx43 hemichannels on shear-induced calcein efflux. Using Cx43 cKO mice, we confirmed that shear-stress-induced calcein efflux was negligible in atrial myocytes lacking Cx43 (Fig. 3H, I). In contrast, in atrial cells from control mice, shear stress continued to induce significant dye efflux, which was not altered by probenecid ("2nd shear, prob") (Fig. 3H, I). These results suggest the activation of Cx43 hemichannels by shear stress in atrial myocytes.

Autocrine activation of P2 receptors mediates $I_{S,Cs}$: a major role of P2X4R

Since Cx43 hemichannels mediate ATP release from atrial myocytes under shear stress [14], P2 receptors can be activated in an autocrine mode. Therefore, we tested whether P2X receptors that carry cations are activated in this way and contribute to $I_{S,Cs}$. When ATP was removed by external application of apyrase $I_{S,Cs}$ were completely suppressed (Fig. 4A). When P2 receptors were suppressed by suramin (10-30 mM), $I_{S,Cs}$ were reduced by ~75% (Fig. 4B, G). Application of the P2XR-selective antagonist iso-PPADS at the concentrations of 10-100 mM reduced $I_{S,Cs}$ by 55%-60% (Fig. 4C, G). Inhibition of the P2Y1 receptor (P2Y1R) using MRS2179 (400 nM) reduced the current by 20-30% (Fig. 4D, G). Additional application of 9-PT did not further suppress the current, suggesting that TRPM4 may be a downstream effector molecule under the activation of P2Y1R. These results suggest that autocrine activation of P2XR and P2Y1R may be responsible for most of cation influx at diastole under shear stress, with a major contribution by P2XRs.

Moreover, to identify the P2XR subtype to carry the $I_{S,Cs}$ pharmacological interventions were further used. Since P2X4R and P2X5R are abundant in rat atrial myocytes [14], their contributions to $I_{S,Cs}$ were examined with dialyzing their antibodies (400 ng/ml) into the myocytes through the patch pipettes. Results in Figure 4E show that introduction of anti-P2X4R antibodies into the cells suppressed $I_{S,Cs}$ by ~50% within 15 min of dialysis. In contrast, dialysis of cells with the P2X4R antibodies together with their antigenic peptides as a control did not significantly alter the currents (Fig. 4E). When the same experiments were carried out using anti-P2X5R antibodies, $I_{S,Cs}$ were not significantly reduced by introduction of the antibodies (Fig. 4G; Fig. S3).

Furthermore, selective inhibition of P2X4R with its antagonist 5-BDBD reduced $I_{S,Cs}$ by 50%-60% (Fig. 4F, G) at its maximally effective concentration (10 mM) [27, 28]. Consistently, PSB-12054, another P2X4R antagonist [29], inhibited $I_{S,Cs}$ in a concentration-dependent manner with a maximal suppression of ~40%

at 3 μM (Fig. S4, Fig. 4G). Altogether, these results suggest that P2X4R is a major subtype responsible for P2XR component (~50%) of $I_{S,Cs}$.

Atrial hypertrophy and dilation induced by short- and long-term TAC

PO such as hypertension is one of leading causes of atrial hypertrophy that leads to depressed atrial contractility in patients and represents an important risk factor for atrial fibrillation, thromboembolic stroke, and HF. Under these pathologic conditions, atria are subjected to higher shear stress. Next, we investigated whether $I_{S,Cs}$ and Cx43-P2X4R signaling under shear stress are altered as atrium undergoes remodeling with gradual increase in afterload using a TAC rat model. Transthoracic echocardiography results represent that TAC for 5 weeks produced typical LV hypertrophy, in which ejection fraction (EF; +20%, $P < 0.01$), fractional shortening (FS; +40%, $P < 0.01$), and wall thickness (+40%, $P < 0.001$) were increased (Fig. S5). At 5-week-TAC, the LA chamber was slightly, but significantly, dilated compared with the sham (+30%, $P < 0.05$; Fig. 5A, B). The FS of LA in the 5-week-TAC rats were not altered (Fig. 5B). Measurement of membrane capacitance using whole-cell patch clamp showed ~two-fold increase in atrial cell surface area after TAC for 5 weeks (+100%, $P < 0.001$; Fig. 5B).

At ~20 weeks after TAC surgery, left ventricle showed HF characteristics, such as lower ventricular EF and wall thickness, and larger LV inner diameter (Fig. S5). After 20-week-TAC, LA dilation became more severe (+70%, $P < 0.01$ *vs.* sham; Fig. 5A, B) and contractility of LA was lower than that in the age-matched sham group (-50%, $P < 0.05$; Fig. 5B). The capacitances of LA cells were not further changed after 20-week-TAC (Fig. 5B).

$I_{S,Cs}$ and its P2X4R component increase during compensatory hypertrophy but decrease with depressed atrial contractility under chronic TAC

Comparison of LA $I_{S,Cs}$ revealed approximately two-fold increase in $I_{S,Cs}$ density during compensatory hypertrophy (upper panels of Fig. 5C; Fig. 5D, “Control”). Inhibition of $I_{S,Cs}$ by 5-BDBD (10 μM) (by ~50%) was not altered in these hypertrophied LA cells (Fig. 5C, D, upper panels), which results larger 5-BDBD-sensitive $I_{S,Cs}$ in these LA cells (sham, 0.81 ± 0.21 pA/pF, n = 12; TAC, 1.4 ± 0.33 pA/pF, n = 11, $P < 0.05$) (Fig. 5C, D). During HF $I_{S,Cs}$ of LA cells were decreased again (lower panels in Fig. 5C, D), resulting in no difference in the $I_{S,Cs}$ from LA cells from sham rats (Fig. 5C, D). Furthermore, $I_{S,Cs}$ in 20-week-sham LA cells were larger than those from LA cells of 5-week-sham rats. At this stage, ~50% of $I_{S,Cs}$ in LA cells were still sensitive to 5-BDBD in sham group while smaller (~40%) fraction of $I_{S,Cs}$ was suppressed by 5-BDBD in TAC group (Fig. 5C, 5), resulting in a reduction of 5-BDBD-sensitive $I_{S,Cs}$ (P2X4R current) in failed LA cells (sham, 0.97 ± 0.18 pA/pF, n = 15; TAC, 0.71 ± 0.22 pA/pF, n = 10, $P < 0.05$). Altogether, these

results suggest that $I_{S,Cs}$ may be a compensatory mechanism that is enhanced during LA remodeling under PO, and that this shear response is ameliorated with severe LA dilation during HF.

Next, we investigated whether P2X4R protein expression in LA muscle is changed in short- and long-term-TAC rats. Results on Figure 5E and 5F show that the expression of P2X4Rs in RA and LA muscles significantly increased during hypertrophy, consistent with larger P2X4R-sensitive currents at this stage. Note that the P2X4R expression is significantly higher in old (20 week) sham atrial muscle compared to that of younger sham (5 week). In contrast, the levels of P2X4R proteins in LA- and RA-muscles during HF were significantly lower than those from the age-matched control group (Fig. 5E, F, lower panels), consistent with the reversal of $I_{S,Cs}$ and decrease of 5-BDBD-sensitive P2X4R current in failed LA myocytes. We observed a similar tendency in the changes of magnitudes of $I_{S,Cs}$ and 5-BDBD-sensitive currents in RA myocytes by short- and long-term TAC, although the fraction of 5-BDBD-senstivive current was significantly larger in RA myocytes compared to LA myocytes (Fig. S6). Taken together, the results suggest that short-term PO increases P2X4R-mediated cation currents, at least in part, via upregulation of P2X4R protein expression, and that long-term PO attenuates $I_{S,Cs}$ and P2X4R function. In addition, expression of P2X4Rs and their functions may increase with aging, which may cause a reduction in internal P2X4R pool to be immediately regulated by shear stress.

Attenuation of shear-activated Cx43 hemichannel activity in atrial myocytes during HF

The changes of $I_{S,Cs}$ in LA myocytes by PO can be caused by alterations in the activity of Cx43 hemichannels. Next, we examined whether Cx43 hemichannel-mediated calcein efflux under shear stress is altered during compensatory hypertrophy and HF. The results shown in Figure 6A and 6B present calcein signal-averages measured in multiple RA- and LA- myocytes from 5- and 20-week-TAC rats and from their age-matched sham rats. During PO-induced hypertrophy shear-induced calcein efflux was not altered in RA and LA myocytes.

During HF, LA myocytes showed significantly lower shear-induced calcein efflux than the age-matched sham group (“20-wk, shear”, Fig. 6B, D). The RA cells from 20-week-TAC rats also showed a similar tendency, with less reduction in the calcein efflux under shear stress (“20-wk, shear”, Fig. 6A, C). Notice that the shear-induced calcein efflux rate was generally 20~30% higher in RA cells compared with LA myocytes (Fig. 6). There were no age-dependent changes in the magnitude of calcein efflux in LA and RA myocytes from sham groups (compare 5-week and 20-week sham in Fig. 6). The results indicate that shear-induced Cx43 hemichannel activity may be maintained during compensatory phase, and that this response is ameliorated with severe atrial dilation under chronic PO.

Alteration in expression and phosphorylation of Cx43 by short- and long-term TAC

Next, we investigated whether the alterations of shear-induced Cx43 hemichannel function by PO are caused by remodeling in Cx43 protein expression using immunoblotting method. Results in Figure 6E and 6F show that total Cx43 expressions were not changed in RA and LA muscles during hypertrophy, while they were significantly decreased under HF. This result was consistent with the functional changes in Cx43 hemichannels in atrial cells under hypertrophy and HF (Fig. 6 A-D). Note that the levels of ANP expression were gradually increased in both RA- and LA-tissues by PO (Fig. 6E, F; “ANP”). Overall ANP protein expressions were higher in LA tissue than RA tissue.

We further assessed the phosphorylated form of Cx43 at Ser368 (pS368-Cx43), a known protein kinase C (PKC) site [30-32], because PKC is a downstream signaling molecule activated by shear-P2Y1R signaling in atrial myocytes [21]. This phosphorylation is also thought to contribute to the localization and remodeling of Cx43 in cardiac muscle [33]. The levels of pS368-Cx43 were increased only in the LA muscles by PO (Fig. 6E, F) and much lower in older LA muscles (Fig. 6E, F). The results on the alteration of pS368-Cx43 indicates that this phosphorylation is PO-dependently regulated in LA muscle.

Discussion

The results obtained in the present study demonstrate a novel depolarization mechanism of Cx43 hemichannel-P2X4R signaling via ATP release in atrial myocytes under shear stimulus and provide new evidence for its association with compensatory atrial hypertrophy and atrial failure under progressive increase in afterload. It has been consistently shown that shear stress induces ATP release via Cx43 hemichannels, and subsequent P2 receptor activation, thereby triggering AP and global Ca^{2+} waves in atrial myocytes [14, 21]. Therefore, the identification of depolarization mechanism under shear stimulus at diastole, and its characteristic enhancements during compensatory remodeling against increased afterload, with a reversal by prolonged PO, might open new avenues toward the establishment of therapeutic strategies to prevent hypertension-induced atrial remodeling and progression to severe atrial dilation.

The physiological function and activation mechanisms of Cx hemichannels in the heart have been poorly understood. Using a dye flux assay combined with pharmacological and genetic tools to selectively suppress Cx43 hemichannels we demonstrate, for the first time, activation of Cx43 hemichannels by shear stress in atrial myocytes (Fig. 3). Cx43 expressed in mammalian expression systems have shown a significant current mostly at positive potentials [34]. Consistently, in the presence of apyrase, the whole-cell cation currents recorded in atrial myocytes at −70 mV under shear stress were negligible (Fig. 4A). Therefore, in the present study, dye flux assay was adopted to measure Cx hemichannel activation in intact resting atrial cells under shear stress. A line of recent reports in *ventricular* myocytes support that Cx43 hemichannels can be activated at negative potentials under other types of interventions including a genetic defect in plakophilin-2 [18], metabolic stress [35] and Ca^{2+} releases (by caffeine, adrenergic stress and pacing) [36]. In addition to these stimuli, mechanical activation of Cx43 hemichannels and Cx46 has been reported in *other* cell types or in the mammalian expression systems [37, 38]. Direct

mechanosensitivity by Cx43 hemichannels, however, has not been proven, although this is the case for Cx46 hemichannels [37].

The shear stress-induced whole-cell cation currents in resting atrial myocytes were dramatically inhibited by Cx43 hemichannel blockade or Cx43 cKO (Fig. 2). However, the currents were eliminated by apyrase, and also suppressed by P2XR- or P2X4R-blockers, suggesting a major role of autocrine P2X4R activation (Fig. 4). P2X4R-mediated cation (most likely Na^+) influx under physiological conditions may cause membrane depolarization and subsequent increase in cytosolic Ca^{2+} concentrations (Fig. 7). In addition, P2X4R-mediated Na^+ accumulation in the cytosol can cause cytosolic Ca^{2+} increase via modulation of the Na^+–Ca^{2+} exchanger, which may result in positive inotropy. Such cellular mechanism as well as large enhancements in $I_{S,Cs}$ and P2X4R current fraction during LA compensatory hypertrophy suggest that shear-mediated cation currents via P2X4R may improve LA contractility and beating to effectively empty LA chamber against ventricular hypertrophy at end-diastole. Amelioration of this compensatory response with more prolonged hemodynamic overload appears to be associated with downregulations of P2X4R and Cx43 in dilated LA muscle (Figs. 5, 6 and 7). In vitro examination to test the effects of prolonged (20 to 50 min) laminar shear stress in a field of isolated rat atrial cells and cultured HL-1 atrial cells showed significant increase in P2X4R expression (Fig. S6), supporting remodeling of shear-P2X4R signaling in short-term TAC rats is caused by afterload-associated shear stress. A role of P2X4R as a positive regulator of cardiac muscle function has been previously suggested in *ventricle*. In this regard, P2X4R-overexpressing mice, ventricular ejection function was improved, and ventricle cell current was increased by external ATP application [39]. Conditional cardiac-specific P2X4R KO has consistently shown depressed ventricular contraction in post-infarct and PO models of heart failure [40].

The shear-stress-induced immediate cation currents and dye efflux in atrial myocytes were not sensitive to Px blockades (Figs. 2C, 3B, 3C, and 3D), which is distinguished from the stretch response in cardiac muscle involving Px activation [41]. Activation of Px can also mediate ATP- and UTP-release in stretched cardiac cells [41, 42], which has been suggested to induce ventricular fibrosis via P2Y6 receptors [42] and recruitment of immune cells [41]. Possible role of Px in ventricular shear signaling remains to be explored.

Total Cx43 protein expressions were not altered under compensatory hypertrophy when there was no significant change in shear-induced calcein efflux (Fig. 6). The levels of total Cx43 proteins were reduced in both RA and LA muscles under HF (Fig. 6), where shear-induced calcein efflux was decreased (Fig. 6). Therefore, the level of total Cx43 expression appeared to be correlated with the level of Cx43 hemichannel activity under shear stress. However, the profile of the magnitudes of calcein flux in RA- versus LA-myocytes under shear was different from their profiles on the levels of Cx43 phosphorylation at S368 (Fig. 6). Overall age-dependent reduction in Cx43 phosphorylation were also observed upon comparison between 5- and 20-week atrial muscles (Figs. 6 and S7), suggesting decrease of phosphorylation machinery or increase in dephosphorylation mechanism by aging. Such changes in phosphorylation/dephosphorylation capacity may be one of the reasons for less increase in Cx43 phosphorylation status under HF (Figs. 6 and S7).

In the failed rat LA myocytes, the Cx43 hemichannel activity induced by *shear stress* was significantly lower than that in the sham-operated atrial myocytes (Fig. 6). However, recent report by De Smet et al. [36] showed increased Cx43 hemichannel activity, induced by Ca^{2+} releases by caffeine, pacing and adrenergic stimulation, in intercalated disc of failing *human ventricular* myocytes [36]. In fact, the intracellular Ca^{2+} buffering using high concentrations of BAPTA did not suppress Cx43 hemichannel-mediated ATP release under shear stress, such that shear-dependent Cx43 hemichannel is not necessarily preceded by intracellular Ca^{2+} release [15], although it rather induces Ca^{2+} waves and depolarizations [15, 21]. The different observations in the hemichannel activity in failing human ventricular myocytes under Ca^{2+} increases [36] and rat atrial myocytes by shear stress may be related to different stimuli (shear *vs.* Ca^{2+} release)-dependent signaling compartmentalization and/or distinct cardiac area (atrium vs ventricle) and to species. Similar to ventricular myocytes, atrial cells have abundant gap junctions with step-like and straight-end intercalated discs, but often also show side-to-side junctions with lateral distribution [43] that could be the regions to be more strongly altered during shear stress.

The present results on the difference in the magnitudes of shear-induced dye flux (Fig. 6) suggest that Cx43 hemichannel activity under shear stress may be higher in RA cells than that of LA cells. Consistently, overall total Cx43 expressions were somewhat higher in RA cells compared to LA cells (Fig. 6). Shear-induced dye flux was more strongly attenuated in LA myocytes than RA cells after long-term TAC (Fig. 6), consistent with larger changes in ANP expression by TAC in LA muscle. Cell capacitance increased to ~200% in the LA cells at 5 weeks following TAC when those of RA cells were not altered significantly (Fig. S5, S8A), although ANP increased in both RA and LA cells after short-term PO (Fig. 6). The sizes of RA cells increased to ~180% after 20-week TAC, which was also accompanied with dramatic increase in ANP (Figs. 6 and S8). These suggest that RA cells are affected by TAC from the early period of TAC even with no significant hypertrophic cell growth. Consistently, $I_{S,Cs}$ in RA cells as well as their P2X4R current component were increased after 5-week TAC, which was even more dramatic compared to those of LA cells (Fig. S8). We consistently found evidence on a bigger contribution by P2X4R to $I_{S,Cs}$ in TAC RA- versus LA-cells, and in old- versus young-sham RA cells (Fig. S8), which may be, in part, due to higher abundance in P2X4R expression in RA cells [14] and larger alteration in its expression in RA cells by TAC and/or age compared to LA cells (Fig. 5F).

Our results and previous reports suggest that cardiac myocytes show significant response to shear stress in the range of 1~25 dyn/cm^2 [44, 45]. Shear force to elicit maximal $I_{S,Cs}$ was about 10 dyn/cm^2 (Fig. 1) that was similar strength showing maximal ATP release in atrial cells [15]. Significant $I_{S,Cs}$ was observed at shear stress of 1.4 dyn/cm^2 (Fig. 1) which is lower than the threshold to elicit TRPM4-mediated ramp current in atrial cells [16]. It is difficult to measure exact shear strength to which atrial myocytes are exposed in working heart *in vivo*. Roughly approximated shear force in beating rat atrial tissue is reported to be ~0.4 dyn/cm^2 [6] and the value is slightly lower than the magnitude (~1 dyn/cm^2) of the current experimental threshold. In the single cell preparations, it is expected that there were more hemichannels than in intact tissue. However, it should be also noted that gap junctions in dissociated adult cardiac myocytes are maintained for up to 22 hours in media and have a longer half-life than its counterpart in

other organ such as the liver [46]. In addition, a recent report has shown Cx43 hemichannel function in intact heart using whole-heart dye flux assay [18]. It has been shown that failing hearts have significantly less physical coupling between myocytes [47, 31], suggesting that the impact of such hemichannel function may be significantly enhanced in such disease. Nevertheless, comparison of Cx43 hemichannel function under shear stress between control group and HF group demonstrated that shear-dependent hemichannel function was attenuated in failing atrial myocytes (Fig. 6).

Cardiac shear stress could gradually increase with hypertrophy (thicker wall), fibrosis (smaller gap distance) and severe dilation (enhanced slippage). During progressive increase of afterload by TAC, the capacity of atrial cells to elicit significant cation influx via Cx43 hemichannels-P2X4R signaling in response to shear stress appeared to increase during compensatory hypertrophy, which was reversed with severe atrial dilation and failure. The results presented in this study bring important insights not only to the shear stress signaling pathway involving Cx43 hemichannels and P2X4R as an intrinsic pressure adaptation mechanism in atrial myocytes to compensate atrial function but also a pathologic role of shear signaling remodeling under prolonged increase in afterload.

Abbreviations

ANP atrial natriuretic peptide

CALHM1 calcium homeostasis modulator 1

AP action potential

cKO conditional knockout

Cx43 connexin43

EF ejection fraction

FS fractional shortening

HF heart failure

I_S shear-activated current

$I_{S,Cs}$ shear-activated Cs^+ current

KD knockdown

LA left atrial

LAID,d left atrial inner diameter at diastole

LAID,s left atrial inner diameter at systole

LV left ventricular

P2XR P2X receptor

P2X4R P2X4 receptor

P2Y1R P2Y1 receptor

PKC protein kinase C

PO Pressure overload

pS368-Cx43 phosphorylated Cx43 at Serine 368

RA right atrial

TAC transverse aortic constriction

TAM tamoxifen

TRPM4 transient receptor potential melastatin 4

Declarations

Ethics approval

This study was performed in line with the principles of the Guiding Principles for the Care and Use of Experimental Animals published by the Korean Food and Drug Administration and Animal and Plant Quarantine Agency in South Korea. The experimental protocols were approved by the Animal Care and Use Committees of the Chungnam National University, South Korea (No. CNU-00992).

Consent to participate

'Not applicable' for that section

Content for publication

Authors confirm that all the authors have seen and been given the opportunity to read both the Material and the Article (as attached) to be published in Cellular and Molecular Life Sciences by Springer.

Acknowledgements

This work was supported by National Research Foundation of Korea (NRF) grants funded by the Korean Government (MEST) (2017R1E1A1A01074504).

Authors' contributions

All authors contributed to the study conception and design. Material preparations, data collection and analysis were performed by Joon-Chul Kim, Long Nguyen Hoang Do, Qui Anh Le, Tran Nguyet Trinh, Luong Phuong Kim, and Sun-Hee Woo. The first draft of the manuscript was written by Joon-Chul Kim and Sun-Hee Woo and all the authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Competing interest

The authors have no relevant financial or non-financial interests to disclose.

Data Availability

The datasets generated during and/or analyzed during the current study are available in the repository.

References

1. Lakatta EG (1993) Cardiovascular regulatory mechanisms in advanced age. Physiol Rev 73:413-467. http://doi.org/10.1152/physrev.1993.73.2.413
2. Nazir S, Lab MJ (1996). Mechanoelectric feedback and atrial arrhythmias. Cardiovasc Res 32:52-61.
3. Goldsmith I, Kumar P, Carter P, Blann AD, Patel RL, Lip GY (2000) Atrial endocardial changes in mitral valve disease: a scanning electron microscopy study. Am Heart J 140:777-784. http://doi.org/10.1067/mhj.2000.110284
4. Gülan U, Saguner AM, Akdis D, Gotschy A, Tanner FC, Kozerke S, Manka R, Brunckhorst C, Holzner M, Duru F (2019) Hemodynamic changes in the right ventricle induced by variations of cardiac output: A possible mechanism for arrhythmia occurrence in the outflow tract. Sci Rep 9:1-11. http://doi.org/10.1038/s41598-018-36614-7
5. Saffitz J. Acquired valvular and endocardial diseases. Essentials of Rubin's Pathology. 2009:232-237.
6. Boycott HE, Barbier CS, Eichel CA, Costa KD, Martins RP, Louault F, Dilanian G, Coulombe A, Hatem SN, Balse E (2013) Shear stress triggers insertion of voltage-gated potassium channels from intracellular compartments in atrial myocytes. Proc Natl Acad Sci 110:E3955-E3964. http://doi.org/10.1073/pnas.1309896110
7. Costa KD TY, McCulloch AD, and Covell JW (1999) Laminar fiber architecture and three-dimensional systolic mechanics in canine ventricular myocardium. Am J Physiol Heart Circ Physiol 276:H595-H607. http://doi.org/10.1152/ajpheart.1999.276.2.H595
8. LeGrice I, Takayama Y, Covell J (1995) Transverse shear along myocardial cleavage planes provides a mechanism for normal systolic wall thickening. Circ Res 77:182-193. http://doi.org/10.1161/01.res.77.1.182
9. Paul S (2003) Ventricular remodeling. Crit Care Nurs Clin North Am 15:407-411. http://doi.org/10.1016/s0899-5885(02)00089-8

10. Woo S, Risius T, Morad M (2007). Modulation of local Ca^{2+} release sites by rapid fluid puffing in rat atrial myocytes. Cell Calcium 41:397-403. http://doi.org/10.1016/j.ceca.2006.09.005
11. Lee S, Kim JC, Li Y, Son MJ, Woo SH (2008) Fluid pressure modulates L-type Ca^{2+} channel via enhancement of Ca^{2+}-induced Ca^{2+} release in rat ventricular myocytes. Am J Physiol Cell Physiol 294:C966-C976.
12. Kong CR, Bursac N, Tung L (2005) Mechanoelectrical excitation by fluid jets in monolayers of cultured cardiac myocytes. J Appl Physiol 98:2328-2336. http://doi.org/10.1152/japplphysiol.01084.2004
13. Lorenzen-Schmidt I, Schmid-Schönbein GW, Giles WR, McCulloch AD, Chien S, Omens JH (2006) Chronotropic response of cultured neonatal rat ventricular myocytes to short-term fluid shear. Cell Biochem Biophys 46:113-122. http://doi.org/10.1385/CBB:46:2:113
14. Le QA, Kim JC, Kim KH, Van VAT, Woo SH (2020) Distinct shear-induced Ca^{2+} signaling in the left and right atrial myocytes: Role of P2 receptor context. J Mol Cell Cardiol 143:38-50. http://doi.org/10.1016/j.yjmcc.2020.04.018
15. Kim JC, Son MJ, Woo SH (2018) Ca^{2+} signaling triggered by shear-autocrine P2X receptor pathway in rat atrial myocytes. Cell Physiol Biochem 50:2296-2313. http://doi.org/10.1159/000495089
16. Son MJ, Kim JC, Kim SW, Chidipi B, Muniyandi J, Singh TD, So I, Subedi KP, Woo SH (2016) Shear stress activates monovalent cation channel transient receptor potential melastatin subfamily 4 in rat atrial myocytes via type 2 inositol 1,4,5-trisphosphate receptors and Ca^{2+} release. J Physiol 594:2985-3004. http://doi.org/10.1113/JP270887
17. Liao Y, Day K, Damon D, Duling B (2001) Endothelial cell-specific knockout of connexin 43 causes hypotension and bradycardia in mice. Proc Natl Acad Sci 98:9989-9994. http://doi.org/10.1073/pnas.171305298
18. Kim JC, Perez-Hernandez M, Alvarado FJ, Maurya SR, Montnach J, Yin Y, Zhang M, Lin X, Vasquez C, Heguy A, et al. (2019) Disruption of Ca^{2+}_i homeostasis and connexin 43 hemichannel function in the right ventricle precedes overt arrhythmogenic cardiomyopathy in plakophilin-2–deficient mice. Circulation 140:1015-1030. http://doi.org/10.1161/CIRCULATIONAHA.119.039710
19. Rockman HA, Ross RS, Harris AN, Knowlton KU, Steinhelper ME, Field LJ, Ross J, Chien KR (1991) Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. Proc Natl Acad Sci 88:8277-8281. http://doi.org/10.1073/pnas.88.18.8277
20. Olesen SP, Clapham D, Davies P (1988) Haemodynamic shear stress activates a K^+ current in vascular endothelial cells. Nature 331:168-170. http://doi.org/10.1038/331168a0
21. Kim JC, Woo SH (2015). Shear stress induces a longitudinal Ca^{2+} wave via autocrine activation of P2Y1 purinergic signalling in rat atrial myocytes. J Physiol 593:5091-5109. http://doi.org/10.1113/JP271016

22. Locovei S, Wang J, Dahl G (2006) Activation of pannexin 1 channels by ATP through P2Y receptors and by cytoplasmic calcium. FEBS Lett 580:239-244. http://doi.org/10.1016/j.febslet.2005.12.004
23. Bruzzone R, Barbe MT, Jakob NJ, Monyer H (2005) Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in Xenopus oocytes. J Neurochem 92:1033-1043. http://doi.org/10.1111/j.1471-4159.2004.02947.x
24. Ma Z, Siebert AP, Cheung K-H, Lee RJ, Johnson B, Cohen AS, Vingtdeux V, Marambaud P, Foskett JK (2012) Calcium homeostasis modulator 1 (CALHM1) is the pore-forming subunit of an ion channel that mediates extracellular Ca^{2+} regulation of neuronal excitability. Proc Natl Acad Sci 109:E1963-E1971. http://doi.org/10.1073/pnas.1204023109
25. Wang N, De Vuyst E, Ponsaerts R, Boengler K, Palacios-Prado N, Wauman J, Lai CP, De Bock M, Decrock E, Bol M, et al. (2013) Selective inhibition of Cx43 hemichannels by Gap19 and its impact on myocardial ischemia/reperfusion injury. Basic Res Cardiol 108:1-16. http://doi.org/10.1007/s00395-012-0309-x
26. Abascal F, Zardoya R (2013) Evolutionary analyses of gap junction protein families. Biochim Biophys Acta 1828:4-14. http://doi.org/10.1016/j.bbamem.2012.02.007
27. Balázs B, Dankó T, Kovács G, Köles L, Hediger MA, Zsembery Á (2013) Investigation of the inhibitory effects of the benzodiazepine derivative, 5-BDBD on P2X4 purinergic receptors by two complementary methods. Cell Physiol Biochem 32:11-24. http://doi.org/10.1159/000350119
28. Coddou C, Sandoval R, Hevia MJ, Stojilkovic SS (2019) Characterization of the antagonist actions of 5-BDBD at the rat P2X4 receptor. Neurosci Lett 690:219-224. http://doi.org/10.1016/j.neulet.2018.10.047
29. Hernandez-Olmos V, Abdelrahman A, El-Tayeb A, Freudendahl D, Weinhausen S, Müller CE (2012) N-substituted phenoxazine and acridone derivatives: structure–activity relationships of potent P2X4 receptor antagonists. J Med Chem 55:9576-9588. http://doi.org/10.1021/jm300845v
30. Lampe P, TenBroek E, Burt JM, Kurata WE, Johnson RG, and Lau AF (2000) Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. J Cell Biol 149:1503-1512. http://doi.org/10.1083/jcb.149.7.1503
31. Rucker-Martin C, Milliez P, Tan S, Decrouy X, Recouvreur M, Vranckx R, Delcayre C, Renaud J-F, Dunia I, Segretain D, et al (2006) Chronic hemodynamic overload of the atria is an important factor for gap junction remodeling in human and rat hearts. Cardiovasc Res 72:69-79. http://doi.org/10.1016/j.cardiores.2006.06.016
32. Brissette J, Kumar N, Gilula N, Dotto G (1991) The tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the ras oncogene modulate expression and phosphorylation of gap junction proteins. Mol Cell Biol 11:5364-5371. http://doi.org/10.1128/mcb.11.10.5364-5371.1991
33. Jain SK SR, Saffitz JE (2003) Mechanisms of delayed electrical uncoupling induced by ischemic preconditioning. Circ Res 92:1138-1144. http://doi.org/10.1161/01.RES.0000074883.66422.C5
34. Contreras JE, Sáez JC, Bukauskas FF, Bennett MV (2003) Gating and regulation of connexin 43 (Cx43) hemichannels. Proc Natl Acad Sci 100:11388-11393.

http://doi.org/10.1073/pnas.1434298100

35. John SA, Kondo R, Wang S-Y, Goldhaber JI, Weiss JN (1999) Connexin-43 hemichannels opened by metabolic inhibition. J Biol Chem 274:236-240. http://doi.org/10.1074/jbc.274.1.236
36. De Smet MA, Lissoni A, Nezlobinsky T, Wang N, Dries E, Pérez-Hernández M, Lin X, Amoni M, Vervliet T, Witschas K, Rothenberg E, Bultynck G, Schulz R, Panfllov AV, Delmar M, Sipido KR, Leybaert L (2021) Cx43 hemichannel microdomain signaling at the intercalated disc enhances cardiac excitability. J Clin Invest 131:e137752. http://doi.org/10.1172/JCI137752
37. Bao L, Sachs F, Dahl G (2004) Connexins are mechanosensitive. Am J Physiol Cell Physiol 287:C1389-C1395. http://doi.org/10.1152/ajpcell.00220.2004
38. Batra N, Burra S, Siller-Jackson AJ, Gu S, Xia X, Weber GF, DeSimone D, Bonewald LF, Lafer EM, Sprague E, et al (2012) Mechanical stress-activated integrin α5β1 induces opening of connexin 43 hemichannels. Proc Natl Acad Sci 109:3359-3364. http://doi.org/10.1073/pnas.1115967109
39. Hu B, Mei QB, Yao XJ, Smith E, Barry WH, Liang BT (2001) A novel contractile phenotype with cardiac transgenic expression of the human P2X4 receptor. FASEB J 15:1-15. http://doi.org/10.1096/fj.01-0445fje
40. Yang T, Shen J-b, Yang R, Redden J, Dodge-Kafka K, Grady J, Jacobson KA, Liang BT (2014) Novel protective role of endogenous cardiac myocyte P2X4 receptors in heart failure. Circ Heart Fail 7:510-518. http://doi.org/10.1161/CIRCHEARTFAILURE.113.001023
41. Oishi S, Sasano T, Tateishi Y, Tamura N, Isobe M, Furukawa T (2012) Stretch of atrial myocytes stimulates recruitment of macrophages via ATP released through gap-junction channels. J Pharmacol Sci 120:296-304. http://doi.org/10.1254/jphs.12202fp
42. Nishida M, Sato Y, Uemura A, Narita Y, Tozaki-Saitoh H, Nakaya M, Ide T, Suzuki K, Inoue K, Nagao T, et al. (2008) P2Y6 receptor-Gα12/13 signalling in cardiomyocytes triggers pressure overload-induced cardiac fibrosis. EMBO J 27:3104-3115. http://doi.org/10.1038/emboj.2008.237
43. Severs NJ, Bruce AF, Dupont E, Rothery S (2008) Remodelling of gap junctions and connexin expression in diseased myocardium. Cardiovasc Res 80:9-19. http://doi.org/10.1093/cvr/cvn133.
44. Belmonte S, Morad M (2008) 'Pressure flow'-triggered intracellular Ca^{2+} transients in rat cardiac myocytes: possible mechanisms and role of mitochondria. J Physiol 586:1379-1397. http://doi.org/10.1113/jphysiol.2007.149294
45. Zhang Y, Sekar RB, McCulloch AD, Tung L (2008) Cell cultures as models of cardiac mechanoelectric feedback. Prog Biophys Mol Biol 97:367-382. http://doi.org/10.1016/j.pbiomolbio.2008.02.017
46. Severs NJ, Shovel KS, Slade AM, Powell T, Twist VW, Green CR (1989) Fate of gap junctions in isolated adult mammalian cardiomyocytes. Circ Res 65:22-42. http://doi.org/10.1161/01.RES.65.1.22.
47. Polontchouk L, Haefliger JA, Ebelt B, Schaefer T, Stuhlmann D, Mehlhorn U, Kuhn-Regnier F, De Vivie ER, Dhein S (2001). Effects of chronic atrial fibrillation on gap junction distribution in human and rat atria. J Am Coll Cardiol 38:883-891. http://doi.org/10.1016/S0735-1097(01)01443-7.

Figures

Figure 1

Nonselective inward cation current activated by shear stress in resting atrial myocytes and its sensitivity to external Ca^{2+}. **A**, Protocol for fluid shear application and voltage-clamp showing shear (~16 dyn/cm^2)-induced inward current recorded in normal Tyrode's solutions (I_S) at −80 mV that is preceded by a series of conditioning pulses. High-KCl internal solutions with 1 mM EGTA, and Tyrode's external solutions were used in panel **A**–**F** (see Online Data Supplement). **B**, Shear-induced currents at −80 mV in 0, 2, and 10 mM external Ca^{2+} concentrations (left) and comparison of mean peak I_S (right). $^{**}P$<0.01, $^{*}P$<0.05 *vs*. "2 mM $[Ca^{2+}]_o$" (n=7). **C** and **D**, Superimposed representative I_S at −70 mV recorded with zero Ca^{2+} puffing solutions in different external Na^+ and K^+ compositions. **D**, Mean peak I_S measured at different concentrations of external Na^+ and K^+. $^{***}P$<0.001, $^{**}P$<0.01, $^{*}P$<0.05 *vs*. control ("C": "137Na_o, 5.4K_o") (n=9). **E** and **F**, Representative I_S recorded at 0 and 75 mM external Ca^{2+} concentration with equimolar Na^+ concentrations, showing significant increase of peak I_S (n=7). $^{***}P$<0.001 *vs*. "0 $[Ca^{2+}]_o$, 25 $[Na^+]_o$". **G**–**I**: Symmetrical CsCl solutions were used with zero Ca^{2+} puffing solution (see Methods). **G**, Shear-induced Cs^+ current ($I_{S,Cs}$) measured at −70 mV in 2 mM and 0 mM external Ca^{2+} containing CsCl-rich solutions and CsCl-rich internal solution including zero EGTA (left). Comparison of peak $I_{S,Cs}$ (right). $^{**}P$<0.01 *vs.* 2 mM Ca_o (Paired *t*-test; n=8). **H**, Representative $I_{S,Cs}$ measured at different shear strength. **I**, Plots of mean peak $I_{S,Cs}$ and time-to-peak versus shear forces (n=6).

Figure 2

Primary role of Cx43 hemichannel in triggering cation influx in atrial cells under shear stress. **A-D**, Upper panels: Representative $I_{S,Cs}$ activated at 16 dyn/cm^2 measured from rat atrial myocytes at -70 mV before (CON) and after applications of 2 mM La^{3+} (**A**), 50 μM carbenoxolone (CBX; **B**), pannexin blocker probenecid (800 μM; **C**), or the TRPM4 blocker 9-phenanthrol (9-PT, 30 μM; **D**). Lower panels: Comparison of mean $I_{S,Cs}$ recorded without and with La^{3+} (n=9; **A**), CBX (n=7; **B**), probenecid (n=6; **C**), and 9-PT (n=8; D). $^{****}P$<0.0001 and $^{**}P$<0.01 *vs.* CON (paired *t*-test). **E**, Representative $I_{S,Cs}$ traces measured at 3-min, 10 min and 15-min after dialysis with control internal solutions, or internal solutions containing scramble (400 ng/ml) or anti-Cx43 antibodies (Ab) (400 ng/ml). **F**, Mean time course of the effects of dialysis of Cx43 Ab (n=14) compared to those of dialysis with control internal (n=9) or scramble containing internal solutions (n=7). $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001, $^{****}P$<0.0001 *vs.* scramble. One-way ANOVA with post hoc test. **G**, Representative $I_{S,Cs}$ traces measured in atrial myocytes isolated from wild-type (CON) and cKO of

Cx43 (left) and mean peak $I_{S,Cs}$ measured in the atrial cells from WT (three mice, n=9) and Cx43 cKO (three mice, n=14) (right). $^{**}P$<0.01 *vs.* CON. One-way ANOVA with post hoc test. **H**, Superimposed $I_{S,Cs}$ traces measured in representative atrial myocytes in the absence (CON) and presence of TAT-Gap19 (left) and mean $I_{S,Cs}$ measured in the presence of 20-μM (n=7)- and 60-μM (n=7)-TAT-Gap16 (right). $^{*}P$<0.01, $^{**}P$<0.01 *vs.* CON. One-way ANOVA with post hoc test.

Figure 3

Activation of Cx43 hemichannels by shear stress in atrial myocytes: evidence from dye flux assay. **A**, Confocal calcein (red-orange) images measured from rat atrial myocytes before (0 s) and after 210-s application of shear stress (~16 dyn/cm^2) under control condition (CON) and in the solutions containing zero Ca^{2+}, 2 mM La^{3+} or 800 μM probenecid. **B**, Time courses of averaged calcein signals under shear application, measured as % decrease (efflux), in the control solutions (2 mM external Ca^{2+}; n=7), Ca^{2+}-free solutions, La^{3+} (n=5)- or probenecid-containing solutions (n=6). **C**, Averaged shear-induced calcein efflux per min in the absence and presence of La^{3+} (n=5) or probenecid ("proben"; n=6) and in Ca^{2+}-free solutions (n=7). $^{**}P$ < 0.01, $^{***}P$<0.001 *vs.* control (CON) (unpaired *t*-test). **D** and **E**, Time courses of averaged calcein (red-orange) signals (**D**) and mean peak efflux per min (**E**) under no stimulus ("no shear") and after the 1st- and 2nd-shear application (n=7; $^{**}P$<0.01). One-way ANOVA with post hoc test. The 2nd shear application was done after the application of 10 μM CBX for 10-min. **F**, Time course of averaged dye fluxes measured under control condition (no shear) and during repetitive shear stress applications with 4 min intervals in the atrial myocytes incubated with Gap19 (600 μM) for 30 min (n=5). **G**, Average dye flux induced by repetitive shear applications in the presence of Gap19 compared to that with no shear application (n=5). $^{\#}P$<0.05 *vs.* "no shear" in the absence of Gap19 (panel **E**). $^{‡}P$<0.05 *vs.* "1st shear (control) in panel **E**. One-way ANOVA with post hoc test. **H**, Time courses of averaged dye flux under control condition and shear applications without and with probenecid (200 μM) in control mouse atrial myocytes (n=9). Right: Averaged dye flux measured in the absence and presence of shear stress in Cx43 cKO atrial myocytes (n=9). **I**, Summary of dye efflux on 1-min shear applications in control mice (three mice, n=9) and Cx43 cKO mice atrial myocytes (three mice, n=13) under control conditions and after applications of shear stress. $^{**}P$<0.01 *vs.* "no shear". $^{\#}P$<0.05 *vs.* "Control, no shear". $^{‡}P$<0.05 *vs.* "1st shear, control". Two-way ANOVA with repeated measures.

Figure 4

Shear-activated cation current is mainly mediated by autocrine ATP action on P2 receptors: major role of P2X4Rs. **A**, Superimposed $I_{S,Cs}$ detected in a representative atrial cell clamped at -70 mV under control puffing (zero external Ca^{2+} solutions) and after the addition of apyrase (2 U/ml for 8-10 min) (left). Mean

peak $I_{S,Cs}$ in control condition and after application of apyrase (right) (n=6). $^{****}P<0.0001$ *vs.* Control (paired *t*-test). **B**, Representative $I_{S,Cs}$ detected before and after application of 10 μM suramin in a representative atrial cell (left) and comparison of peak $I_{S,Cs}$ (right). $^{**}P<0.01$ *vs.* Control (n=7). **C**, Representative $I_{S,Cs}$ detected before and after application of 30 μM iso-PPADS (5 min) in a representative atrial cell (left) and comparison of peak $I_{S,Cs}$ (right). $^{***}P<0.001$ *vs.* Control (n=7). **D**, Superimposed $I_{S,Cs}$ detected in a representative atrial cell under control conditions, after the addition of 200 nM MRS2179, and 200 nM MRS2179 plus 30 μM 9-PT (left), and mean peak $I_{S,Cs}$ under each conditions (right; n=6; $^{**}P<0.01$ *vs.* Control; paired *t*-test). **E**, Representative currents show $I_{S,Cs}$ measured following different dialysis duration in the same cells with anti-P2X4R antibodies (Ab; 400 ng/ml) with or without its antigenic peptides (Ag) (left). Right, Plots of means of peak $I_{S,Cs}$ versus dialysis durations (Ab+Ag, n=11; Ab, n=13). $^{**}P<0.01$ *vs.* "P2X4R Ab+Ag". **F**, Representative $I_{S,Cs}$ measured before and after application of 5-BDBD (10 μM) in a same cell (left) and summary of the effects of 5-BDBD on peak $I_{S,Cs}$ (n=8). $^{*}P < 0.05$ *vs.* Control. **G**, Summary of the effects of various pharmacological agents on peak $I_{S,Cs}$. The number above each bar indicates number of cells tested. $^{****}P<0.0001$, $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ *vs.* control. $^{\#}P<0.05$ *vs.* "0.3 PSB-12054".

Figure 5

Alterations in $I_{S,Cs}$ and P2X4R-function and -expression with TAC-induced LA adaptive hypertrophy and LA failure. **A**, LA echocardiogram showing progressive increases in LA diameters (arrows) in systole and diastole following 5- (left) and 20-week-TAC (right). AscAo: ascending aorta; AoR: Aortic root; LV: Left ventricle; RVOT: right ventricular outflow tract. **B**, comparisons of mean LA inner diameter at diastole (LAID,d), fractional shortening (FS) and cell capacitance between LA cells from sham and TAC at 5- and 20-week after surgery. Number of rats: 5-week sham, 12; 5-week TAC, 15; 20-week sham, 15; 20-week TAC, 20. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* sham. $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* 5-wk TAC. **C** and **D,** Representative $I_{S,Cs}$ (**C**) and their mean peak values (**D**) obtained under the control solutions and after pretreatment of 5-BDBD (10 μM) in LA myocytes isolated from 5-week-sham (12 cells from 3 rats) and -TAC rats (11 cells from 3 rats) as well as 20-week-sham (15 cells from 3 rats) and -TAC rats (10 cells from 3 rats), showing large increase in $I_{S,Cs}$ and 5-BDBD-sensitive currents after 5-wk-TAC and a reversal of such effects after 20-wk-TAC. $^{*}P<0.05$ *vs.* sham. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* each control. $^{\ddagger}P<0.05$ *vs.* 5-wk TAC, control. **E** and **F**, Immunoblots from LA and RA tissue lysates of sham- and TAC-treat-hearts (**E**). Protein expression changes are summarized by bar graphs (**F**). Blots are representative of 3 independent experiments. $^{**}P<0.01$ *vs.* sham. $^{\#}P<0.05$ *vs.* sham, RA. $^{\ddagger}P<0.05$ *vs.* 5-week sham (two-way ANOVA with repeated measures).

Figure 6

Reductions in shear-mediated Cx43 hemichannel activity and Cx43 expression in RA and LA of long-term pressure overload. **A** and **B**, Signal-averaged calcein fluorescence traces normalized to control level without shear stress (0 dyn/cm^2) and with 1-min application of shear stress (16 dyn/cm^2) in RA (**A**; 5 wk: 3 sham rats, 12 cells; 3 TAC rats, 15 cells; 20-wk: 3 sham rats, 11 cells; 3 TAC rats, 14 cells) and LA myocytes (**B**; 5 wk: 3 sham rats, 10 cells; 3 TAC rats, 19 cells; 20-wk: 3 sham rats, 14 cells; 3 TAC rats, 16 cells) from 5-week- (left) and 20-week-TAC rats (right), showing a significant reduction after 20-week-TAC. **C** and **D**, Averages of shear-induced calcein efflux per min measured in the RA (**C**) and LA myocytes (**D**) from 5-week-TAC and 20-week-TAC rats were summarized. $^{*}P$<0.05 *vs.* sham. $^{\#\#\#}P$<0.001, $^{\#\#\#\#}P$<0.0001 *vs.* "No shear". $^{\ddagger}P$<0.05 *vs.* RA "shear" for the same group (two-way ANOVA with repeated measures). Alterations of Cx43 expression and its phosphorylation status by progressive pressure overload in RA and LA muscles. **E,** Immunoblots of ANP, Cx43 and pS368-Cx43 from RA and LA tissue lysates of 5-week- (left; "hypertrophy") and 20-week (right; "HF")-sham and TAC-treated hearts. **F**, The changes in protein expression at 5-week-sham and TAC rats (left) and 20-week-sham and TAC rats (right) were normalized to GAPDH bands shown in Fig. 5E (same preparations) and are summarized by bar graphs. Blots are representative of 3 independent experiments (rats). $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs.* sham. $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 *vs.* sham, RA, $^{\ddagger}P$<0.05, $^{\ddagger\ddagger}P$<0.01 *vs.* corresponding 5-wk group (two-way ANOVA with repeated measures).

Figure 7

A schematic diagram showing a signaling pathway to underlie shear-stress-induced nonselective cation influx in atrial myocytes at negative potential, and its alteration under PO induced compensatory hypertrophy (blue arrows) and atrial failure (red arrows). HC: hemichannel. Shear stress activates cation influx via mainly P2X4R in atrial myocytes at negative potentials and this response is governed by ATP release via Cx43 hemichannels. A quarter of such cation influx under shear stress appears to be mediated by TRPM4 channels that possibly linked to P2Y1R. The cation entry into the myocytes will further increase Ca^{2+} concentration due to reduced Na^{+} gradient and subsequent mobilization of Na^{+}-Ca^{2+} exchange and membrane depolarizations. Atrial hypertrophy develops during PO with increase of P2X4 receptor expression, which may result in large enhancement in shear-dependent cation influx with no significant change in Cx43 hemichannel function. Shear stress stimulus may also induce such upregulation of P2X4R during this period (Fig. S6). Prolonged PO induces severe left atrial dilation with reduced contraction. During this period, P2X4R-meidated cation influx and Cx43 hemichannel function under shear decrease again with downregulations of P2X4R and Cx43 proteins.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplements4CMLS.docx