Regulation of gap-junction protein connexin 43 by β-adrenergic receptor stimulation in rat cardiomyocytes

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Aim: β-adrenergic receptor (β-AR) agonists are among the most potent factors regulating cardiac electrophysiological properties. Connexin 43 (Cx43), the predominant gap-junction protein in the heart, has an indispensable role in modulating cardiac electric activities by affecting gap-junction function. The present study investigates the effects of short-term stimulation of β-AR subtypes on Cx43 expression and gap junction intercellular communication (GJIC) function.

Methods: The level of Cx43 expression in neonatal rat cardiomyocytes (NRCM) was detected by a Western blotting assay. The GJIC function was evaluated by scrape loading/dye transfer assay.

Results: Stimulation of β-AR by the agonist isoproterenol for 5 min induces the up-regulation of nonphosphorylated Cx43 protein level, but not total Cx43. Selective β2-AR inhibitor ICI 118551, but not β1-AR inhibitor CGP20712, could fully abolish the effect. Moreover, pretreatment with both protein kinase A inhibitor H89 and Gi protein inhibitor pertussis toxin also inhibited the isoproterenol-induced increase of nonphosphorylated Cx43 expression. Isoproterenol-induced up-regulation of nonphosphorylated Cx43 is accompanied with enhanced GJIC function.

Conclusion: Taken together, β2-AR stimulation increases the expression of nonphosphorylated Cx43, thereby enhancing the gating function of gap junctions in cardiac myocytes in both a protein kinase A- and Gi-dependent manner.

Keywords: connexin43; gap junction; β-adrenergic receptor; cardiac myocyte

Introduction
In the mammalian heart, efficient intercellular communication is essential for normal electromechanical coupling by the transmission of signaling molecules and sequenced propagation of the action potential through the myocardial gap junction[1]. Connexins (Cx) are membrane proteins that oligomerize to form gap-junction channels, through which ions and small molecules diffuse between cells[2]. At least 8 homologous connexin isoforms – Cx31.9, Cx37, Cx40, Cx43, Cx45, Cx46, Cx50, and Cx57 – have been characterized in the heart[3]. In the cardiomyocytes, the most abundant isoform is Cx43, whereas Cx40 is mainly found in atrial tissue and the conduction system. Cx45 has been detected predominantly during early development of the heart. Numerous studies have shown that alterations in the amount and distribution of Cx43 affect current conduction, induce arrhythmias and uncoordinated contraction, and even alter myocardial function[4, 5]. Phosphorylation and dephosphorylation of Cx43 are also regulators of gap-junction function, as phosphorylation of Ser 368 is needed to keep the gap junctions in a closed state[6].

Sympathetic nervous system activation is a common pathophysiologic feature of cardiovascular diseases such as hypertension and chronic heart failure. Transient activation of the sympathetic nervous system usually causes lethal arrhythmias in a diseased heart. Interestingly, disrupted gap-junction structure and decreased expression of Cx43 are also frequently seen in cardiac remodeling in response to various pathologic stimuli, such as ischemia, chronic pressure and volume overload in dogs[7], guinea pigs[8] and humans[9]. However, little information concerning the relationship between sympathetic
nervous system activation and functional regulation of myocardial gap junctions is available. Responses to sympathetic activation are mediated through the action of the endogenous catecholamines norepinephrine and epinephrine on adrenergic receptors (ARs). The heart expresses all three subtypes of β-AR, as well as three types of α1-AR: α1A-AR, α1D-AR, and α1β-AR[10]. A recent report demonstrated that α-adrenergic receptor agonist phenylephrine enhanced Cx43 expression, but not Cx40 and Cx45 expression, in neonatal rat cardiac myocytes (NRCMs), resulting in enhanced gap-junction conductance. These effects were fully suppressed by a selective α1D-antagonist BMY7378, suggesting that the α1D-adrenergic receptor mediated this effect[11]. A recent report showed that β-AR blockade elicited larger reductions in the isoproterenol-mediated increases in ventricular contractility in dogs that were susceptible to VF than in dogs that were resistant to these malignant arrhythmias. The mechanism may be that β-AR activation increases the Ca2+ current without altering Ca2+ reuptake by the sarcoplasmic reticulum, which could trigger arrhythmias. Thus, β-AR activation would tend to reduce the cardiac electrical stability, increasing the propensity for arrhythmias[12]. However, little is known about the relationship between β-adrenergic receptor subtypes, cardiac Cx43 regulation and myocardial gap junction function, which regulates the current between the cells.

In this study, we investigate the effect of β-adrenergic receptor stimulation on Cx43 expression and the gating function of gap junctions. We also study the possible receptor subtype and mechanism involved in this effect.

Materials and methods

Materials
Multiple reagents and antibodies, including isoproterenol (ISO), propranolol, ICI 118551, CGP 20712A, pertussis toxin (PTX), H89, clenbuterol, okadaic acid (OA), Lucifer Yellow dye, polyclonal rabbit anti-Cx43 antibody and horseradish peroxidase-labeled secondary antibodies, and chemiluminescence reagents were from Pierce (Rockford, IL). TRITC-conjugated anti-rabbit IgG were from Beijing Zhongshan Golden Bridge Biotechnology (Beijing, China). The doses of ISO, propranolol, ICI 118551, CGP20712A, PTX, H89, clenbuterol were used according to the international concentration[10, 13].

Cell culture
Cardiomyocytes were isolated and cultured as described previously[14]. Briefly, ventricles of new-born Sprague-Dawley rats were digested in collagenase II solution and centrifuged. After a preplating period to remove noncardiac cells, the cardiomyocytes were resuspended in DMEM (Hyclone, Logan, UT) medium containing 100 mg/mL streptomycin and penicillin, 10% fetal calf serum. To inhibit non-cardiac myocyte growth, 100 µmol/L BrdU was also added. The cells were seeded in 35-mm dishes. After incubation at 37 °C in humidified air with 5% (v/v) CO2 for 24 h, the cardiac myocytes were then deprived of serum and incubated for another 24 h before treatment. Experiments using animals were approved by the Committee on the Ethical Aspects of Research Involving Animals of the Peking University Health Science Center. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals.

Western blotting assay
The lysis buffer used for Western blot analysis consisted of 150 mmol/L NaCl, 20 mmol/L Tris-hydrochloride, pH 7.5, 1.5 mmol/L MgCl2, 1 mmol/L Na3VO4, 1% Triton X-100, 10 mmol/L NaF, and a protease inhibitor cocktail (Roche Applied Science). The cell lysates were mixed with gel-loading buffer, and 70 µg of each protein sample was fractionated through a 5% stacking and 10% running SDS-polyacrylamide gel for electrophoresis. Proteins were then transferred electrically onto nitrocellulose membranes and blocked with 5% low-fat milk blocker at room temperature for 1 h. Primary antibody to Cx43 (diluted 1:2000) or to Cx43-NP (diluted 1:1000) was applied for 4 °C overnight. The blots were then washed 3 times with TBST and incubated with secondary horseradish peroxidase-labeled antibody diluted 1:1000 for 1 h at room temperature. Bands were visualized by use of a super Western sensitivity chemiluminescence detection system (Pierce, IL, USA). Autoradiographs were quantitated by densitometric analysis using a Science Imaging system (Bio-Rad, Hercules, CA).

Scrape loading (SL)/dye transfer (DT) assay
Gap junction intercellular communication (GJIC) levels of control and treated cells were determined by the SL/DT technique, as previously reported[15]. Cardiomyocytes, cultured on glass cover-slips in six-well plates, were grown to 80% confluence. The cells were treated with ISO for 5 min and washed thoroughly with PBS. Scrape loading was performed by two cuts on the cell mono-layer with a razor blade before 500 mL of 0.05% Lucifer Yellow CH (LY) solution (Sigma) was added on the cover-slip to imbue the cells for 3 min. Cells were rinsed three times with PBS, fixed with 4% formaldehyde in PBS, and detected by fluorescence emission with an inverted fluorescence microscope (Olympus, Japan). The distances of Lucifer Yellow diffusion after scrape loading were compared between the differently treated cell groups. Three experiments were carried out for each treatment.

Statistical analysis
All the experiments were repeated at least three times. The data were expressed as mean±SEM. The statistical differences between groups were determined by one-way ANOVA or Student t-test. $P<0.05$ was considered statistically significant.
Results

β-adrenergic receptor stimulation up-regulates Cx43 expression

Serum-starved NRCMs were stimulated with β-AR agonist ISO (1 μmol/L) for 0 to 30 min. As shown in Figure 1A, ISO significantly increases Cx43-NP expression by 2.16±0.71 fold at 5 min, whereas the total Cx43 expression remains unchanged. The increased Cx43-NP expression is inhibited by a non-selective β-AR blocker propranolol, suggesting that β-AR mediates this effect (Figure 1B). To further define which subtype of β-adrenergic receptor is responsible, cells were preincubated with the highly selective β2-AR inhibitor ICI 118551 (1 μmol/L) and the β1-AR inhibitor CGP 20712 (1 μmol/L) for 30 min before stimulation with ISO. Figure 1C shows that pretreatment of ICI 118551, but not CGP 20712, effectively attenuates ISO-induced Cx43-NP expression. A specific β2-AR agonist clenbuterol (1 μmol/L) could fully mimic these effects (Figure 1D). These results suggest that the ISO-induced up-regulation of Cx43-NP expression is caused by β2-AR stimulation.

Protein kinase A (PKA) is involved in the up-regulation of ISO-induced Cx43-NP expression

Following ligand binding, β2-AR activates the cAMP/PKA pathway, via classic coupling to Gs, to regulate a variety of biological responses[16]. To explore the role of PKA in ISO-induced elevation of Cx43-NP expression, NRCMs were pretreated with 10 mmol/L H89, a highly selective PKA inhibitor. As shown in Figure 2, H89 significantly inhibits ISO-induced up-regulation of Cx43-NP expression. This result suggests that PKA is essential for ISO-induced up-regulation of Cx43-NP expression.

Pertussis toxin (PTX)-sensitive pathway is also required for the up-regulation of Cx43 expression by ISO stimulation

Apart from Gs, numerous studies have confirmed that chronic β2-AR stimulation facilitates the switching from Gs to Gi coupling, leading to ERK1/2 activation. In NRCMs, coupling of β2-AR to Gi helps protect cells from hypoxia-induced and reactive oxygen species-induced apoptosis by activating the PI3K/Akt dependent cell-salvage pathway[17]. Therefore, we

Figure 1. Isoproterenol enhanced the expression of cardiac Cx43-NP. Serum-starved NRCMs were stimulated with 1 μmol/L isoproterenol for 0 to 30 min (A). Cells were preincubated with 1 μmol/L propranolol (B), ICI 118551 (1 μmol/L) or CGP 20712 (1 μmol/L) (C) for 30 min, and then stimulated with ISO for 5 min. The expressions of Cx43-NP, or eIF-5 were determined by Western blotting with appropriate antibodies. (D) NRCMs were stimulated with 1 μmol/L isoproterenol or β2-AR agonist clenbuterol for 5 min. n=5. Mean±SEM. *P<0.05 vs no ISO and clenbuterol.
explored the role of the PTX-sensitive pathway in the ISO-dependent up-regulation of Cx43-NP expression. After preincubation with 200 ng/mL PTX for 16 h, NRCMs were exposed to ISO for 5 min. The level of Cx43-NP expression in PTX-preincubated cells is similar to that in control cells (Figure 3), suggesting that the PTX-sensitive pathway plays a part in the ISO-induced up-regulation of Cx43-NP expression.

Because the phosphorylation of proteins is determined by dynamic modulation of both kinases and phosphatases, it is possible that the accumulation of Cx43-NP could be attributed to reduced phosphorylation by kinase inactivation and/or increased dephosphorylation by phosphatase activation. Protein phosphatase PP2A appears to serve as a potent Cx43 phosphatase\[20\], and ISO could increase the activation of PP2A in the rat heart\[19\]. Thus, we explored the influence of protein phosphatases on the level of ISO-induced Cx43-NP expression. NRCMs were pretreated with a PP2A inhibitor OA (10 to 100 nmol/L) for 30 min and then exposed to ISO for 5 min. Pretreatment with OA fails to block the increase of Cx43-NP expression by ISO (Figure 4). This result suggests that the increase of Cx43-NP expression by β₂-AR may not depend on the activation of PP2A in response to ISO stimulation.

ISO stimulation also facilitates the enhanced function of GJIC
To further determine whether the up-regulation of Cx43-NP by ISO is related to the change in GJIC level, we used SL/DT assays with the gap junction permeable fluorescent dye LY to evaluate GJIC function. After NRCMs were treated with 1 μmol/L ISO for 5 min, the diffused distance of dye transfer was measured. ISO increases the LY transfer by 170% compared with the control, suggesting that ISO could enhance the GJIC of NRCMs (Figure 5). Furthermore, propranolol, H89, PTX and ICI 118551, but not CGP 20712, effectively abolished the increase in distance of dye transfer (Figures 5 and 6). These results indicate that β₂-AR mediates the enhancement of GJIC levels in response to ISO stimulation.

Discussion
Abnormal activation of β-AR, the prominent AR in the heart, results in a variety of cardiac arrhythmias\[20\]. However, the underlying mechanism of altered cardiac electrophysiological properties by the activation of β-AR remains largely unknown. In this study, we demonstrated that β₂-AR stimula-
tion increased Cx43-NP expression and enhanced the GJIC, providing new insight into the mechanism of β₂-AR-induced arrhythmias. Furthermore, we established that β₂-AR/Gi coupling was implicated in the elevation of Cx43-NP protein level in response to ISO stimulation.

Based on the role of Cx43 in the regulation of GJIC function, we investigated how β₂-AR stimulation affected Cx43 protein expression. As shown in our results, the protein level of Cx43-NP, rather than total-Cx43, is up-regulated upon ISO treatment. Because ICI 118551 abolished this effect, we believe that β₂-AR regulates Cx43 activity. The up-regulation of Cx43-NP expression is concomitant with the enhancement of function of GJIC. Because the function of GJIC in the heart depends on the number of gap junctions between neighboring cells and the gating function of the individual gap junction[21], and an increase in Cx43-NP implies enhancement of gap-junction function, our results suggest that β₂-AR alters the electrical stability by enhancing the opening of gap junctions. In fact, previous studies show that long-term treatment with β₂-AR agonists significantly increases the risk of sudden cardiac death due to arrhythmias[22, 23]. For example, inhaled β₂-AR agonist salbutamol might contribute to the generation of spontaneous arrhythmias by enhancing atrioventricular nodal conduction, decreasing atrioventricular nodal, atrial and ventricular refractoriness and increasing QT dispersion[24, 25].

Figure 5. Isoproterenol stimulation enhanced GJIC function. The distance of Lucifer Yellow diffusion after scrape loading was viewed with an inverted fluorescence microscope (×200), and compared between differently treated cell groups. (A) Control. (B) Incubated with isoproterenol (1 μmol/L) for 5 min. (C) Incubated with isoproterenol for 5 min after pretreatment with propranolol (1 μmol/L) for 30 min. (D) Incubated with isoproterenol for 5 min after pretreatment with ICI 118551 (1 μmol/L) for 30 min. (E) Incubated with isoproterenol for 5 min after pretreatment with H89 (10 mmol/L) for 30 min. (F) Incubated with isoproterenol for 5 min after pretreatment with PTX (200 ng/mL) for 16 h. A representative figure for each treatment from three independent experiments is shown. 'P<0.05 compared with ISO alone.

Figure 6. CGP 20712 could not abolish the increased dye transfer. The distance of Lucifer Yellow diffusion after scrape loading was viewed with an inverted fluorescence microscope (×200), and compared between differently treated cell groups. (A) Control. (B) Incubated with isoproterenol (1 μmol/L) for 5 min. (C) Incubated with isoproterenol for 5 min after pretreatment with CGP 20712 (1 μmol/L) for 30 min. A representative figure for each treatment from three independent experiments is shown. 'P<0.05 compared with ISO alone.
After agonist binding, β2-AR generally couples to Gs, which activate adenyl cyclases (AC) to increase intracellular cAMP levels. PKA, the direct substrate of increased cAMP, has been implicated in the various biological responses of β2-ARs. Here we demonstrated that the up-regulation of Cx43-NP expression was dependent on PKA, as H89, the PKA inhibitor, suppressed the above-mentioned effect. In fact, several authors have shown that activation of the cAMP/PKA pathway can regulate Cx43 phosphorylation, thereby altering cell coupling and communication. For example, Maithili et al reported that phosphorylation of Cx43 at the Ser 364 site by PKA was important for subsequent phosphorylation of the Ser 368 site by protein kinase C, which then inhibited the GJIC. Conversely, Mochizuki et al found that the gating function of GJIC was enhanced by PKA activation and Cx43-NP was not affected by PKA. The differences between their findings and ours may be due to different durations of PKA activation.

In addition, we observed that PTX could also block ISO-induced up-regulation of Cx43-NP, suggesting that β2-ARs couple to Gi to carry out the effect. Until now, β2-AR coupling to Gi is generally regarded as a result of the switching of Gs, in which PKA-mediated receptor phosphorylation acts as an essential mechanism. Therefore, we suggest that PKA is involved in the Gs/Gi switching and initiates Gi signaling. The subsequent inhibition of the AC/cAMP/PKA pathway by Gi signaling decreases the amount of phosphorylated Cx43, increasing the expression of Cx43-NP.

Under short-term ISO treatment (as short as 5 min), the up-regulation of Cx43-NP expression could not be attributed to the increased transcription level of Cx43. Therefore, it is possible that reduced phosphorylation of Cx43 is due to either reduced kinase (ie, PKA, PKC) activity or increased dephosphorylation by protein phosphatases. For example, a previous study demonstrated that increased PP2A, which colocalizes with Cx43, could contribute to the augmentation of Cx43-NP in failing myocardium. The potent PP2A inhibitor OA fails to reduce the ISO-induced increase in Cx43-NP. Nevertheless, it remains unclear whether other protein phosphatases are involved in the up-regulation of Cx43-NP.

As the most abundant connexin in the working myocardium, Cx43 has important roles in the morphogenesis and developmental remodeling of heart. Recent studies in cardiac-restricted silencing of the Cx43 gene have demonstrated some characterized phenotypes with ventricular outflow tract defects, lethal ventricular tachycardias, and sudden cardiac death. Cultured cardiac myocytes from homozygote Cx43 knockout mice displayed very slow conduction, and loss of Cx43 resulted in increased susceptibility to ischemia-induced arrhythmias. To our knowledge, increased β-AR activity also results in arrhythmias, especially under diseased conditions. In the present study, we demonstrated a new mechanism through which β2-AR regulates the Cx43 activity and gap-junction function. Further in vivo studies are needed to investigate the interaction between β2-AR, Cx43, and electrophysiological activity.

Conclusion
In summary, β2-AR mediates the up-regulation of Cx43-NP in a Gi/PKA-dependent manner, thereby enhancing the function of GJIC in neonatal rat cardiac myocytes. This helps explain how β2-AR agonists may alter the cardiac electrophysiological properties, consequently causing arrhythmias.

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Author contribution
Ping ZHANG, You-yi ZHANG, and Ji-hong GUO designed research; Yi XIA and Yao SONG performed research; Ming XU contributed new analytical tools and reagents; Yi XIA, Kai-zheng GONG, and Ping ZHANG analyzed data; Yi XIA and Yao SONG wrote the paper.

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