The α_{1D}-adrenoreceptor antagonist BMY 7378 reverses cardiac hypertrophy in spontaneously hypertensive rats.

Short tittle: Effect of BMY 7378 on Myocardial Remodeling

Jessica E. Rodríguez[1*], Alberto D. Saucedo-Campos[2], Ana V. Vega[3], Diana Ramírez-Hernández[4], Luisa Martínez-Aguilar[4], J. Rafael Jiménez-Flores[2], Erik Andrade-Jorge[1,5], Samuel E. Estrada-Soto[6], Rafael Villalobos-Molina[1], Rhian M. Touyz[7], Itzell A. Gallardo-Ortíz[1*]

[1]Unidad de Biomedicina, [2]Unidad de Morfofisiología, [3]Carrera de Medicina, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla, México. [4]Laboratorio de Farmacología del Miocardio, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, Cuautitlán, México. [5]Laboratorio de Investigación en Bioquímica, Sección de Estudios de Posgrado e Investigación. Escuela Superior de Medicina del Instituto Politécnico Nacional, Plan de San Luis y Díaz Mirón. [6]Laboratorio de Farmacognosia, Facultad de Farmacia, Universidad Autónoma del Estado de Morelos, Morelos, México. [7]Institute of Cardiovascular and Medical Sciences, University of Glasgow, United Kingdom.

Corresponding Authors*:

Jessica E. Rodríguez, PhD; Itzell A. Gallardo-Ortíz, PhD

Unidad de Biomedicina, FES Iztacala, UNAM

Av. de los Barrios 1, Los Reyes Iztacala, Tlalnepantla 54090, México

Tel. (52 55) 56231333 ext. 39795

e-mail. jessrr23@outlook.com

e-mail. itzellg@gmail.com

Abstract

The α_{1D}-adrenergic receptor (α_{1D}-AR) is involved in angiotensin II (Ang II)-induced vascular remodeling and hypertension. Whether α_{1D}-AR plays a role in hypertension-associated cardiac hypertrophy is unclear. Here we investigated effects of BMY 7378, an α_{1D}-AR antagonist, on cardiac status in aged spontaneously hypertensive rats (SHR). Male SHR were studied during the phase of developing hypertension (5 and 10-weeks-old) and once hypertension was established (20 and 30 weeks old) to assess the evolution of cardiac hypertrophy. Age-matched WKY rats were studied as controls. 30 week-old SHR were treated for 4 weeks with BMY 7378 (10 $mg.kg^{-1}.day^{-1}$, o.a.), or captopril (angiotensin converting enzyme inhibitor, 40 $mg.kg^{-1}.day^{-1}$, o.a.) (as a positive control). Blood pressure and cardiac function were measured *in vivo*, cardiac hypertrophy by histology, and α_{1D}-AR protein expression by immunofluorescence. By 30 weeks of age, SHR exhibited significant hypertension and cardiac hypertrophy. BMY 7378 and captopril decreased blood pressure and improved hemodynamic parameters and cardiac function in treated SHR versus untreated SHR ($p<0.05$). Histology showed increased cardiomyocyte size, fibrosis, and left ventricular hypertrophy in SHR hearts. BMY 7378 ameliorated fibrosis and cardiac hypertrophy, but had no effect on cardiomyocyte size in SHR. Effects of BMY 7378 were associated with increased α_{1D}-AR protein expression in SHR. Our data indicate that pharmacological inhibition of α_{1D}-AR reduces blood pressure and associated cardiac hypertrophy in aged SHR. These findings suggest that the α_{1D}-AR system plays a pathophysiological role in the development of hypertension and cardiac target organ damage in SHR.

Keywords

α_{1D}-Adrenoreceptors, Cardiac Remodeling, Heart Failure, Hypertension, Left Ventricular Hypertrophy.

Introduction

Hypertension is a chronic non-communicable disease that affects 1.3 billion people worldwide (1). Hypertension promotes hypertensive heart disease (HHD), which manifests clinically as functional and structural changes with systolic and diastolic ventricular dysfunctiis associated with poor prognosis and patients are at risk of myocardial infarction, congestive heart failure, stroke and sudden death (2).

Spontaneously hypertensive rats (SHR), a genetic model of human hypertension, develop target organ damage as they age. Undelying pathophysiological processes include activation of the renin angiotensin system (RAS) (3,4) because treatment with angiotensin converting enzyme inhibitors prevented hypertension, left ventricular hypertrophy and vascular remodeling in young SHR (5), and reduced BP, fibrosis and cardiac weight in older SHR (6). Activation of the sympathetic nervous system has also been implicated in SHR (reference). It is possible that functional and molecular interactions between the RAS and the adrenergic system could modulate cardiovascular responses in SHR (7,8). For example, angiotensin II (Ang II) promotes expression of the α_{1D}-adrenergic receptor (α_{1D}-AR) in vascular smooth muscle cells (9,10), while inhibiting ACE decreased both α_{1D}-AR expression and vascular function in pre-hypertensive SHR (11), and in aryl hydrocarbon receptor null mice (another model of hypertension) (12).

α_{1D}-ARs, expressed primarily in vascular cells (13,14), plays a role in vascular hypertrophy, as evidenced in vivo and in vitro by increased media thicknes and protein synthesis (10,15). Ang II infusion in rats promoted an increase in media thickness and media-to-lumen ratio in aorta, where α_{1D}-AR predominates, but not in tail arteries where α_{1A}-AR is the main receptor subtype expressed (16). BMY 7378, an α_{1D}-AR antagonist reversed Ang II-induced aortic remodeling (16). α_{1D}-AR knockout mice exhibit thinner vascular walls and cardiac ventricles than control wildtype mice (17,18). These mice also have lower blood pressure than control counterparts. Together these findings suggest that the α_{1D}-AR system is involved in vascular remodeling in hypertension.

Despite the growing evidence that signaling through α_{1D}-ARs is important in vascular changes in hypertension, there is a paucity of information regarding its significance in hypertension-associated cardiac remodeling. To address this, we questioned whether SHR myocardium expresses α_{1D}-AR and whether BMY 7378, a pharmacological inhibitor of α_{1D}-AR, influences cardiac remodeling in hypertension.

Materials and Methods

Animals

Male Wistar Kyoto rats (WKY) and SHR of 5, 10, 20 and 30 weeks of age were obtained from the breeding colony of the Institute of Cell Physiology (UNAM). Three or four rats of each age were housed per cage and maintained in a pathogen-free environment under controlled conditions (22 ± 2°C, 40-60% humidity, 12-h/12-h light/dark cycle), with food and water *ad libitum*. The Bioethics Committee of our institution approved all procedures

and we followed Mexican Official Norm about technical specifications for the production, care, and use of laboratory animals (NOM-062-ZOO-1999, Ministry of Agriculture).

Blood Pressure and Heart Rate Recordings

A time-course of the increase in BP was conducted in rats of 5, 10, 20 and 30 weeks of age to observe the progression of hypertension, measuring BP through a non-invasive, indirect method with a tail-cuff device (Automatic Blood Pressure Computer, Model LE5007; Letica, Panlab, Spain). Rats were trained for 1 week inside a plastic restrainer at 37°C; then, a sensor and an inflatable latex ring were placed on the rat's tail to obtain BP recordings. An average of five readings was used to obtain heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP).

Histology

After BP recordings, histological analysis to examine development of LVH in SHR at different ages was conducted. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused with phosphate buffered saline solution (0.88 % NaCl, 0.14 % Na_2HPO_4, and 0.02 % $NaHPO_4$, pH 7.4), and then with a 4% formaldehyde-phosphate buffer solution. The heart was removed and maintained in a cryoprotective 4% formaldehyde-10% sucrose-0.1M DMSO solution for at least 24 h. This solution was replaced by a 30% sucrose solution at 4°C for at least 3 h. Thereafter hearts were embedded in Tissue-Tek O.C.T. mounting media compound (Sakura Finetek, Inc., Torrance, CA, USA), and frozen in acetone cooled with dry ice. Six-micrometer sections were cut on a cryostat at -25°C (Leica Microsystems, Wetzlar, Germany) with a cross-sectional orientation, all slices were taken at the same height and we took as reference the papillary-muscle of the left ventricle. Sections were applied to 1% gelatinized slides and stored at -20°C until use or were stained with Masson's Trichrome

(for detection of fibrosis in 30-week-old rats) or hematoxylin and eosin (H&E); slices were analyzed in an optical microscope. The left ventricular thickness was measured using ImageJ software, ver.2.1.4.5 (National Institutes of Health, Bethesda, MD, USA), and the area (μm^2) of cardiomyocyte was measured in fields that include papillary muscle and the vertically aligned muscle layers adjacent to it, measuring the major and minor transverse axes of cardiomyocytes to estimate the cross-sectional area using the ZEN 2012 software (Carl Zeiss Laser Scanning systems LSM 510, Oberkochen, Germany) (19).

BMY 7378 and Captopril Administration

30 weeks-old rats were randomly separated, and four groups were formed (n = 3-4 rats each): Control groups of WKY and SHR were orally administered with 0.5 mL of saline solution; SHR + BMY 7378 group (selective α_{1D}-AR antagonist, 10 $mg.kg^{-1}.day^{-1}$; 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione; Sigma-Aldrich, St. Louis, MO, USA), and the SHR + captopril group (ACE inhibitor, 40 $mg.kg^{-1}.day^{-1}$; S-1-(3-mercapto-2-methyl-1-oxopropyl)-L-proline; Sigma-Aldrich). Drugs were dissolved in saline solution and administered with a cannula daily for 4 weeks.

Cardiac Function Measurement

After 4 weeks of treatment, rats were anesthetized with sodium pentobarbital (50 $mg.kg^{-1}$ i.p.), and the right carotid artery was exposed at a length of 5 mm. A heparin-coated catheter (PE10) was advanced into the left ventricle through the right carotid artery; the correct position of the catheter tip in the left ventricle was then confirmed by visualizing the waveform of the left ventricular (LV) pressure. The arterial line was connected to the computerized data-acquisition system (model MP100WSW; Biopac Systems Inc., Santa

Barbara, CA, USA) to record left ventricular end diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP) and maximal rates of LVP rise and fall (± dP/dt) (20).

A catheter was also inserted into the left jugular vein for drug administration, and a dose-response curve to isoproterenol ((−)-isoprenaline hydrochloride; Sigma-Aldrich) was constructed in graded doses ranging from 0.75 - 6 $ng.kg^{-1}$ i.v. Isoproterenol doses were administered after each LV hemodynamic measurement returned to baseline (21). The recording and reading of data were analyzed using AcqKnowledge software, ver.3.9.1 (Biopac Systems, Inc.). At the end of each experiment, the catheter was cut, and the heart was removed to verify that the catheter was inside the ventricle. After dose-response curves, the histological procedure was developed as mentioned.

Immunofluorescence

Slices were washed 10 minutes with PBS and incubated with collagenase II (cat. CLS 2, Worthington, Lakewood, NJ, USA) for 3 hours at 37°C to decrease extracellular matrix; then, slices were washed 3 times 10 minutes each, and subjected to a heat-induced epitope retrieval in a solution of Tris 20 mM and 2% of SDS, pH 9 at 90°C for 15 minutes, and then exposed to UV radiation for at least 6 hours to decrease background fluorescence. Slices were washed 3 times 10 min each with PBS and incubated with blocking solution (3% fetal bovine serum and 0.3 % Triton X100 in PBS) at room temperature for 1 hour. After removal of the blocking solution, primary rabbit anti- α_{1D}-AR was applied at 1:1500 in blocking solution and left overnight at 4°C in a humidity chamber (22). The slices were washed with PBS followed by exposure of a secondary goat anti-rabbit antibody coupled to Alexa Fluor 488 (cat. A11034, Invitrogen, Eugene, OR, USA) at 1:1000, and incubated 1 hour at room temperature in humidity chamber. Secondary antibody was removed by washing with PBS and slices were

covered with Vectashield antifade mounting medium (cat. H-1000, Vector laboratories, Burlingame, CA, USA) added with 0.1% of Hoechst 33342 nucleic acid stain (cat. H1399, Thermo Fisher Scientific, Waltham, MA, USA), then a glass coverslip was applied. The tissue sections were examined with a fluorescence microscope (Carl Zeiss Laser Scanning systems LSM 510, Oberkochen, DE) with 200X amplification. Fluorescence was determined adjusting a threshold to exclude background fluorescence, and gated to include intensity measurements only from positively stained tissues using the area of the whole stained tissue section, measurements were obtained using ImageJ software, ver.2.1.4.5 (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM) of 3-4 rats per group.
A Student's-*t* test was developed for comparisons between WKY and SHR; analysis of repeated measures (analysis of variance, ANOVA) for dose-response curves; and one-way ANOVA for histological and immunofluorescence analysis were carried out, both ANOVA with a Bonferroni *post-hoc* test. Differences were considered statistically significant when $p < 0.05$ ($^{\#}p < 0.05$ *vs.* WKY or $^{*}p < 0.05$ *vs.* SHR). All the images and the statistical analysis were performed employing the Systat SigmaPlot Statistical Software, ver.12.0 (SSI, San Jose, CA, USA).

Results

Blood Pressure

Mean base line SBP, DBP, and MAP were not significantly different between WKY and SHR at 5 and 10 weeks of age. In contrast, all of these parameters were significantly higher

in SHR of 20 and 30-week-old compared with control WKY ($^{\#}p < 0.05$) (Table 1). In SHR, (5 to 30 weeks of age), HR was significantly higher versus age-matched WKY ($^{\#}p < 0.05$) (Table 1).

Four week oral administration of BMY 7378 or captopril to SHR of 30 weeks of age decreased BP within 1 week, with the BP decrease being even greater from week 2 post-treatment (*$p < 0.05$). Neither BMY 7378 nor captopril modified HR ($p > 0.05$) (Table 2).

Histology

The cross-sectional slices at the papillary-muscle level revealed the right and left cardiac ventricles. In SHR from 20 weeks of age, wall thickness of the left ventricle was increased versus WKY and by 30 weeks there was clear concentric hypertrophy (Figure 1a). Quantitative analysis of cardiomyocyte size (Figure 1b) and left ventricular wall thickness (Figure 1c) demonstrated that both were greater in SHR versus WKY ($p < 0.05$).

Treatment with either BMY 7378 or captopril, during 4 weeks, diminished left ventricular thickness (Figure 2a) and reduced fibrosis in 30-week-old SHR (Figure 2b). Quantitative analysis showed that BMY 7378 or captopril decreased the left ventricular wall in comparison with control SHR ($p < 0.05$) (Figure 2c); there was no difference between WKY and SHR + BMY 7378 groups ($p > 0.05$) (Figure 2c). However, the ventricular wall of SHR rats treated with captopril was even thinner than in WKY ($p < 0.05$) (Figure 2c). There were no significant changes in cardiomyocyte size in the groups treated with BMY 7378 or captopril *vs*. SHR ($p > 0.05$) but there were significant differences between treatments groups *vs.* WKY ($p < 0.05$) (Figure 2d).

Cardiac Function

Hemodynamic parameters of 30-week-old rats are shown in Table 3. LVSP as well as the values of (±) dP/dt were significantly reduced in the untreated control SHR group ($p < 0.05$); LVEDP was higher in SHR *vs.* WKY ($p < 0.05$). Oral administration of BMY 7378 or captopril improved hemodynamics in SHR ($p < 0.05$).

Dose-response curves to isoproterenol were used to test cardiac function. Isoproterenol increased LVSP (Figure 3a) and LVEDP (Figure 3b) values in a dose-dependent manner in WKY rats, while in SHR isoproterenol had no effect at any dose ($p < 0.05$). BMY 7378 recovered the response to isoproterenol ($p < 0.05$). However, the change in LVSP was lower in comparison with that of WKY ($p < 0.05$) (Figure 3a), but LVEDP was not different ($p > 0.05$) (Figure 3b). In a similar manner, captopril recovered the response to isoproterenol in LVSP and LVEDP ($p < 0.05$) (Figures 3a, 3b); but LVEDP was higher in comparison with WKY ($p < 0.05$) (Figure 3b). Absolute values of LV (±) dP/dt were significantly reduced in SHR ($p < 0.05$). Both were improved after treatment with BMY7 378 or captopril ($p < 0.05$), however there were still difference between treatments groups and normotensive control WKY ($p < 0.05$) (Figure 3c).

Immunofluorescence

The α_{1D}-AR expression in cardiac muscle was abundant in SHR versus age-matched WKY ($p < 0.05$). Treatment with BMY 7378 induced expression of the receptor in SHR ($p < 0.05$ versus untreated group). Captopril did not modify expression of α_{1D}-AR in comparison with SHR control ($p > 0.05$) (Figure 4).

Discussion

The SHR is a genetic model of human hypertension (23). It is known that SBP increases in SHR at around 5-10 weeks of age, while cardiac hypertrophy develops at 9-12 weeks of age (24). We found that SHR developed high blood pressure after week 10 and it was higher than those of WKY after 20 weeks of age. This was associated with development of cardiac hypertrophy, which was clearly evident at 20 weeks and severe at 30 weeks.

After a stable period of compensatory hypertrophy supporting contractility in hypertension, SHR progress to heart failure with senescence (25). In the adaptation stage, LVH occurs as a response to normalize wall stress and to maintain pumping ability. If pressure overload persists, hypertrophy becomes maladaptive with a progressive decline in LV contractility and diastolic function, as evidenced in SHR at 30 weeks where there was diminished LVSP and (+) dP/dt. These functional changes indicate damage in cardiac muscle and loss in the ability of the heart to contract (26). SHR had a higher value of LVEDP than WKY. In addition there was a decrease in (-) dP/dt, which is the maximal descent velocity (speed at which intraventricular pressure decreases during isovolumetric relaxation) and reflects the speed of relaxation of muscle fibers (27). Both parameters were modified in SHR indicating diastolic dysfunction. These phenomena were associated with reduced responses to isoproterenol and may predisopose to heart failure (28). It is known that intrinsic impairment in the hypertrophied myocardial fibers, or diminished β-ARs sensitivity due to high norepinephrine, are possible causes for this reduced cardiac responsiveness (29). In this context, we did not observe isoproterenol-induced contractility of SHR myocardium in contrast with the marked response in WKY rats, probably because 30-week-old SHR had severe concentric hypertrophy and great deposition of collagen between cells. We think that

this excess of ECM led to the formation of a fibrotic scar, avoiding the interaction of isoproterenol with β_1-AR.

To assess whether α_{1D}-AR is involved in myocardial remodeling in hypertension, we compared effects of BMY 7378 *vs*. captopril (as positive control) in 30-week-old SHR with severe LVH, during 4 weeks. As expected, BMY 7378 decreased blood pressure, as well as left ventricular thickness and fibrosis, it regenerated the response to isoproterenol and improved heart´s contraction-relaxation as determined by (±) dP/dt, and significantly increased α_{1D}-AR expression, which indicates that the antagonism of this receptor produces, at least in part, these reductions. Captopril showed the same actions as BMY 7378, which are in agreement with those reported in clinical studies, where ACE inhibitors and AT_1R blockers revert LVH, decrease TGF-β_1 and reduce the ECM (30).

Processes underlying these effects likely involve the RAS, important in HHD (3,4,31,32), The RAS and adrenergic receptors interact as previously demonstrated (16). This may involve dimerization between α_{1D}-AR and AT_1R (33). Accordingly in conditions where Ang II is elevated, as in SHR and in the aryl hydrocarbon receptor null mouse (3,12,31), vascular α_{1D}-AR expression and function are increased, suggesting that both systems might be related with the onset of hypertension. Furthermore, BMY 7378, which has high affinity for α_{1D}-AR ($pA_2 = 8.9$) (34), reversed the vascular hypertrophy generated by the continuous infusion of Ang II. It is thus possible that interaction between α_{1D}-AR and the actions of Ang II-AT_1R also play a role in LVH in SHR (16).

In conclusion these findings demonstrate that α_{1D}-AR is involved in hypertension-associated cardiac hypertrophy. Mechanisms underlying this may involve the RAS and processes linked

to cardiac fibrosis. Since inhibition of α_{1D}-AR with BMY 7378 also had blood pressure-lowering effects, we can not exclude the possibility that prevention of LVH may be secondary to decreased blood pressure in SHR. Nevertheless, cardiac expression of α_{1D}-AR was increased in SHR myocardium suggesting a possible direct effect in the heart. Our study defines a role for α_{1D}-AR in cardiac remodeling in hypertension.

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Conflict of interest statement

Authors report no conflicts of interest.

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