Caduet enhances connexin 43 phosphorylation in left ventricular and thoracic aorta of SH model rats

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Abstract. Caduet, also known as amlodipine besylate and atorvastatin calcium (AM + AT) tablet, can improve cardiac and vascular remodeling in patients with spontaneous hypertension (SH), but the underlying mechanism remains unknown. The present study aimed to explore whether AM + AT improved hypertensive left ventricular and thoracic aortic remodeling by regulating connexin 43 (Cx43) phosphor-ylation. A total of 32 male spontaneous hypertension model rats (SHR) were randomly divided into four groups: SHR control group, amlodipine-alone group (SHR-AM), atorvastatin-alone (SHR-AT) and AM + AT group (SHR-AM + AT); 8 Wistar-Kyoto (WKY) rats with normal blood pressure were used as the normal control. Drugs were orally administered for 8 weeks; subsequently, body weight, heart rate (HR), left ventricular mass index (LVMI), blood pressure (BP), plasma lipid levels and morphological changes of myocardial tissue in each group were analyzed. The expression of total (T)-Cx43 and phosphorylated (P)-Cx43 protein in the left ventricular and thoracic aorta tissues was determined using western blotting and immunofluorescence double labeling. The results revealed that AM + AT significantly decreased LVMI and cardiomyocyte cross-sectional area compared with SHR-AM and SHR-AT group. The western blotting results demonstrated that AM + AT could inhibit the expression of T-Cx43 protein, but increased the expression of P-Cx43 in the left ventricular and thoracic aorta. Moreover, immunofluorescence results indicated AM + AT could also decrease the expression of T-Cx43, and increase that of P-Cx43 in the left ventricular and thoracic aorta compared with AM and AT alone. Therefore, it was concluded that AM + AT may mitigate left ventricular and thoracic aorta remodeling in SH rats by enhancing Cx43 phosphorylation, and the efficacy of AM + AT was superior to that of AM and AT alone.

Introduction

Spontaneous hypertension (SH) is one of the most critical factors in leading to cardiovascular diseases (1,2). Persistently elevated blood pressure in patients who are hypertensive results in vascular tonel increasement and vascular smooth muscle systolic dysfunction, leading to myocardial ischemia and ventricular and vascular remodeling (3,4). Furthermore, left ventricular and aorta remodeling is a frequent pathological change in hypertension, which contributes to arrhythmia, heart failure and cardiovascular mortality (5-7).

Caduet is a single pill containing a combination of amlodipine besylate and atorvastatin calcium tablet (AM + AT), which is widely used in the clinical treatment of cardiovascular diseases, such as hypertension and coronary heart disease (8). In addition, previous experimental and clinical studies have reported that AM + AT can prevent cardiac hypertrophy and remodeling (9,10).

The gap junction (GJ) channel is the structural basis for cellular electric coupling and signal transmission, which mainly functions though regulating the GJ protein connexin (11,12). Connexin 43 (Cx43)-associated GJ protein in ventricular cardiomyocytes and vascular smooth muscle cells is involved in cardiac and vascular remodeling (13). Moreover, left ventricular remodeling is closely associated with the expression and distribution of Cx43 in cardiomyocytes (9,14), and our previous study revealed that the expression of Cx43 was enhanced in the thoracic aorta of SH model rats (SHR) (15). Previous studies have also reported that Cx43
phosphorylation contributes to ischemia-associated remodeling of Cx43 channels in cardiomyocytes (16,17), and Cx43 dephosphorylation contributes to arrhythmias and cardiomyocyte apoptosis in ischemia/reperfusion hearts (18). Based on these clinical data and laboratory reports, it was hypothesized that Cx43 phosphorylation may be involved in the process of hypertensive left ventricular and thoracic aorta remodeling, and AM + AT may improve this remodeling by enhancing Cx43 phosphorylation.

Therefore, the aim of the present study was to investigate the effect of Cx43 phosphorylation in the process of hypertensive left ventricular and thoracic aorta remodeling, and to examine whether AM + AT improved this remodeling by regulating Cx43 phosphorylation.

Materials and methods

Experimental reagents. A ProteoPrep Total Extraction Sample Kit was obtained from Sigma-Aldrich (Merck KGaA), the BCA protein assay kit was from Beyotime Institute of Biotechnology and nitrocellulose membrane was purchased from Thermo Fisher Scientific, Inc. Primary antibody against rat total (T)-Cx43 was obtained from Thermo Fisher Scientific, Inc. (1:1,000 for western blotting; 1:100 for immunofluorescence; cat. no. 3D8A5). Primary antibody against rat phosphorylated (P)-Cx43 was obtained from Cell Signaling Technology (1:1,000 for western blotting; 1:100 for immunofluorescence; cat. no. 52559). Horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:1,000; cat. no. G-21040) and goat anti-rabbit polyclonal (1:1,000; cat. no. 31466) secondary antibodies were obtained from Thermo Fisher Scientific, Inc. ECL reagents were purchased from Thermo Fisher Scientific, Inc. (cat. no. 35055). The FITC-conjugated goat anti-mouse secondary antibody was obtained from Thermo Fisher Scientific, Inc. (1:50; cat. no. 62-6511), and the tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit secondary antibody was purchased from Thermo Fisher Scientific, Inc. (1:100; cat. no. A16101). Bovine serum albumin (BSA) was obtained from Thermo Fisher Scientific, Inc. (cat. no. 37520).

Drugs and animals. The Chinese drug administration license nos. of AM, AT and AM + AT were H10950224, J20070061 and J20171045, respectively, which were from Betriebsstätte Freiburg, Inc.. All procedures and ethics associated with animal use were reviewed and approved by Medical School of X'ian Jiaotong University (approval no. 2018-898). In total, 32 male SHR (age, 8 weeks; weight, 210-265 g) were obtained from Weitong Lihua Experimental Animal Technology Co., Ltd, with clean grade certificate [SCXK (Shanghai) 2007-0005]. The AM group (SHR-AM; n=8), AT group (SHR-AT; n=8) and AM + AT group (SHR-AM + AT; n=8) were orally administered 10 mg/kg/day of AM, AT or AM + AT as treatment groups for 8 weeks (19,20), respectively. The WKY control group (n=8) and SHR control group (n=8) were orally administrated with the same volume of water as the treatment control group (n=8) and SHR control group (n=8) were orally administered 10 mg/kg/day of AM, AT or AM + AT as treatment and AM + AT group (SHR-AM + AT; n=8) were orally administered 10 mg/kg/day of AM, AT or AM + AT as treatment. The AM group (SHR-AM; n=8), AT group (SHR-AT; n=8) were obtained from Jiaotong University (approval no. 2018-898). In total, 32 male SHR (age, 8 weeks; weight, 210-265 g) were obtained from Weitong Lihua Experimental Animal Technology Co., Ltd, with clean grade certificate [SCXK (Beijing) 2007-0001], and eight Wistar-Kyoto (WKY) rats were purchased from Skerries Laboratory Animal Center, Ltd. as the WKY control group, with clean grade certificate [SCXK (Beijing) 2007-0001], and eight Wistar-Kyoto (WKY) rats were purchased from Skerries Laboratory Animal Center, Ltd. as the WKY control group, with clean grade certificate [SCXK (Beijing) 2007-0001].

A total of 2 ml blood was collected from heart of every rat, and the plasma lipid levels of each group were measured using a Hitachi 7170 biochemical analyzer (Hitachi, Inc.); these included total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and triglycerides (TG). Then the chest of the rat was opened, the heart was quickly removed and the left ventricular free wall was cut along the atrioventricular ring and weighed as the LVMI (left ventricular mass/body weight; mg/g).

Comparison of pathological changes in cardiac tissues. The free wall of the left ventricle was fixed in 10% formaldehyde for 2 h at room, dehydrated in an ethanol gradient (80, 90, 95 and 100%), then embedded in paraffin. Sections (3 µm thick) were cut and conventionally de waxed to water in a series, including xylene I, xylene II, 100% alcohol I and 100% alcohol II (10 min each), then a 95, 90, 80, 70% ethanol series (10 min each) and finally distilled water. Sections were stained with hematoxylin for 1 min, rinsed with water once, stained with eosin for 2 min, then dehydrated with conventional gradient alcohol (70, 80, 90 and 95% ethanol, 2 min each; 100% ethanol I, 100% alcohol II, 10 min each), cleared with xylene, and finally mounted with neutral gum and observed with Olympus BX41 fluorescent microscope (magnification, x400; Olympus Corporation). Images were captures and used to quantitatively analyze the myocardial cell cross-sectional area using Image-Pro Plus analysis software 6.0 (Media Cybernetics, Inc.).

Analysis of T-Cx43 and P-Cx43 protein expression levels in the left ventricular and thoracic aortic tissue using western blotting. Western blotting was performed as reported previously (21). Proteins of the left ventricular free wall and thoracic aortic ascending tissue were extracted using ProteoPrep Total Extraction Sample kit and measured using a BCA protein assay kit. Each sample was mixed with 40 µl 1X SDS electrophoresis sample buffer and boiled for 2-3 min. Protein samples (30 µg) were separated by 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% skimmmed milk for 2 h at room temperature, and washed twice with Tris-buffered saline with 0.1% Tween-20. Membranes were incubated with T-Cx43 and P-Cx43 primary antibodies overnight at 4°C, then probed with HRP-conjugated secondary antibody for 2 h at room temperature. Protein bands were visualized using ECL reagents and imaged using an Alpha Innotech FluorChem FC2 Imaging System (Alpha Innotech Inc.), the densitometric analysis was used ImageJ.
The SHR-AT + AM group were significantly lower compared with the SHR-AM and SHR-AM + AT group (P>0.05; Fig. 1A). However, there was no difference between SHR and SHR-AM group (P<0.05; Fig. 1A). Moreover, compared with the SHR group, the cardiomyocyte was arranged regularly and the cross-sectional area was reduced in the treatment groups (P<0.05; Fig. 2A and B). And the P-Cx43/T-Cx43 ratio demonstrated significantly increased in treatment groups compared with the ratio in the SHR group (P<0.05), especially in SHR-AM + AT group (Fig. 2A and D). The results suggested that drug treatment could enhance P-Cx43 protein expression, and that AM + AT was superior to either AM or AT alone.

In thoracic aortic tissue, the expression of T-Cx43 was decreased in the treatment groups compared with the SHR group (P<0.05), especially in SHR-AM + AT group (Fig. 2E and F). In addition, compared with SHR group, P-Cx43 expression was significantly increased in the treatment groups, with the highest upregulation in the SHR-AM + AT group (P<0.05; Fig. 2E and G). The P-Cx43/T-Cx43 ratio was also increased to a greater extent in the SHR-AM + AT group compared with the SHR-AM and SHR-AT groups (P<0.05; Fig. 2E and H). The plasma lipid results identified that TC, LDL and TG in the SHR-AT + AM group were significantly lower compared with the SHR group (P<0.05), and TC and LDL was lower in the SHR-AM + AT compared with levels in the SHR-AM group (P<0.05; Fig. 1B). Furthermore, there was no significant difference in HDL in all groups (P>0.05).

Table I. Effects of the different drugs on body weight, HR and LVMI (n=8).

| Parameter          | WKY            | SHR            | SHR-AM         | SHR-AT         | SHR-AM + AT |
|--------------------|----------------|----------------|----------------|----------------|-------------|
| Body weight, g     | 299.91±9.90    | 298.33±8.52    | 292.00±10.58   | 288.72±10.68   | 288.72±10.68 |
| HR, bpm            | 346.61±6.92a   | 384.88±8.26    | 372.44±8.79    | 376.50±5.96    | 360.77±6.56  |
| LVMI, mg/g         | 2.06±0.43ab    | 2.92±0.24      | 2.55±0.28ab    | 2.65±0.38ab    | 2.31±0.49ab  |

Data are presented as the mean ± SD (n=8). *P<0.05 vs. SHR; †P<0.05 vs. SHR-AM + AT. AM, amlodipine; AT, atorvastatin; HR, heart rate; LVMI, left ventricular mass; SHR, spontaneous hypertension model rat; WKY, Wistar-Kyoto.

Comparison of body weight, HR and LVMI in each group.

As presented in Table I, no significant differences were identified in the body weight in all groups after 8 weeks of drug treatment (P>0.05). It was demonstrated that HR and LVMI were significantly lower in treatment groups compared with the respective SHR group (P<0.05); and LVMI was especially lower in the SHR-AM + AT group.

Comparison of blood pressure, plasma lipid level and myocardial tissue morphology. After treatment for 8 weeks, SBP and DBP in the SHR-AM and SHR-AM + AT groups were significantly decreased compared with the SHR group (P<0.05; Fig. 1A). However, there was no difference between SHR-AM and SHR-AM + AT group (P>0.05; Fig. 1A). The plasma lipid results identified that TC, LDL and TG in the SHR-AT + AM group were significantly lower compared with the SHR group (P<0.05), and TC and LDL was lower in the SHR-AM + AT compared with levels in the SHR-AM group (P<0.05; Fig. 1B). Furthermore, there was no significant difference in HDL in all groups (P>0.05).

Expression of T-Cx43 and P-Cx43 in myocardial tissue using immunofluorescence double labeling. The frozen tissue embedded with optimum cutting temperature compound was cut into 8-µm thick tissue sections and fixed for 15 min with pre-cooled acetone at 4°C. Then, the samples were blocked with 1% BSA at 37˚C for 1 h and then incubated with primary antibodies against rat T-Cx43 and P-Cx43 at 4˚C overnight, followed by incubation with the FITC-conjugated secondary antibody in 1% fetal bovine serum at 37˚C for 2 h at room temperature, and incubated with the primary antibodies against rat T-Cx43 and P-Cx43 at 4˚C overnight, followed by incubation with the TRITC-conjugated secondary antibody in 1% fetal bovine serum at 37˚C for 1 h in the dark. After washing three times with PBS (5 min each), five random fields of each section were examined under an Olympus BX41 fluorescent microscope (magnification, x400; Olympus Corporation), and the fluorescence intensity was determined using ImageJ software.

Statistical analysis. The data were analyzed with SPSS 21.0 statistical software (IBM Corp.). Data are presented as the mean ± SD; the experiments were repeated three times. Comparisons between groups were performed using one-way ANOVA followed Tukey’s test. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of body weight, HR and LVMI in each group. As presented in Table I, no significant differences were identified in the body weight in all groups after 8 weeks of drug treatment (P>0.05). It was demonstrated that HR and LVMI were significantly lower in treatment groups compared with the respective SHR group (P<0.05); and LVMI was especially lower in the SHR-AM + AT group.

Comparison of blood pressure, plasma lipid level and myocardial tissue morphology. After treatment for 8 weeks, SBP and DBP in the SHR-AM and SHR-AM + AT groups were significantly decreased compared with the SHR group (P<0.05; Fig. 1A). However, there was no difference between SHR-AM and SHR-AM + AT group (P>0.05; Fig. 1A). The plasma lipid results identified that TC, LDL and TG in the SHR-AT + AM group were significantly lower compared with the SHR group (P<0.05), and TC and LDL was lower in the SHR-AM + AT compared with levels in the SHR-AM group (P<0.05; Fig. 1B). Furthermore, there was no significant difference in HDL in all groups (P>0.05).

Comparison of T-Cx43 and P-Cx43 protein expression levels in the left ventricular and thoracic aortic tissue using western blotting in each group. Compared with the WKY group, the expression of T-Cx43 in the free wall of the left ventricle was significantly increased in the SHR group (P<0.05). After treatment, compared with the SHR group, the expression of T-Cx43 was significantly decreased in SHR-AM and SHR-AM + AT (P<0.05), especially in SHR-AM + AT group (Fig. 2A and B). Moreover, compared with the SHR group, the expression of P-Cx43 was significantly increased in treatment groups (Fig. 2A and C). And the P-Cx43/T-Cx43 ratio demonstrated significantly increased in treatment groups compared with the ratio in the SHR group (P<0.05), especially in SHR-AM + AT (Fig. 2A and D). The results suggested that drug treatment could enhance P-Cx43 protein expression, and that AM + AT was superior to either AM or AT alone.

Expression of T-Cx43 and P-Cx43 in myocardial tissue using immunofluorescence double labeling. Compared with the SHR group, the expression of T-Cx43 in the left ventricular myocardium was significantly decreased in other groups.
(P<0.05; Fig. 3A and B), whereas the expression of P-Cx43 was significantly increased (P<0.05), particularly in the SHR-AM + AT group (P<0.05; Fig. 3A and C).

Expression and distribution of T-Cx43 and P-Cx43 in the thoracic aorta using immunofluorescence double labeling. Compared with WKY group, T-Cx43 expression was increased and mainly distributed at the inner and medial membrane of the thoracic aorta. Moreover, P-Cx43 expression was decreased and mainly distributed at the medial and outer membrane in the SHR group. Compared with the SHR group, the expression of T-Cx43 was significantly decreased in the treatment groups, especially in the SHR-AM+AT group (all P<0.05; Fig. 4A and B), and the expression of P-Cx43 was significantly increased in treatment groups (P<0.05), especially in the SHR-AM+AT group (P<0.05; Fig. 4A and C).

Discussion

Hypertension is one of the most critical factors to cause left ventricular and vascular remodeling (22,23). Furthermore, ventricular remodeling is a risk factor of various severe arrhythmias, as well as sudden mortality (24). Owing to improved compliance, AM + AT have been used for treating hypertension and hyperlipidemia, and can protect vascular smooth muscle and myocardium from remodeling (25,26). Caduet is a single pill containing AM and AT, which blocks Ca²⁺ transmembrane influx, inhibits β-Hydroxy β-methylglutaryl-coenzyme A reductase, decreases vascular resistance and reverses ventricular remodeling (27). In addition, our previous study revealed that the expression of Cx43 was enhanced in the ventricular and thoracic aorta of SHR (15). However, the exact mechanism of AM + AT improving ventricular and thoracic aorta remodeling by regulating Cx43 phosphorylation it yet to be fully elucidated.

The present results indicated that AM + AT enhanced Cx43 phosphorylation in the left ventricular and thoracic aorta of SHR, and that AM + AT was superior to AM and AT treatment alone.

GJs serve an important role in vascular tone and blood pressure regulation (28). The GJ protein Cx43 is a sensitive pressure receptor that is closely related to the development of hypertension (29). Cardiovascular diseases have been reported to affect the expression and localization of Cx43, and dysregulated Cx43 is related to the loss of cardioprotection (30,31).
Figure 2. Comparison of T-Cx43 and P-Cx43 protein expression levels in left ventricular and thoracic aortic using western blotting. (A) Representative results of western blotting analysis on left ventricular tissue, and subsequent densitometric analysis of (B) T-Cx43 and (C) P-Cx43 protein expression levels; (D) P-Cx43/T-Cx43 ratio. (E) Results of western blotting analysis on thoracic aortic tissues, and subsequent densitometric analysis of (F) T-Cx43 and (G) P-Cx43; (H) P-Cx43/T-Cx43 ratio. Data are expressed as the mean ± SD; n=8; *P<0.05 vs. SHR; #P<0.05 vs. SHR-AM + AT. AM, amlodipine; AT, atorvastatin; Cx43, connexin 43; P-, phosphorylated; SHR, spontaneous hypertension model rat; T-, total; WKY, Wistar-Kyoto.

Figure 3. Comparison of T-Cx43 and P-Cx43 protein expression levels in left ventricular tissue using immunofluorescence double labeling. (A) Results of immunofluorescence double labeling analysis in left ventricular tissue. Green fluorescence, T-Cx43; red fluorescence, P-Cx43. Arrows indicate the intercalated disc. Magnification, x400. Fluorescence intensity for (B) T-Cx43 and (C) P-Cx43. Data are expressed as the mean ± SD; n=8; *P<0.05 vs. SHR; *P<0.05 vs. SHR-AM + AT. AM, amlodipine; AT, atorvastatin; a.u., arbitrary unit; Cx43, connexin 43; P-, phosphorylated; SHR, spontaneous hypertension model rat; T-, total; WKY, Wistar-Kyoto.
Furthermore, ischemic preconditioning may reduce the incidence of arrhythmias by increasing Cx43 expression and altering GJ channel remodeling (32). In the current study, it was suggested that the upregulation of T-Cx43 and downregulation of P-Cx43 in myocardial tissue of SHR may be an adaptive response for increasing blood pressure and cardiac load. Moreover, the expression of P-Cx43 was increased and T-Cx43 was decreased after AM + AT treatment, which could contribute to enhancing GJ communication among vascular smooth muscle cells in resistant arteries of SHR group, and causing an increased response to vasoconstrictors and hypertension (33). Thus, it was concluded that AM + AT may mitigate ventricular and vascular remodeling via increased phosphorylation of Cx43.

In summary, AM + AT could improve ventricular and vascular remodeling by inhibiting T-Cx43 expression and enhancing Cx43 phosphorylation in SHR, indicating that AM + AT is superior to AM and AT alone.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XH, JY and GC conceived and designed the experiments; XH, JY, BS, MM, HW, SW and GC performed the experiments; XH, NW and GC analyzed the data; XH, SH and NW made data interpretation and critical manuscript revisions; XH and JY wrote the manuscript. All authors read and approved the final manuscript.
Ethics approval and consent to participate

All procedures and ethics associated with animals were reviewed and approved by Medical School of Xi’an Jiaotong University (approval no. 2018-898).

Patient consent for publication

Not applicable.

Competing interests

The authors declared that they have no competing interests.

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