

ORIGINAL ARTICLE

Is overall blockade superior to selective blockade of adrenergic receptor subtypes in suppressing left ventricular remodeling in spontaneously hypertensive rats?

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To test the hypothesis that nonselective blockade of adrenergic receptor (AR) subtypes is superior to selective blockade of AR subtypes in suppressing left ventricular (LV) remodeling induced by hypertension. Sixty-four spontaneously hypertensive rats (SHR) were randomly divided into four groups: bisoprolol-treated, propranolol-treated, carvedilol-treated and no treatment groups ($n=16$, each). Sixteen Wistar–Kyoto (WKY) rats served as a control group. Echocardiography and cardiac catheterization were carried out to record the mitral flow velocity ratio of E wave to A wave (E/A), LV mass index (LVMI), maximal rising (dp/dt_{max}) and falling ($-dp/dt_{max}$) rate of the LV pressure and LV relaxation time constant (τ). The mRNA and protein expression levels of AR, protein kinase (PK) and G-protein subtypes, intracellular free calcium (Ca^{2+}) concentration and cardiocyte apoptosis rate were determined. Three drug-treated groups showed higher velocity ratio of E wave to A wave (E/A) and $-dp/dt_{max}$ and lower systolic blood pressure (SBP), LVMI, τ , apoptosis rate and intracellular free Ca^{2+} concentration than the no treatment group. The mRNA expression levels of AR- α_{1B} in the carvedilol group were significantly lower than the other two drug-treated groups. The mRNA expression levels of AR- β_1 , AR- β_2 and G α were significantly higher in the three drug-treated groups than in the no treatment group, with the expression levels of AR- β_2 being the highest in the carvedilol-treated group. The protein expression levels of PKA and PKC subtype α and δ were lower in the three drug-treated groups than in the no treatment group. Overall blockade of AR subtypes is not superior to selective blockade of AR subtypes in suppressing LV remodeling in SHR. Although carvedilol is the most effective in attenuating cardiocyte apoptosis, normalizing AR- α_{1B} and G α expression and increasing AR- β_2 expression.

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INTRODUCTION

Left ventricular (LV) remodeling is the one of most important target organ damages in patients suffering with hypertension. Previous studies have shown that LV remodeling is an independent risk factor of cardiovascular events.¹ An ideal antihypertensive therapy should be able to suppress LV remodeling, as well as to lower blood pressure. Although most current antihypertensive drugs are successful in achieving these goals, atenolol, a β_1 -adrenoceptor antagonist, has been criticized for less successful protection of target organs and recent British and European guidelines have even withdrawn β -adrenoceptor antagonists from antihypertensive therapy simply because these drugs lack clinical evidence for reducing cardiovascular events.^{2,3} However, with the advent of carvedilol with adrenergic receptor (AR)- α_1 , AR- β_1 and AR- β_2 antagonism and antioxidative property, there has been a continuing dispute about whether overall

blockade is superior to selective blockade of AR subtypes in suppressing LV remodeling induced by hypertension, which has spurred further investigation on ‘drug effects’ with respect to ‘class effects’ among adrenoceptor antagonists.

Adrenergic receptors in the cardiovascular system include α and β receptors, in which α receptor comprises AR- α_1 and AR- α_2 subtypes and β receptor contains AR- β_1 and AR- β_2 subtypes. Multiple studies have confirmed the importance of the AR system in the development of LV remodeling secondary to hypertension.⁴ In experimental studies, norepinephrine has been reported to induce myocyte proliferation through its stimulation of AR- α_1 and AR- β in cultured cardiocytes.⁵ Consequently, suppression of AR- α_1 and AR- β has been considered important for attenuating the progress of cardiac remodeling. A major concern with the use of selective AR- β antagonists is that AR- β blockade may lead to intrinsic agonist stimulation of the AR- α subtype

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through feedback mechanisms, which may aggravate LV remodeling. For these reasons, overall blockade of AR subtypes seems to be superior to selective blockade of AR subtypes in the treatment of hypertension. This study, therefore, was designed to test the hypothesis that overall blockade of AR subtypes is superior to selective blockade of AR subtypes in suppressing LV remodeling secondary to hypertension by comparing the effects of AR- β_1 antagonist bisoprolol, AR- β_1 and AR- β_2 antagonist propranolol and AR- α_1 , AR- β_1 and AR- β_2 antagonist carvedilol in spontaneously hypertensive rats (SHR).

METHODS

Animal model and experimental protocol

Ten-week-old SHR ($n=64$) and Wistar-Kyoto (WKY) rats ($n=16$) (Beijing University, Beijing, China) were housed at a constant temperature (24 °C) in a room under 12 h dark/12 h light cycles. The SHR were randomly divided into bisoprolol-treated group, propranolol-treated group, carvedilol-treated group and no treatment group ($n=16$ for each group) that received daily oral dose of 2 mg kg⁻¹ per day bisoprolol, 6 mg kg⁻¹ per day propranolol, 6 mg kg⁻¹ per day carvedilol and an equal amount of distilled water, respectively. WKY rats as a control group were given an equal amount of distilled water. On the basis of pharmacological properties, we selected bisoprolol (Merck Pharmaceuticals, Darmstadt, Germany) as an AR- β_1 antagonist, propranolol (Dong Feng Pharmaceuticals, Jinan, China) as an AR- β_1 and AR- β_2 antagonist and carvedilol (Qilu Pharmaceuticals, Jinan, China) as an AR- α_1 , AR- β_1 and AR- β_2 antagonist. All rats were treated for 24 weeks to ensure a sufficient time-window for LV remodeling and therapeutic effects to take place.⁶

As the purpose of this study was to compare the efficacy of different AR antagonists in suppressing LV remodeling secondary to hypertension, the prerequisite for a chosen drug is to lower blood pressure to a normal level. The drug doses were determined by referring to previous studies on animal models of LV remodeling⁷⁻¹⁰ and by a preliminary study in which the drug doses were increased gradually from a low dose until the systolic blood pressure (SBP) in each drug-treated group reached the level of SBP as that of WKY rats. Our preliminary results showed that bisoprolol, propranolol and carvedilol at a dose of 2 mg kg⁻¹ per day, 6 mg kg⁻¹ per day and 6 mg/kg/day, respectively, were effective in normalizing SBP in SHR and thus, these doses were finally chosen for comparing the therapeutic effects of bisoprolol, propranolol and carvedilol in suppressing LV remodeling in SHR.

All animal care and experimental protocols complied with the Animal Management Rule of the Ministry of Public Health, People's Republic of China (documentation 55, 2001), and were approved by the Animal Care Committee of Shandong University.

Blood pressure measurement

During the entire period of treatment, body weight, heart rate and SBP were measured once a week in all the rats in a conscious state. SBP was measured using the tail-cuff method with a Rat Tail Manometer Tachometer System (RBP-1, Institute of Clinical Medicine, China-Japan Friendship Hospital, Beijing, China).

Echocardiographic imaging

Echocardiographic imaging involved use of an 8-MHz transducer connected with an ultrasonic system (HP SONOS 5500, Hewlett Packard, Andover, MA, USA). All rats underwent general anesthesia with the chest shaved. Echocardiographic measurements were taken before treatment (at 10 weeks of age) and at the end of the treatment (at 34 weeks of age). LV end-diastolic and end-systolic diameters, LV ejection fraction, interventricular septum thickness and LV posterior wall thickness were measured by M-mode echocardiography, and LV mass index (LVMI) was calculated by the Devereux method and adjusted for body weight.¹¹ The mitral flow velocities were recorded by the pulsed Doppler technique and the ratio of (E/A) velocities was calculated.

Integrated backscatter analysis

The integrated acoustic backscatter data from the interventricular septum, the posterior and the lateral walls of LV and the LV chamber were analyzed by the

acoustic densitometry technique inherent in the ultrasonic system. The mean and peak ultrasonic intensity, cardiac cycle variation of integral backscatters and s.d. of the intensity of the LV wall and chamber were measured. The percentage of integrated backscatter was derived by calculating the ratio of the integrated backscatters of the LV wall to the integrated backscatters of the LV chamber.

Cardiac catheterization

Cardiac catheterization was carried out in all rats at the end of the treatment (at 34 weeks of age). With the animals under general anesthesia, a cardiac catheter (PE-50, Becton Dickinson, Franklin Lakes, NJ, USA) was introduced into the LV through the right common carotid artery and connected to a polygraph system (RM-6000, Nihon Kohden, Tokyo, Japan) to obtain the LV end-diastolic and the maximum rising (dp/dtmax) and falling (-dp/dtmax) rate of the LV pressure. The LV relaxation time constant (τ) was calculated by the Weiss method.¹²

LVMI measurement

Rats were euthanized by an overdose of intravenous pentobarbital. The LV mass was weighed after the LV was dissected from the heart, and LVMI was calculated by dividing LV mass by the body weight. Then the left ventricle was cut into four pieces at the papillary muscle level for histopathological, immunohistochemical, electronic microscopic and molecular biochemical studies.

Histopathological and immunohistochemical analysis

The LV samples were fixed in 1% formalin overnight and then cut into serial 5- μ m thick tissue sections for hematoxylin and eosin staining to observe morphological changes in myocardial cells and for Van Gieson staining to detect the degree of fibrosis. Stereology involving a video-microscopic system (Olympus BX50, Olympus, Tokyo, Japan) was used for quantitative analysis of the interstitial collagen in a given slide. Five high-power microscopic fields were randomly selected in each slide using a test-system composed of 36 test points (P_T) to derive volume density (V_{vi})= P_p/P_T (%) where P_p represents the points hitting the structure.¹³

The primary antibodies used were monoclonal antibodies for goat anti-mouse type I and III collagen fiber, goat anti-mouse protein kinase A (PKA) and protein kinase C (PKC) subtype α and δ (PKC- α and PKC- δ) and goat anti-mouse p38-mitogen-activated protein kinase (p38-MAPK) (Sigma, St Louis, MO, USA). Positive staining was measured and expressed as mean percentage of the myocardium area in at least ten high-power fields ($\times 400$ magnification) by use of Image pro plus 5.1 software.

Electronic microscopy

The LV samples were fixed in 3% glutaral, post-fixed with 1% osmium tetroxide and examined by scanning electron microscopy (TEM-100, Electron, Joel, Tokyo, Japan) under 15kV accelerating voltage. Quantitative evaluation of the ultrastructural characteristics of interstitial collagen fibers (relative volumes) was carried out using the point-counting method.¹⁴ The ultrastructure of the myocardial cells was observed with transmission electron microscopy (JEM-1200EX, Electron, Joel, Tokyo, Japan). Samples were fixed with 0.2 mol/l glutaraldehyde and osmium tetroxide and embedded into epon by standard procedures. Ultrathin sections were cut and doubly stained with uranyl acetate and lead citrate.

Flow cytometry

The heart was mounted on a Langendorff apparatus and perfused retrogradely with a Ca²⁺-free Joklik medium (Sigma) containing 0.01 mmol l⁻¹ ethylene glycol tetraacetic acid, followed by perfusion with isolation solution containing 0.8 mg ml⁻¹ collagenase (type I, Worthington Biochemical, Lakewood, NJ, USA), and 0.08 mg ml⁻¹ protease (type XIV, Sigma). Tissues were minced and poured through 250 μ m nylon mesh to collect the myocytes, which were resuspended in isolation solution and then washed with phosphate-buffered saline (PBS). Cell pellets were collected by centrifugation and resuspended in 0.5 ml PBS.¹⁵ Then, 0.5 ml of propidium iodide solution (100 μ g ml⁻¹ in PBS) was added and the mixture was allowed to stand on ice for 30 min. The myocardial cells (1 $\times 10^6$ ml⁻¹) were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and distribution of the cell cycle phases

was determined by means of ModFit LT software (Verity Software House, Topsham, ME, USA). The population of apoptotic cells was quantified by annexing with V-FITC staining (Bender Medsystems, Boehringer Mannheim, Germany) and the apoptosis rate was calculated.

Intracellular Ca²⁺ concentration assay

Intracellular Ca²⁺ concentration was measured in Fluo-3/AM-loaded myocardial cells by use of flow cytometry as previously described.¹⁶ The myocardial cells were harvested and resuspended in PBS buffer without Ca²⁺ and Mg²⁺. A 490 µl myocardial cell (4 × 10⁶ ml⁻¹) suspension was then incubated with 10 µl Fluo-3/AM buffer (1.0 mM, Dojindo, Kumamoto, Japan) for 30 min at 37 °C, then washed twice and resuspended in PBS buffer. A FACScan flow cytometer (Becton Dickinson) was used to measure the fluorescence intensity. With the same process, the fluorescence intensity was measured in another sample containing 500 µl of myocardial cell suspension. Intracellular free Ca²⁺ concentration was calculated as mean fluorescence intensity with Fluo-3/AM divided by mean fluorescence intensity without Fluo-3/AM.

Quantitative RT-PCR

Tissue samples were frozen with liquid nitrogen. Total RNA was extracted and mRNA expression of α_{1A}-AR, α_{1B}-AR, α_{1D}-AR, β₁-A and β₂-AR and G-protein (Gα, Gβ and Gγ) of the myocardium was determined by quantitative RT-PCR with use of LightCycler (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's instruction. The mRNA sequences were obtained from the GenBank and the primer sequences of each gene were shown in Table 1. Quantitative values were obtained from the threshold cycle value, the point at which a significant increase of fluorescence is first detected, and the data were analyzed with the 2^{-ΔΔCT} method.¹⁷ Experiments were carried out in triplicate for each data point. The transcript number of glyceraldehyde 3-phosphate dehydrogenase was quantified as an internal control.

Western blotting analysis

Total protein was extracted and detected by western blot. The protein expression of type I and III collagen, PKC-α, PKC-δ and p38-MAPK was shown by the ratio of integral optical density between these values and β-actin.

Statistical analysis

Values are expressed as mean ± s.d. Comparison of continuous variables among multiple groups was performed by analysis of variance with ANOVA and *post hoc* comparisons were made using lysergic acid diethylamide test. The

relation between variables was estimated with use of Pearson correlation. A *P* < 0.05 was considered significant. All analyses involved use of SPSS version 13.0 (SPSS, Chicago, IL, USA).

RESULTS

Effects on body weight and blood pressure

In total, nine rats (three in WKY group, one each in the bisoprolol-treated and carvedilol-treated groups and two in the propranolol-treated and no treatment groups) died of anesthetic accident, aspirated pneumonia or upper gastrointestinal bleeding during the experiment.

There was no significant difference in body weight and heart rate among WKY, no treatment and three drug-treated groups either at baseline or 24-weeks after treatment. The SBP did not differ among all five groups of rats at baseline, but was significantly lower in the WKY and three drug-treated groups than in the no treatment group 24-weeks after treatment. In contrast, SBP did not differ between the WKY and three drug-treated groups 24-weeks after treatment (Table 2).

Effects on echocardiographic measurements

There was no significant difference in LV end-diastolic and end-systolic diameters and LV ejection fraction among five groups of rats before and after treatment. Twenty-four weeks after treatment, LVMI was significantly higher but the E/A ratio was lower in the no treatment group than in the WKY group (*P* < 0.01, Figure 1). The LVMI was significantly lower, but E/A ratio was higher in the three drug-treated groups than in the no treatment group (all *P* < 0.05). The LVMI and E/A ratio did not significantly differ among the three drug-treated groups.

Effects on integrated backscatter measurements

Percentage of integrated backscatter in the interventricular septum and LV posterior and lateral walls was significantly higher, whereas cardiac cycle variation of integral backscatters in the interventricular septum and LV posterior wall was lower in the no treatment group than in the WKY group (Table 3). The three drug-treated groups did not differ from the no treatment group or from each other in percentage of integrated backscatter and cardiac cycle variation of integral backscatters in the interventricular septum and LV posterior and lateral walls.

Effects on hemodynamic measurements

Compared with the WKY group, the no treatment group showed higher LV end-diastolic and τ and lower -dp/dt_{max}, the bisoprolol-treated and propranolol-treated groups showed higher τ and lower -dp/dt_{max} and the carvedilol-treated group showed only lower -dp/dt_{max}. The three drug-treated groups showed higher -dp/dt_{max} and lower τ than the no treatment group (Figure 2). In contrast, none of these hemodynamic parameters showed significant difference among the three drug-treated groups.

Effects on histopathological changes

Pathological measurement of LVMI of the no treatment group was significantly higher than that of the WKY and three drug-treated groups, with no significant difference among the three drug-treated groups (Figure 1). In the no treatment group, LVMI measured by echocardiography and pathology showed a good correlation (*r* = 0.69, *P* < 0.01).

Hematoxylin and eosin staining of the myocardium showed increased volume of myocardial cell nuclei in the no treatment

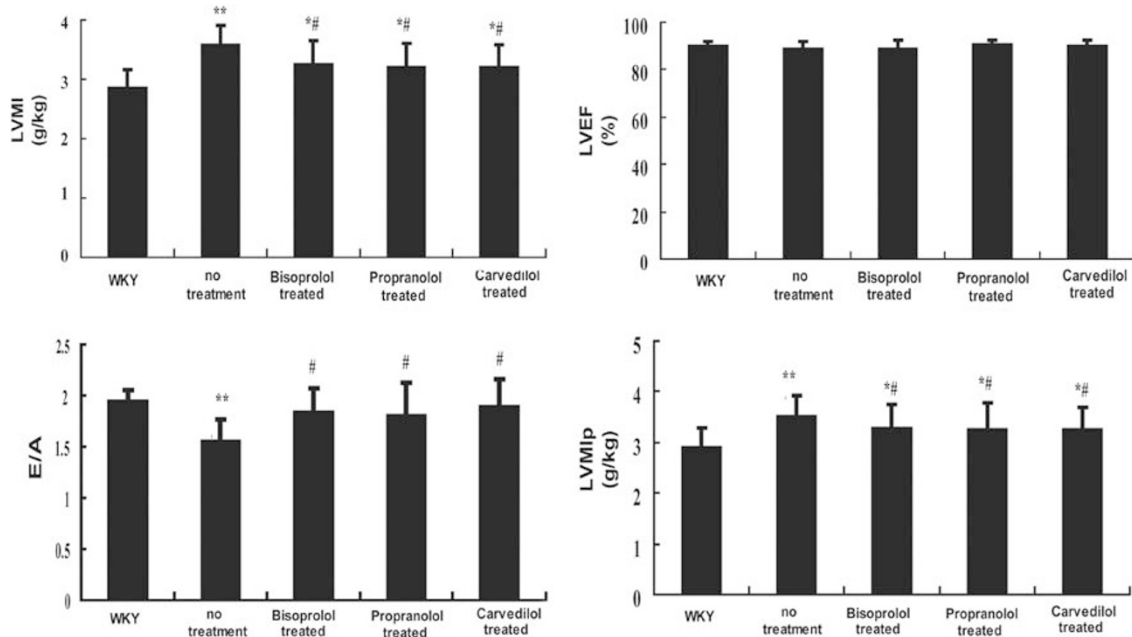
Table 1 Primer sequences and gene lengths for RT-PCR

Genes	Primer sequences	Primer length (bp)
β-actin	5'-CAGCACTGTGTTGCCATAGAG-3'	327
	5'-AAGATCCTGACCGAGCGTGG-3'	
β ₁ adrenoceptor	5'-CTCACCAACCTCTTCATCATG-3'	522
	5'-GAAGCGGGCTCGCAGCTGTCG-3'	
β ₂ adrenoceptor	5'-GAGACCCTGTGCGTGATTGC-3'	387
	5'-CCTGCTCCACCTGGCTGAGG-3'	
α _{1A} adrenoceptor	5'-CGAGAGAAGAAAGCTGCCAAG-3'	299
	5'-TCCTGGCTGGAGCATGGGTA-3'	
α _{1B} adrenoceptor	5'-ATCGTGGCCAAGAGGACCAC-3'	265
	5'-CGGGAGAGCGATGAAGAAGG-3'	
α _{1D} adrenoceptor	5'-CGTGTGCTCCTTCTACTACC-3'	302
	5'-GCACAGGACGAAGACACCCAC-3'	
G _{sα} -protein	5'-CAGAAGGACAAGCAGGTCTAC-3'	318
	5'-GTTTCATCACGCTCAGAATG-3'	
G _{βγ} -protein	5'-GGAGAATCAGGGAAGAGC-3'	213
	5'-ACAGGACAGTGCAGAACAG-3'	
G _{qα} -protein	5'-AGAGTGGCAAGAGTACCTTC-3'	495
	5'-TTCTCTGACCTTTGGC-3'	
G _β -protein	5'-TGCCTTGTGGGACATTGAG-3'	378
	5'-AAGTCATCATAGCCAGCAAAG-3'	

Table 2 Body weight, heart rate (HR) and systolic blood pressure (SBP) at baseline and after treatment

Groups	Weight (g)		HR (min ⁻¹)		SBP (mm Hg ⁻¹)	
	Baseline	Week24	Baseline	Week 24	Baseline	Week 24
WKY (n=13)	213.09 ± 3.81	287.74 ± 5.37	385.67 ± 41.25	374.78 ± 44.36	123.33 ± 31.03	124.33 ± 28.15
No treatment (n=14)	208.32 ± 3.74	270.78 ± 4.77	362.14 ± 37.82	379.22 ± 56.83	133.21 ± 22.57	218.21 ± 36.68**
Bisoprolol-treated (n=15)	210.17 ± 5.90	276.93 ± 5.98	366.10 ± 35.36	363.41 ± 39.50	137.70 ± 25.14	115.52 ± 37.13#
Propranolol-treated (n=14)	209.70 ± 4.72	283.85 ± 4.95	372.88 ± 36.17	352.57 ± 58.82	134.94 ± 23.09	119.75 ± 41.37#
Carvedilol-treated (n=15)	212.99 ± 4.85	293.13 ± 5.05	369.01 ± 35.55	357.37 ± 46.02	136.09 ± 24.75	124.11 ± 39.25#

Abbreviation: WKY, Wistar-Kyoto.

***P*<0.01, vs. WKY group at week 24; #*P*<0.05, vs. no treatment group at week24.**Figure 1** Echocardiographic and pathological measurements in five groups of rats. Left ventricular mass index (LVMI) was higher in the no treatment and three drug-treated groups than in the Wistar-Kyoto (WKY) group, but was lower in the three drug-treated groups than in the no treatment group. E/A ratio was lower in the no treatment group than in the WKY and three drug-treated groups. Pathological measurement of LVMI showed similar differences among the five groups of rats as LVMI. **P*<0.05, ***P*<0.01, vs. WKY group; # *P*<0.05, vs. no treatment group.**Table 3** Echocardiographic measurements of integrated backscatters of myocardium

Groups	IB%			CVIB (dB)		
	IVS	LVPW	LVLW	IVS	LVPW	LVLW
WKY (n=13)	1.35 ± 0.17	1.39 ± 0.22	1.28 ± 0.18	5.97 ± 1.96	7.86 ± 2.70	6.73 ± 2.07
No treatment (n=14)	1.59 ± 0.34*	1.62 ± 0.29*	1.46 ± 0.21*	4.52 ± 1.83*	5.82 ± 2.05*	5.65 ± 2.13
Bisoprolol-treated (n=15)	1.55 ± 0.38	1.58 ± 0.32	1.44 ± 0.27	4.86 ± 2.12	6.15 ± 2.34	5.98 ± 2.23
Propranolol-treated (n=14)	1.52 ± 0.33	1.56 ± 0.24	1.41 ± 0.35	4.98 ± 1.94	6.18 ± 2.41	5.87 ± 2.04
Carvedilol-treated (n=15)	1.48 ± 0.24	1.54 ± 0.19	1.39 ± 0.34	5.06 ± 2.17	6.17 ± 2.29	6.01 ± 2.35

Abbreviations: CVIB, cycle variation of integral backscatters; IB%, percentage of integrated backscatters; IVS, interventricular septum wall thickness; LVLW, left ventricular lateral wall; LVPW, left ventricular posterior wall thickness; WKY, Wistar-Kyoto.

**P*<0.05, vs. WKY group.

group, which was significantly improved in the three drug-treated groups. The expression level of collagen indicated by Van Gieson staining was significantly higher in the no treatment group than in the WKY group (15.22 ± 4.34 vs. $11.85 \pm 3.21\%$, *P*<0.05), with no significant difference among the three drug-treated groups (13.86 ± 4.63 , 13.11 ± 3.50 and $13.45 \pm 4.15\%$, respectively).

Effects on ultrastructure characterization of LV

Significant ultrastructure changes were found in the no treatment group, including abundant swollen myocardial cells, plentiful tough collagen fibers, dissolved myofilaments, disarrayed Z-line and swollen mitochondria (Figure 3). These abnormalities were markedly ameliorated in the three drug-treated groups. Stereology showed fewer

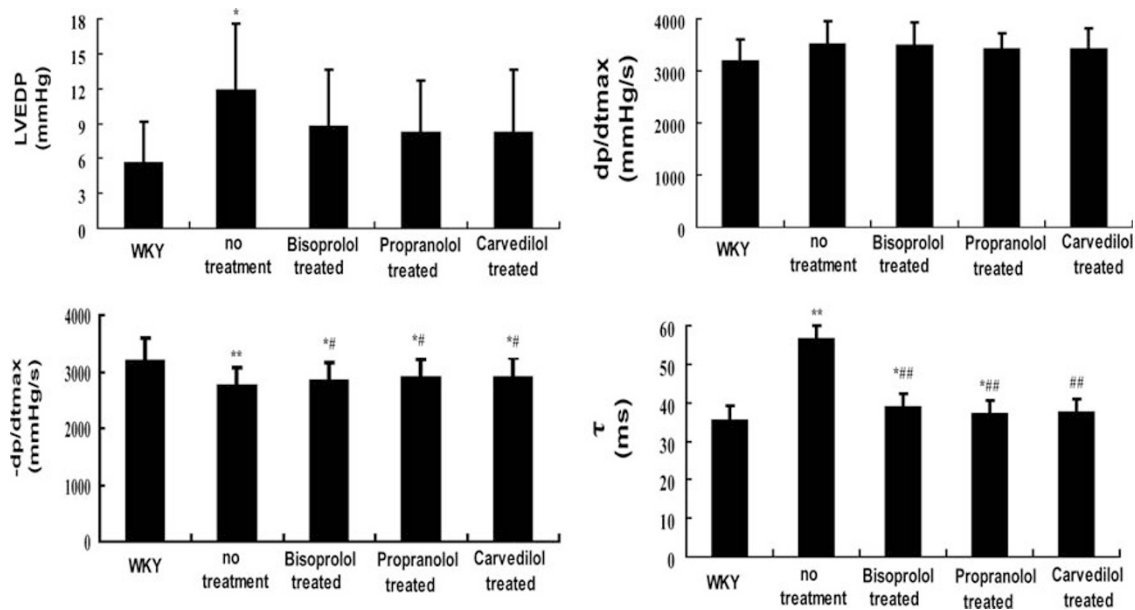


Figure 2 Hemodynamic measurements in five groups of rats. left ventricular(LV) end-diastolic was higher in the no treatment group than in the Wistar–Kyoto (WKY) group. $-\text{dp}/\text{dt}_{\text{max}}$ was lower in the no treatment group than in the WKY and three drug-treated groups. The LV relaxation time constant (τ) was lower in the WKY and three drug treated groups than in the no treatment group. * $P < 0.05$, ** $P < 0.01$, vs. WKY group; # $P < 0.05$, ## $P < 0.01$, vs. no treatment group.

collagen fibers in the three drug-treated groups than in the no treatment group ($P < 0.05\text{--}0.01$, Figure 3).

Effects on cellular apoptosis and intracellular free Ca^{2+} concentration

In comparison with the WKY group, the no treatment and three drug-treated groups showed increased cellular apoptosis rates. All three drug-treated groups had lower apoptosis rates than the no treatment group ($P < 0.05\text{--}0.01$) and the apoptosis rate in the carvedilol-treated group was significantly lower than that of the other two drug-treated groups. The WKY group had lower intracellular free Ca^{2+} concentration than any other group ($P < 0.01$). As compared with the no treatment group, the three drug-treated groups showed decreased intracellular free Ca^{2+} concentrations ($P < 0.05\text{--}0.01$), with no significant difference among the three drug-treated groups (Figure 4).

Effects on mRNA expression of AR subtypes

The mRNA expression of $\text{AR-}\alpha_{1A}$ and $\text{AR-}\alpha_{1D}$ did not differ among all five groups of rats (Figure 5). The mRNA expression of $\text{AR-}\alpha_{1B}$ was significantly higher in the no treatment, bisoprolol-treated and propranolol-treated groups than in the WKY group. After treatment, the mRNA expression of $\text{AR-}\alpha_{1B}$ was significantly lower in the carvedilol-treated group than in the no treatment, bisoprolol-treated and propranolol-treated groups but did not differ from that in the WKY group. The mRNA expression of $\text{AR-}\beta_1$ and $\text{AR-}\beta_2$ was significantly lower in the no treatment and three drug-treated groups than in the WKY group. After treatment, however, the mRNA expression of $\text{AR-}\beta_1$ was higher in the three drug-treated groups than in the no treatment group but still lower than that of the WKY group (all $P < 0.01$). Similarly, after treatment the expression of $\text{AR-}\beta_2$ was higher in the three drug-treated groups than in the no treatment group ($P < 0.05\text{--}0.01$), with a significantly stepwise increase in the bisoprolol-treated, propranolol-treated and carvedilol-treated groups ($P < 0.05\text{--}0.01$).

The mRNA expression of $\text{Gs}\alpha$ protein was significantly lower in the no treatment, bisoprolol-treated and propranolol-treated groups than in the WKY group ($P < 0.01$, Figure 5) but did not differ from that in the carvedilol-treated group. The mRNA expression of $\text{Gq}\alpha$ protein was lower in the propranolol-treated and carvedilol-treated groups than in the no treatment group (all $P < 0.05$) but was higher in the no treatment and bisoprolol-treated groups than in the WKY group. The $\text{Gi}\alpha$ and $\text{G}\beta$ mRNA expression did not differ among the no treatment and three drug-treated groups.

Effects of AR antagonist on expression of collagens and protein kinase

The effects of AR antagonist on the levels of protein expression of collagen and protein kinases were examined by immunohistochemical staining and western blotting. The expression level of type I collagen was significantly lower in the WKY and three drug-treated groups than in the no treatment group ($P < 0.05\text{--}0.01$, Figures 6 and 7), with no significant differences among the three drug-treated groups. The expression level of type III collagen did not differ among any of the five groups of rats. The expression level of PKA, $\text{PKC-}\alpha$ and $\text{PKC-}\delta$ was higher in the no treatment group than in the WKY group ($P < 0.01$), and that of PKA was significantly lower in the three drug-treated groups than in the no treatment group ($P < 0.05\text{--}0.01$, Figures 6 and 7), with no significant difference among the drug-treated groups. $\text{PKC-}\alpha$ and $\text{PKC-}\delta$ expression in the carvedilol-treated group was markedly lower than that of the no treatment group. The expression of p38-MAPK in the no treatment group and three drug-treated groups was higher than that in the WKY group ($P < 0.01$, Figure 6) and was significantly lower in the carvedilol-treated group than in the no treatment group ($P < 0.01$, Figure 7).

DISCUSSION

In this study, we compared the effects of selective $\text{AR-}\beta_1$ antagonist bisoprolol, nonselective $\text{AR-}\beta_1$ and $\text{AR-}\beta_2$ antagonist propranolol and

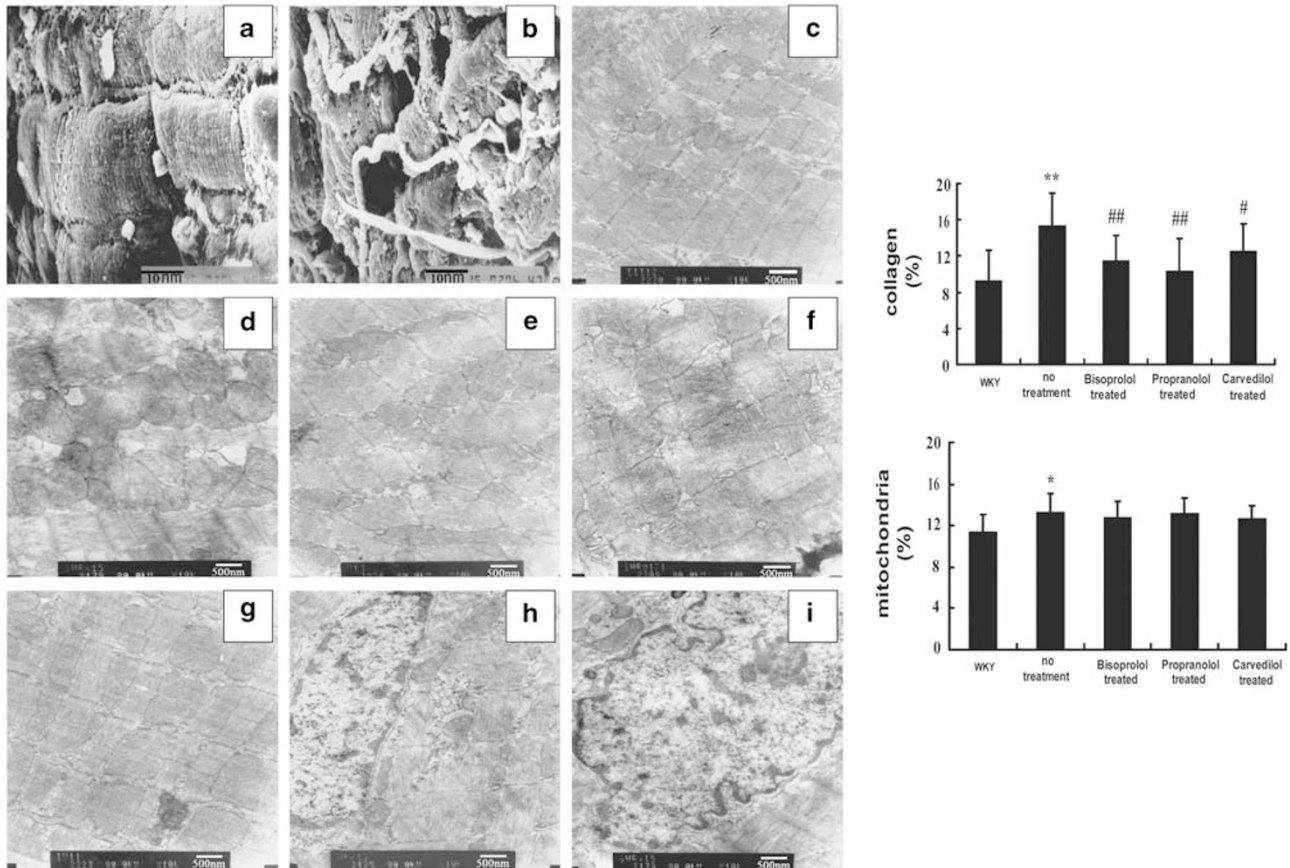


Figure 3 Electron microscopic images. (a) Scanning electron microscopic image of myocardial cells covered by few tough collagen fibers in the Wistar–Kyoto (WKY) group ($\times 3500$); (b) Scanning electron microscopic image of myocardial cells covered by plentiful tough collagen fibers in the no treatment group ($\times 3500$); (c) Transmission electron microscopic image of myocardial cells and mitochondria that line up in order with distinct Z-line in the WKY group ($\times 10000$); (d) Transmission electron microscopic image showing abundant swollen myocardial cells and mitochondria with disordered myofilaments in the no treatment group ($\times 10000$); (e) Transmission electron microscopic image displaying less swollen myocardial cells and mitochondria in the bisoprolol-treated group ($\times 10000$); (f) Transmission electron microscopic image depicting less swollen myocardial cells and mitochondria in the propranolol-treated group ($\times 10000$); (g) Transmission electron microscopic image showing mildly swollen myocardial cells and mitochondria in the carvedilol-treated group ($\times 10000$); (h, e) Transmission electron microscopic image showing normal perinuclear space in a myocardial cell of the WKY group ($\times 10000$); (i) Transmission electron microscopic image showing widened perinuclear space in a myocardial cell of the no treatment group ($\times 10000$). Bar graphs on the right show quantitative analysis of the relative contents of collagen and mitochondria. The relative content of collagen was lower in the three drug-treated groups than in the no treatment group. The relative content of mitochondria was higher in the no treatment group than in the WKY group. * $P < 0.05$, ** $P < 0.01$, vs. WKY group; # $P < 0.05$, ## $P < 0.01$, vs. no treatment group.

combined AR- α_1 , AR- β_1 and AR- β_2 antagonist carvedilol on LV remodeling. Our major finding was that carvedilol was not superior to bisoprolol or propranolol in inhibiting LV remodeling and ameliorating LV diastolic dysfunction even though it had the largest effect on attenuating cardiocyte apoptosis, normalizing AR- α_{1B} and Gs α expression and increasing AR- β_2 expression.

In patients with hypertension, LV remodeling is the key pathological process leading to heart failure. The mechanisms of LV remodeling involves both mechanical and neurohumoral factors. Mechanical factors include hypertrophy of myocardial cells, interstitial fibrosis and collagen accumulation, whereas neurohumoral factors contain catecholamine, angiotensin II, aldosterone, endothelin and cytokines, which accelerate the pathologic processes of LV remodeling. Therefore, identification of antihypertensive drugs that are most effective in inhibiting LV remodeling is of major importance in the prevention of heart failure secondary to hypertension.

AR- β antagonists, either alone or in combination with other classes of antihypertensive agents, have had a long history of use in lowering high blood pressure. Bisoprolol, a highly selective β_1 -adrenoceptor

antagonist, is currently used to treat hypertension, atrial fibrillation, heart failure and coronary artery disease. Weiss *et al.*¹⁸ found that bisoprolol attenuated LV hypertrophy after a 8-week treatment in patients suffering with essential hypertension. Onodera *et al.*¹⁹ reached the same conclusion after a 4-week treatment of a 12-week-old SHR. Propranolol is a nonselective β -adrenoceptor antagonist and blocks both AR- β_1 and AR- β_2 of the myocardium. However, the efficacy of propranolol in abating LV hypertrophy and interstitial fibrosis is controversial. Saragoca *et al.*²⁰ reported that propranolol did not reverse cardiac hypertrophy in renal hypertensive rats. In contrast, Pacca *et al.*²¹ found that the propranolol attenuated cardiomyocyte hypertrophy and myocardial fibrosis in rats under long-term blockade of nitric oxide synthesis. Carvedilol is a combined AR- α_1 , AR- β_1 and AR- β_2 antagonist with potent antioxidant activity and has been used widely in the treatment of hypertension, angina and congestive heart failure. In many clinical and experimental studies, carvedilol has proved effective in reducing LV hypertrophy in humans and SHR.²²

In this study, SHR as the animal model of spontaneous hypertension was used to compare the therapeutic effects of three different

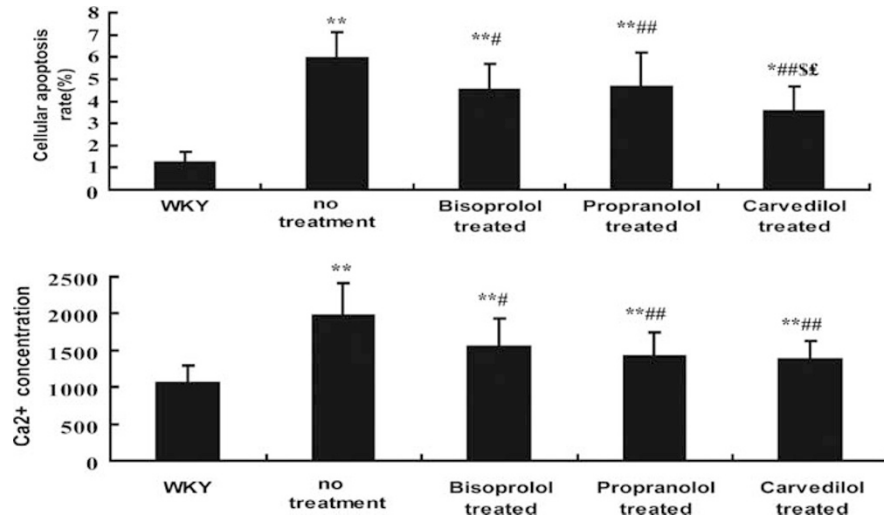


Figure 4 Cellular apoptosis rate and intracellular free Ca²⁺ concentration measured by flow cytometry. Apoptosis rate was higher in the no treatment and three drug-treated groups than in the Wistar–Kyoto (WKY) group but was lower in the three drug-treated groups than in the no treatment group, with the carvedilol-treated group having the lowest apoptotic rate. The intracellular free Ca²⁺ concentration was lower in the WKY group than in any other group, and was lower in the three drug-treated groups than in the no treatment group. **P*<0.05, ***P*<0.01, vs. WKY group; #*P*<0.05, ##*P*<0.01, vs. no treatment group; †*P*<0.05 vs. bisoprolol-treated group; ‡*P*<0.05 vs. propranolol-treated group.

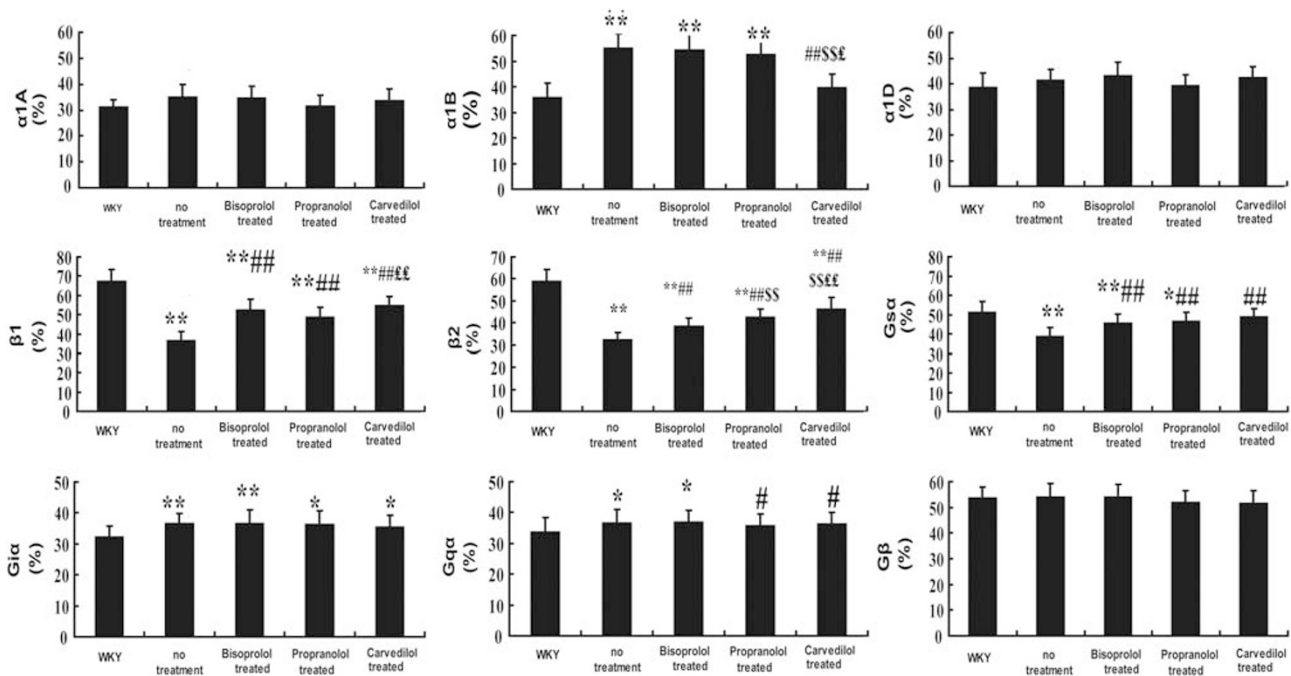


Figure 5 The mRNA expression of adrenergic receptor (AR) and G-protein subtypes. The mRNA expression of AR-α_{1B} was lower in the Wistar–Kyoto (WKY) group than in the no treatment, bisoprolol-treated and propranolol-treated groups but did not differ from that in the carvedilol-treated group. The mRNA expression of AR-β₁ and AR-β₂ was lower in the no treatment and three drug-treated groups than in the WKY group but was higher in the three drug-treated groups than in the no treatment group. The mRNA expression of Gαα expression was higher in the WKY group than in the bisoprolol-treated and propranolol-treated groups but did not differ from that in the carvedilol-treated group. The mRNA expression of Gαα was lower in the WKY group than in the any other group. The mRNA expression of Gαα was lower in WKY group than in the no treatment and bisoprolol-treated groups but did not differ from that in the propranolol-treated and carvedilol-treated groups. **P*<0.05, ***P*<0.01, vs. WKY group; #*P*<0.05, ##*P*<0.01, vs. no treatment group; \$\$\$*P*<0.01, vs. bisoprolol-treated group; †*P*<0.05, ‡*P*<0.01, vs. propranolol-treated group.

AR-β antagonists. Our results showed that SBP was significantly lowered in all three drug-treated groups to the normal level of WKY rats 24-weeks after treatment. In addition, all drug-treated groups, as compared with the no treatment group, showed decreased LVMI and τ and increased E/A ratio and $-dp/dt_{max}$, which indicated that LV

hypertrophy and diastolic dysfunction were greatly improved and all three AR antagonists had equal effects on LV remodeling. Moreover, ultrastructural analysis showed significantly decreased interstitial collagen content after antihypertensive treatment by the three AR antagonists, which was confirmed by histopathology and immuno-

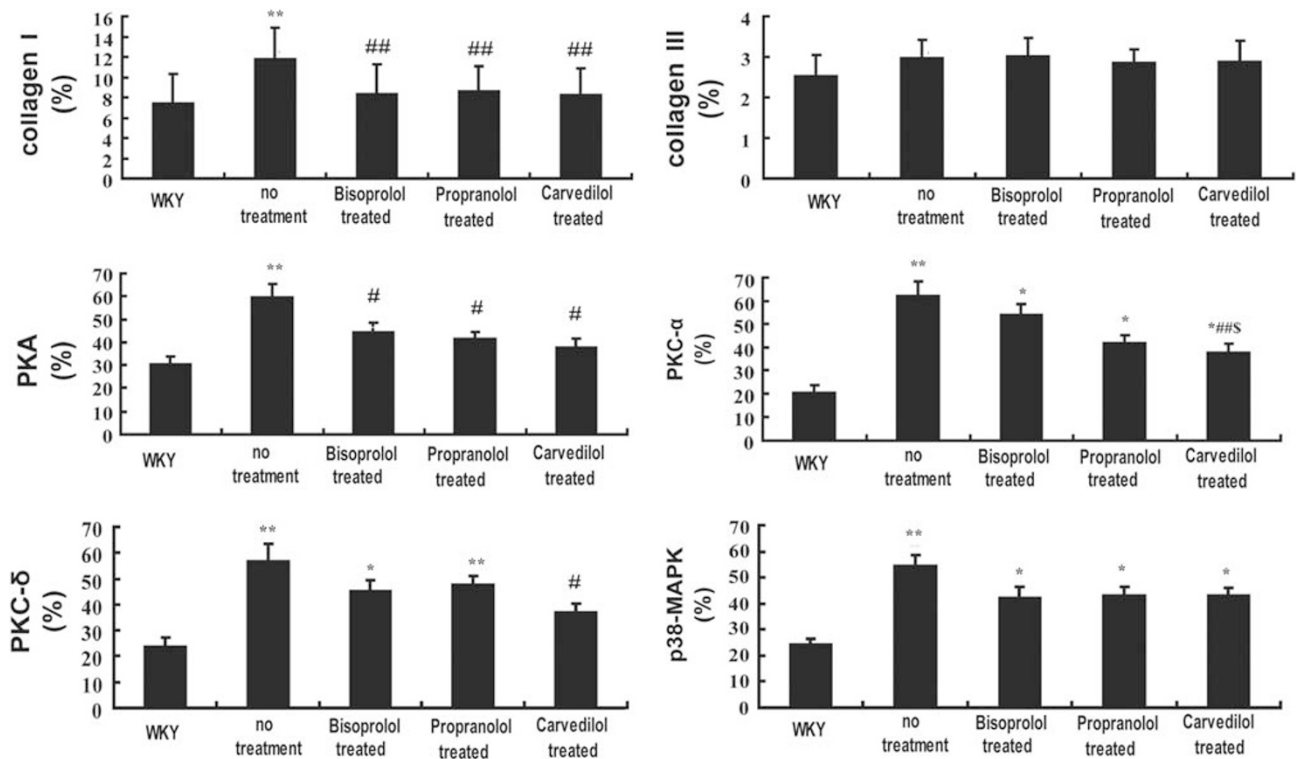


Figure 6 Quantitative analysis of immunohistochemical staining in five groups of rats. Protein expression of type I collagen was significantly lower in the Wistar-Kyoto (WKY) and the three drug-treated groups than in the no treatment group. The expression level of protein kinase(PK) PKA, PKC- α and (PK)C- δ was higher in the no treatment group than in the WKY group. Protein expression of PKA was significantly lower in the three drug-treated groups than in the no treatment group. PKC- α and PKC- δ expression in the carvedilol-treated group was lower than in the no treatment group. The expression of p38-MAPK was significantly lower in the in the WKY group than in the no treatment and three drug-treated groups. * $P < 0.05$, ** $P < 0.01$, vs. WKY group; # $P < 0.05$, ## $P < 0.01$, vs. no treatment group; \$\$\$ $P < 0.05$ vs. bisoprolol-treated group.

histochemistry. Immunohistochemistry also showed markedly decreased expression of type I collagen, with no significant difference in type III collagen content. Thus, AR antagonists inhibited LV remodeling by reducing interstitial fibrosis and accumulation of collagen, especially type I collagen. In addition, cellular apoptosis rate and intracellular free Ca^{2+} concentration were significantly lower in the drug-treated groups than in the no treatment group, suggesting that myocardial cell depletion and intracellular Ca^{2+} overload had important roles in myocardial remodeling. Compared with the other two medications, carvedilol appeared to have no greater effect on improving LV remodeling, except for having a higher effect on inhibition of cardiocyte apoptosis.

The ARs in the cardiovascular system include α and β receptors with the former comprising of AR- α_1 and AR- α_2 and the later mainly of AR- β_1 and AR- β_2 . The AR- α_1 can be further classified into AR- α_{1A} , AR- α_{1B} and AR- α_{1D} subtypes with AR- α_{1B} is found to be dominant in the myocardium of mice.^{23,24} Wang *et al.*²⁵ reported that cardiac expression of constitutively active AR- α_{1B} is detrimental in terms of cardiac hypertrophy and dysfunction after pressure overload and also that increased AR- α_{1A} mRNA expression is not a feature of the hypertrophic response to hypertension. Vecchione *et al.*²⁶ found no occurrence of high blood pressure and LV remodeling in response to noradrenaline stimulation in AR- $\alpha_{1B}^{-/-}$ mice. Chalothorn *et al.*²⁷ showed that AR- α_{1B} was involved in the regulation of cardiac growth and contractile function, whereas AR- α_{1D} was coupled to smooth muscle contraction and blood pressure regulation. A number of studies found that both AR- β_1 and AR- β_2 were downregulated in SHR.^{28–31} Chruscinski *et al.*^{32,33} reported that AR- β_1 had a dominant

role in mediating vasodilation in mice. Hirota *et al.*³⁴ found AR- β_2 stimulation improved inotropic and lusitropic function in the failing heart and Communal *et al.*³⁵ reported that AR- β_2 inhibited cardiocyte apoptosis through a Gi-coupled pathway in rats. Thus, AR- β_1 and AR- β_2 have an important role in the regulation of cardiovascular function in mice and rats.

In this study, we found that the major changes of AR subtypes in the myocardium of SHR were increased expression of AR- α_{1B} and decreased expression of AR- β_1 and AR- β_2 . The mechanisms probably involve decreased AR- β densities in response to enhanced sympathetic stimulation, and increased AR- α densities to compensate for impaired cardiac function in SHR. After treatment, we found mRNA expression of AR- α_{1B} was normalized in the carvedilol-treated group as compared with the WKY group, but was not significantly different among the no treatment, bisoprolol-treated and propranolol-treated groups, which may reflect the fact that carvedilol directly blocks AR- α_1 , whereas bisoprolol and propranolol have no effects on AR- α_1 . On the other hand, bisoprolol, propranolol and carvedilol had no affect the expression of AR- α_{1A} and AR- α_{1D} , which was consistent with previous studies.²⁷ We also used bisoprolol to selectively block AR- β_1 , and propranolol and carvedilol to nonselectively block AR- β_1 and AR- β_2 and found the expression levels of AR- β_1 and AR- β_2 increased significantly after treatment. Of note, carvedilol was superior to bisoprolol and propranolol in elevating the expression levels of AR- β_2 . These therapeutic differences may be explained by the pharmacological discrepancies of the three AR- β antagonists: bisoprolol has strong antagonism of AR- β_1 but weak antagonism of AR- β_2 , propranolol has strong antagonism of both AR- β_1 and AR- β_2 and

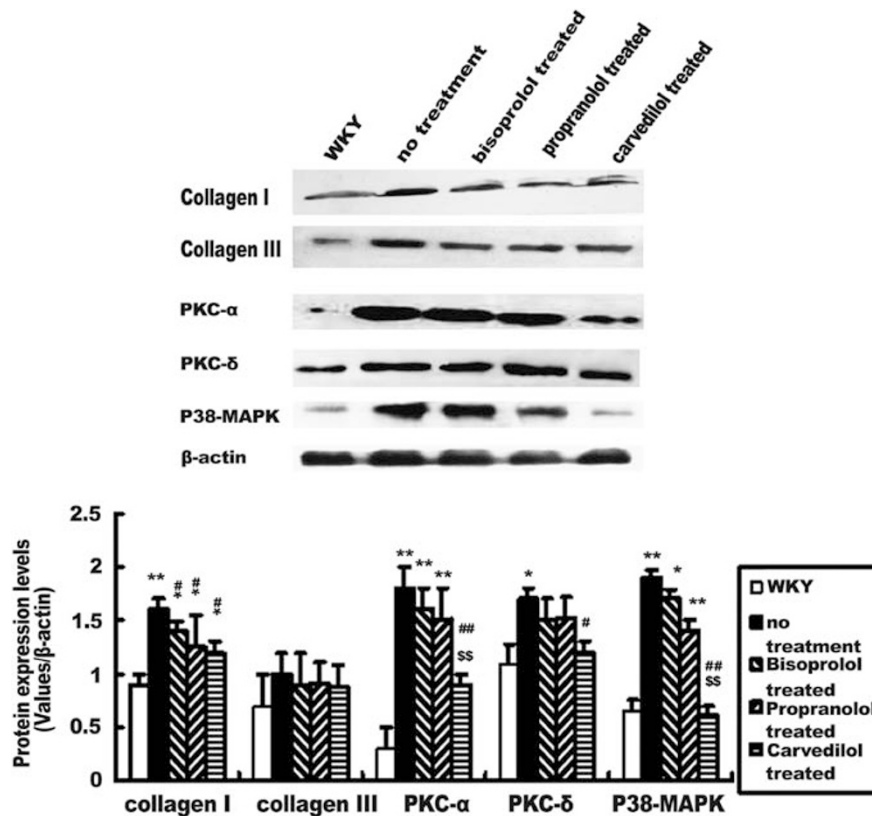


Figure 7 Western blotting analysis. Protein expression level of type I collagen was lower in the Wistar–Kyoto (WKY) and three drug-treated groups than in the no treatment group. Protein expression of PKC- α and PKC- δ was lower in the WKY and carvedilol-treated groups than in the no treatment group. Similarly, protein expression of p38-MAPK was lower in the WKY and carvedilol-treated groups than in the no treatment group. * $P < 0.05$, ** $P < 0.01$, vs. WKY group; # $P < 0.05$, ## $P < 0.01$, vs. no treatment group; \$\$ $P < 0.01$, vs. bisoprolol-treated group.

carvedilol has stronger antagonism of AR- β_1 and AR- β_2 . Blockade of AR- β_1 and AR- β_2 attenuated sympathetic stimulations and resulted in increased myocardial densities of these receptors.

G protein-coupled receptors are important downstream signaling molecules of AR activation.³⁶ Signaling pathways initiated by AR- α_1 involve the activation of the heterotrimeric G-protein, Gq, which elicits the accumulation of diacylglycerol and inositol 1,4,5-triphosphate and activates PKC and MAPKs.³⁷ Dempsey *et al.*³⁸ found that cardiac myocytes contain at least six different PKC isozymes but preconditioning results in activation of only two isozymes, PKC- δ and PKC- ϵ . Quantitation of PKC-isoform expression showed the dominance of the Ca²⁺-dependent PKC α over PKC- δ and PKC- ϵ . PKC- α is the single isozyme that translocates to the contractile system on Ca²⁺ stimulation in the rat heart, suggesting a unique physiological role for PKC α in the Ca²⁺-dependent regulation of myofibrillar contractility.^{39,40} The α -subunit of Gq is a well-established mediator of hypertrophic growth in neonatal cardiomyocytes and in mouse hearts. Studies have found that although aortic AR- α_{1A} , AR- α_{1B} and AR- α_{1D} are associated with Gq α , AR- α_{1B} is also linked to G α and the couple AR- α_{1B} -G α mediates the contractile response of rat aorta.^{41,42} Although AR- β binding to Gs and Gi-coupled receptors stimulate adenylyl cyclase and induce accumulation of cyclic adenosine monophosphate and activation of PKA, the α -subunit of Gs (G α) and Gi (Gi α) has the main role. In the vasculature, these pathways have opposite effects: the Gq cascade results in the constriction of blood vessels, whereas the Gs and Gi signals mediate vasodilation. Both pathways have been shown to mediate hypertrophy in myocardial cells.^{43,44} In this study, after AR- α_1

blockade with carvedilol, the expression of Gq α protein, PKC- α and PKC- δ was decreased significantly in the carvedilol-treated group, which suggested that Gq α , PKC- α and PKC- δ were the main molecules linked to AR- α_1 in the signal transduction pathway. In contrast, the expression of AR- β_1 and AR- β_2 was increased after treatment, followed by a normalized G α protein expression and decreased PKA level, and these effects were the most significant in the carvedilol-treated group. Therefore, carvedilol treatment appeared to be the most effective in counteracting the imbalance between AR- α and AR- β in SHR among the three AR antagonists used in this study.

Recent studies have confirmed that cardiocyte apoptosis is a critical event in the transition between compensatory cardiac hypertrophy and heart failure.³⁴ Catecholamine, released by activated sympathetic system, has been implicated in the progressive deterioration of the failing heart. Communal *et al.*³⁵ showed that norepinephrine, functioning through AR- β_1 pathway, induces cardiac apoptosis by phosphorylation of L-type Ca²⁺ channels by activation of PKA.³⁵ Increased cytosolic Ca²⁺ levels affects mitochondrial membrane permeability and triggers the release of apoptogenic factors, including caspase-9 from damaged mitochondria. In this study, we found abundant swollen mitochondria with disordered myofilaments in the no treatment group, which was markedly ameliorated after treatment together with a reduced intracellular free Ca²⁺ concentration. Reduction of the intracellular Ca²⁺ concentration at resting state by the treatment of AR- β antagonists has been reportedly associated with restoration of the cardiac ryanodine receptor (RyR2)/Ca²⁺ release channel function that regulates cardiac excitation-contraction coupling.⁴⁵ Further

evidence of cardiocyte apoptosis by activation of the AR- β_1 signaling pathway was derived from isoproterenol-treated rat hearts⁴⁶ and transgenic mice overexpressing Gs α ⁴⁷ or Gq α .⁴⁸ In this study, we found that the intracellular free Ca²⁺ concentration and the expression levels of PKA, PKC- α , PKC- δ and p38-MAPK were substantially decreased after administration of AR antagonists, particularly in the carvedilol-treated group, which may have contributed to the anti-apoptotic effects of these medications.

Our study contains several limitations. First, we did not specifically investigate the effects of AR- α_{1A} , AR- α_{1B} and AR- α_{1D} subtypes on hypertension because drugs that selectively block these AR subtypes are not available at this time. Second, the antioxidative effects of carvedilol was not assessed, although these effects may have contributed to the antiapoptotic benefits. Third, we did not measure the protein expression levels of AR subtypes and G proteins although we did measure the protein expression levels of PKA, PKC- α , PKC- δ and p38-MAPK, the downstream signaling molecules of AR subtypes, which correlated well with the mRNA expression levels of AR subtypes and G proteins. Finally, previous studies reported that only the rat heart had high expression of AR- α_{1B} as compared with human and other animal species.²² Therefore, our finding that carvedilol effectively blocks AR- α_1 cannot be extrapolated to human or other animals.

In conclusion, treatment with bisoprolol, propranolol and carvedilol substantially ameliorates LV remodeling and diastolic dysfunction in SHR and carvedilol is not superior to bisoprolol and propranolol in these therapeutic effects, although carvedilol is advantageous in attenuating cardiocyte apoptosis, normalizing AR- α_{1B} and Gs α expression and increasing AR- β_2 expression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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