Original Article

Synergism of Hydrochlorothiazide and Nifedipine on Blood Pressure Variability Reduction and Organ Protection in Spontaneously Hypertensive Rats

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This study was designed to investigate the effects of a hydrochlorothiazide-nifedipine combination on blood pressure (BP), blood pressure variability (BPV), baroreflex sensitivity (BRS), and organ protection in spontaneously hypertensive rats (SHR). The doses used were 10 mg/kg/d for both hydrochlorothiazide and nifedipine, and 10+10 mg/kg/d for the combination of these two drugs. Drugs were mixed into rat chow at the aforementioned doses. SHR were treated for 4 months, and then BP was continuously recorded for 24 h. After the determination of BRS, rats were killed for organ-damage evaluation. It was found that long-term treatment with hydrochlorothiazide, nifedipine or both significantly decreased BP and BPV, enhanced BRS and conferred organ protection in SHR. The combination of hydrochlorothiazide and nifedipine had a significant synergistic effect on BPV reduction, BRS enhancement and organ protection in SHR, whereas no obvious synergism on BP reduction was found. Multiple-regression analysis showed that the decrease in left ventricular and aortic hypertrophy was most closely associated with the decrease in systolic BPV and the increase in BRS, and the amelioration of renal lesions was most closely associated with the increase in BRS. In conclusion, long-term treatment with a combination of hydrochlorothiazide and nifedipine yielded a significantly synergistic effect on BPV reduction, BRS restoration and organ protection in SHR. In addition to BP reduction, the decrease in BPV and the enhancement of BRS may have made important contributions to the observed organ protection. (*Hypertens Res* 2008; 31: 685–691)

***Key Words*: hydrochlorothiazide, nifedipine, hypertension, end-organ damage, blood pressure variability**

Introduction

The usefulness of combination therapy for the treatment of hypertension has been well established (*1*, *2*). Improved blood pressure (BP) control is the main objective of such therapy, since, generally speaking, a combination of two drugs belonging to different classes tends to have a synergistic effect on BP reduction. However, in the case of the combination of a diuretic and calcium antagonist, previous studies have reported rather conflicting results (*3*–*6*).

It is well known that high BP induces organ damage, and conversely, that reduction of BP can prevent end-organ damage. However, high BP is not the only determinant of hypertensive end-organ damage. Recently, it has been proposed that blood pressure variability (BPV) may be another important factor (*7*–*10*), suggesting that antihypertensive treatment should aim at not only reducing BP values but also reducing BPV. In recent studies, we showed that combination therapy might be an optimal way to control BPV, and that the combination of atenolol and nitrendipine has a synergistic effect on both BPV reduction and organ protection in hypertensive rats

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Table 1. Effects of Long-Term Treatment with Hydrochlorothiazide, Nifedipine Alone and in Combination on Hemodynamics in Spontaneously Hypertensive Rats

	SHR (n=16)	Hyd (n=11)	Nif (n=11)	Hyd+Nif (n=10)
SBP (mmHg)	195±2.3	180±4.2**	181±3.7**	176±5.0**
DBP (mmHg)	132±4.5	122±3.2	122±3.6	118±3.5*
HP (ms)	158±3.1	156±3.2	152±3.9	156±2.1
SBPV (mmHg)	14.9±0.6	12.6±0.7**	13.0±0.7*	10.5±0.5**
SBPV-CV (%)	7.6±0.3	7.0±0.4	7.2±0.2	6.0±0.3**
DBPV (mmHg)	11.9±0.5	10.1±0.6*	11.0±0.5	8.5±0.3**
DBPV-CV (%)	9.2±0.6	8.3±0.5	9.0±0.3	7.2±0.2**
HPV(ms)	29.3±2.6	27.1±1.1	27.3±0.8	27.5±0.9
BRS (ms/mmHg)	0.28±0.05	0.50±0.05**	0.44±0.06*	0.65±0.04**

Values are mean±SEM. *p<0.05; **p<0.01 *vs*. SHR. SHR, spontaneously hypertensive rats; Hyd, hydrochlorothiazide-treated rats; Nif, nifedipine-treated rats; Hyd+Nif, combination-treated rats; SBP, systolic blood pressure; DBP, diastolic blood pressure; HP, heart period; SBPV, SBP variability expressed by the standard deviation of SBP; SBPV-CV, SBP variability expressed by the coefficient of variation of SBP; DBPV, DBP variability expressed by the standard deviation of DBP; DBPV-CV, DBP variability expressed by the coefficient of variation of DBP; HPV, heart period variability; BRS, baroreflex sensitivity.

(*7*, *11*). However, it remains to be established whether the combination of a diuretic and a calcium antagonist would have a synergistic effect on either BPV reduction or organ protection in hypertensive rats.

Accordingly, the present work was designed to investigate the effects of combination treatment with the diuretic hydrochlorothiazide and the calcium antagonist nifedipine on BP, BPV, and organ protection in spontaneously hypertensive rats (SHR). We also estimated the baroreflex sensitivity (BRS) of each animal because the main function of arterial baroreflex is to diminish the magnitude of BP fluctuations.

Methods

Animals and Chemicals

Eighteen-week-old male SHR were provided by the animal center of our university. The rats were housed under controlled temperature (22–23°C) and lighting (8:00–20:00 light, 20:00–8:00 dark) conditions with free access to food and tap water. All the animals used in this work received humane care in compliance with institutional animal care guidelines. The antihypertensive drugs used in this study were as follows: nifedipine (Nanjing Pharmaceutical Co., Ltd., Nanjing, China) and hydrochlorothiazide (Shanghai Xinyi Pharmaceutical Co., Ltd., Shanghai, China).

Drug Administration

Studies were performed in four groups of SHR. Hydrochlorothiazide, nifedipine or both were mixed in the rat chow. The rate of consumption of rat chow was determined before the start of the study. The content of drugs in the rat chow was calculated according to the chow consumption, such that the ingested doses of drugs would be approximately 10 mg/kg/d for both hydrochlorothiazide and nifedipine, and 10+10 mg/kg/d for the combination of the two drugs. The control SHR group received the same diet without the drugs. After 4 months of drug administration, the BP was recorded over a period of 24 h, and then BPV was calculated and BRS was determined in conscious, freely moving rats. Histopathological examinations were performed after BP recording and BRS studies.

BP Measurement

The systolic BP (SBP), diastolic BP (DBP) and heart period (HP) of rats were continuously recorded using a previously described technique (*11*, *12*). Briefly, rats were anesthetized with a combination of ketamine (40 mg/kg) and diazepam (6 mg/kg). A floating polyethylene catheter was inserted into the lower abdominal aorta *via* the left femoral artery for BP measurement, and another catheter was placed into the left femoral vein for intravenous injection. The catheters were exteriorized through the interscapular skin. After a 3-d recovery period, the animals were placed in individual cylindrical cages containing food and water for BP recording. The aortic catheter was connected to a BP transducer *via* a rotating swivel that allowed the animals to move freely in the cage. After about 14-h of habituation, the BP signal was digitized by a microcomputer. SBP, DBP and HP values from every heartbeat were determined on line. The mean values and standard deviation of these parameters over a period of 24 h were calculated. The standard deviation of the mean was calculated and defined as the quantitative parameter of BPV, *i.e.*, systolic blood pressure variability (SBPV), diastolic blood pressure variability (DBPV), and heart period variability (HPV). The coefficient of variation (CV) of BP (CV = SD × 100/mean BP value) was taken as the measure of normalized BPV, *i.e.*, CV for SBP (SBPV-CV) and CV for DBP (DBPV-CV).

BRS Measurement

To determine the function of arterial baroreflex in conscious rats, the methods widely used are derived from that of Smyth *et al*., which was first applied in humans (*13*). The principle of this method is to measure the prolongation of HP in response to an elevation of BP. Previous authors have made several modifications to render this method applicable to conscious rats (*14*, *15*). These modifications were used in the present study. A bolus injection of phenylephrine (5 μg/mL) over approximately 1 s was used to induce an elevation of BP. The dose of phenylephrine (2–5 μg/kg) was adjusted to raise the SBP between 20 and 40 mmHg. HP was plotted against SBP for linear regression analysis and the slope of SBP-HP was expressed as BRS (ms/mmHg).

Morphological Examination

The animal was weighed and killed by decapitation. The thoracic and peritoneal cavities were immediately opened. The right kidney, aorta and heart were excised and rinsed in cold physiological saline. The right kidney was blotted, and weighed. The left ventricle was isolated, blotted, and weighed. At the same time, the aorta was cleaned of adhering fat and connective tissue. Just below the branch of the left subclavicular artery, a 30-mm-long segment of thoracic aorta was harvested, blotted, and weighed. Ratios of left ventricular weight to body weight (LVW/BW) and aortic weight to the length of aorta (AW/length) were calculated (*16*, *17*). Histopathological observation was also carried out with our conventional method (*18*). Briefly, immediately after gross detection, all kidney samples were immersed in formalin solution for more than 1 week, dehydrated in ethanol, cleared in dimethylbenzene and embedded in paraffin. Then 5-μm-thick sections were prepared and stained with hematoxylin and eosin for light microscopic evaluation.

Glomerulosclerosis Score

For the semiquantitative evaluation of glomerulular damage, the glomerulosclerosis score (GSS) was defined as previously described (*19*). On the light microscopic specimens, approximately 50 glomeruli from the outer cortex and the same number of glomeruli from the inner cortex for each kidney were graded according to the degree of sclerosis: 0, if no mesangial expansion; 1, if mild mesangial expansion (less than 30% of a glomerular area); 2, if moderate mesangial expansion (30–60% of a glomerular area); 3, if marked mesangial expansion (more than 60% of a glomerular area); and 4, if the sclerosis was global. This was performed by one observer in a blind fashion using coded slides. A weighted composite sclerosis score was then calculated for each kidney according to the following formula: glomerulosclerosis score = [1 × (number of grade 1 glomeruli) + 2 × (number of grade 2 glomeruli) + 3 × (number of grade 3 glomeruli) + 4 × (number of grade 4 glomeruli)] × 100/(number of glomeruli observed).

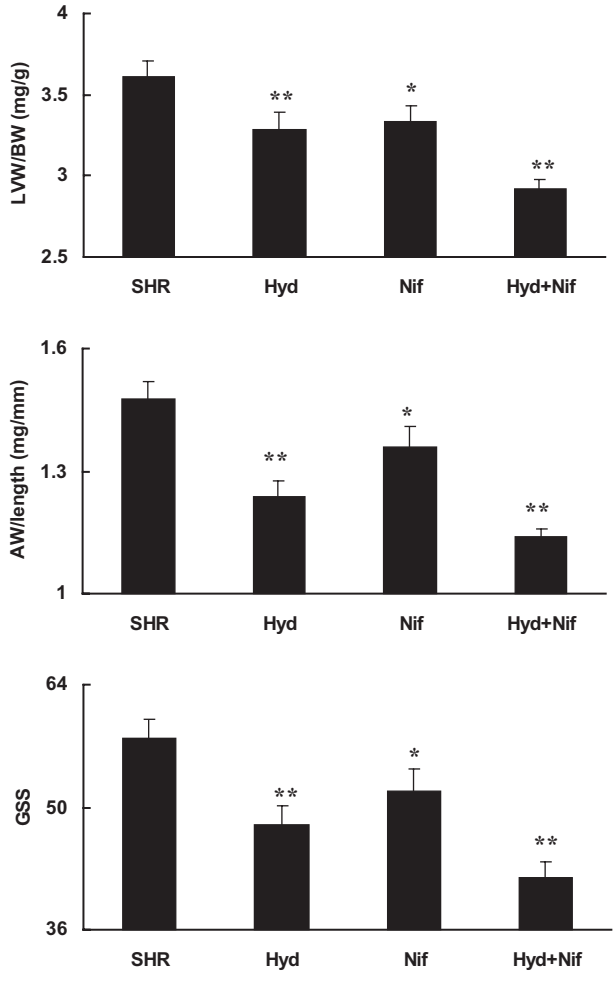

Fig. 1. *Effects of long-term treatment with hydrochlorothiazide, nifedipine, or both on pathological changes in ventricles, kidneys and aortae in spontaneously hypertensive rats. SHR, spontaneously hypertensive rats (n = 16); Hyd, hydrochlorothiazide-treated rats (n = 11); Nif, nifedipine-treated rats (n = 11); Hyd+Nif, combination-treated rats (n = 10); LVW, left ventricular weight; BW, body weight; AW, aortic weight; GSS, glomerulosclerosis score. *p < 0.05; **p < 0.01 vs. SHR.*

Probability Sum Test

To determine whether the combination was synergistic, we used the probability sum test (*q* test). This test derives from classic probability analysis, and several groups have suggested that it may be useful for evaluating the synergism of combinations of two drugs (*11*, *20–22*). In the present work, we used the following criteria. Treated rats that showed a decrease in BP (SBP or DBP) ≥ 20 mmHg compared with the mean value of the control rats were defined as responders. For

Table 2. Results of Probability Sum Test in Spontaneously Hypertensive Rats Treated with Long-Term Hydrochlorothiazide and Nifedipine

			q
SBP	Hyd	P_{Hyd}=36%	
	Nif	P_{Nif}=18%	1.04
	Hyd+Nif	$P_{Hyd+Nif}$=50%	
SBPV	Hyd	P_{Hyd}=36%	
	Nif	P_{Nif}=27%	1.68
	Hyd+Nif	$P_{Hyd+Nif}$=90%	
SBPV-CV	Hyd	P_{Hyd}=18%	
	Nif	P_{Nif}=9%	2.34
	Hyd+Nif	$P_{Hyd+Nif}$=60%	
DBP	Hyd	P_{Hyd}=0%	
	Nif	P_{Nif}=18%	0.91
	Hyd+Nif	$P_{Hyd+Nif}$=20%	
DBPV	Hyd	P_{Hyd}=36%	
	Nif	P_{Nif}=18%	1.88
	Hyd+Nif	$P_{Hyd+Nif}$=90%	
DBPV-CV	Hyd	P_{Hyd}=27%	
	Nif	P_{Nif}=9%	2.07
	Hyd+Nif	$P_{Hyd+Nif}$=70%	
BRS	Hyd	P_{Hyd}=73%	
	Nif	P_{Nif}=36%	1.21
	Hyd+Nif	$P_{Hyd+Nif}$=100%	
LVW/BW	Hyd	P_{Hyd}=18%	
	Nif	P_{Nif}=9%	2.73
	Hyd+Nif	$P_{Hyd+Nif}$=70%	
AW/length	Hyd	P_{Hyd}=36%	
	Nif	P_{Nif}=9%	2.14
	Hyd+Nif	$P_{Hyd+Nif}$=90%	
GSS	Hyd	P_{Hyd}=63%	
	Nif	P_{Nif}=33%	1.39
	Hyd+Nif	$P_{Hyd+Nif}$=100%	

See Table 1 and Fig. 1 for abbreviations. P_{Hyd}, P_{Nif}, and $P_{Hyd+Nif}$ were the percentages of animals possessing an effective decrease in hemodynamic or organ damage parameters produced by hydrochlorothiazide, nifedipine, and a combination of hydrochlorothiazide and nifedipine. $q \geq 1.15$ means synergism.

other parameters, treated rats with a decrease or increase of ≥20% compared with the mean values of the control group were defined as responders. The formula used to calculate the synergism (q) of the drug combination was as follows:

$$q = P_{A+B}/(P_A + P_B - P_A \times P_B).$$

Here, A and B indicate drug A and drug B; P (probability) is the percentage of responders in each group. P_{A+B} is the real percentage of responders and $(P_A + P_B - P_A \times P_B)$ is the expected response rate. $(P_A + P_B)$ indicates the sum of the probabilities when drug A and drug B were used alone. $(P_A \times P_B)$ is the probability of rats responding to both drugs when they were used alone, *i.e.*, assuming the two drugs act independently. When $q < 0.85$, the combination is antagonistic; when $q > 1.15$, it is synergistic; and when q is between 0.85 and 1.15, it is additive.

Table 3. Linear Regression Coefficient between BP, BPV, BRS Values and Organ Damages in Treated and Untreated Spontaneously Hypertensive Rats (n=48)

	LVW/BW	AW/length	GSS
SBP	0.467**	0.556**	0.318*
DBP	0.292*	0.354*	0.206
SBPV	0.778**	0.727**	0.578**
SBPV-CV	0.686**	0.535**	0.516**
DBPV	0.730**	0.705**	0.505**
SBPV-CV	0.599**	0.519**	0.418**
BRS	−0.644**	−0.672**	−0.671**

*$p<0.05$; **$p<0.01$. See Table 1 and Fig. 1 for abbreviations.

Statistical Analysis

Data are expressed as the means±SEM. Comparisons among groups were made by ANOVA followed by Duncan test. The relationships between hemodynamic parameters and organ damage parameters were analyzed by classic univariate correlation analysis. Stepwise multiple-regression analysis was performed to study the independent effect of hemodynamic parameters on organ damage. F to enter and F to remove were set at $p<0.05$ and $p>0.10$, respectively. Values of $p<0.05$ were considered to indicate statistical significance.

Results

Treatment Effects on BP, BPV, and BRS in SHR

As shown in Table 1, long-term hydrochlorothiazide (10 mg/kg/d), nifedipine (10 mg/kg/d) or both all significantly decreased SBP (by −7% [$p<0.01$], −7% [$p<0.01$], and −10% [$p<0.01$], respectively) and SBPV (by −15% [$p<0.01$], −13% [$p<0.05$], and −29% [$p<0.01$], respectively) and significantly enhanced BRS (by +79% [$p<0.01$], +57% [$p<0.05$], and +132% [$p<0.01$], respectively) in SHR. A significant reduction in SBPV-CV (−22% [$p<0.01$]), DBPV-CV (−21% [$p<0.01$]) and DBP (−11% [$p<0.05$]) was found in the rats receiving combination therapy, but not in those receiving monotherapy. DBPV values were markedly decreased in hydrochlorothiazide- and combination-treated rats (by −15% [$p<0.05$] and −29% [$p<0.01$], respectively), but not in nifedipine-treated rats. Moreover, combination therapy clearly had a much greater effect on BPV reduction and BRS improvement than monotherapy. No obvious change in HP and HPV was found in any treatment group.

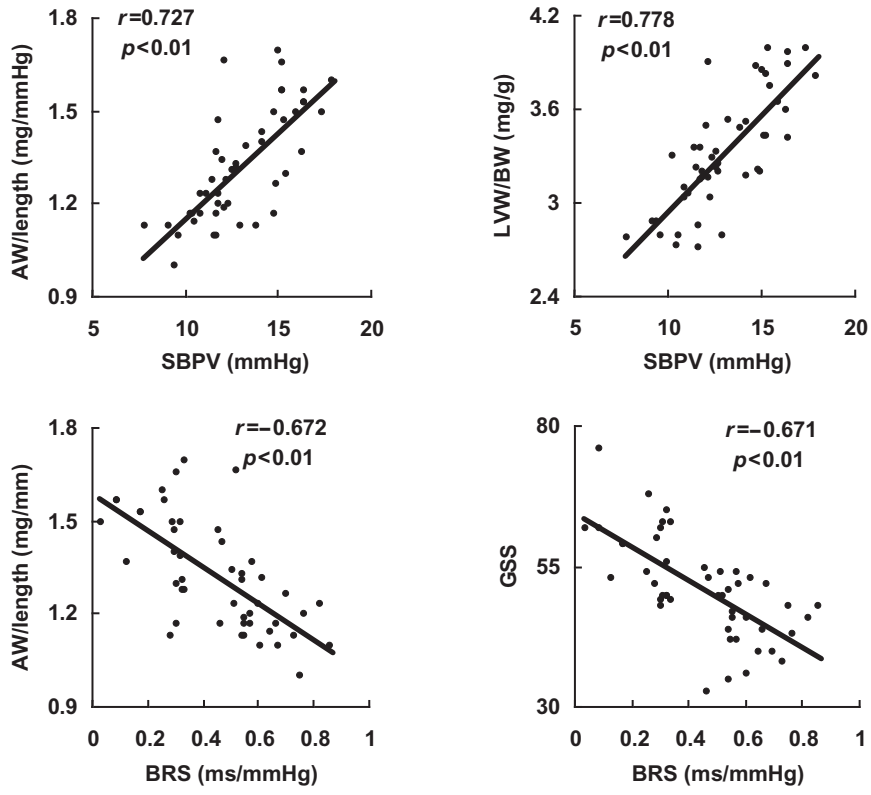

Fig. 2. *Sample correlations between hemodynamic parameters and organ-damage parameters in treated and untreated spontaneously hypertensive rats. r, linear regression coefficient between hemodynamic parameters and organ-damage parameters. n=48. See Table 1 and Fig. 1 for abbreviations.*

Treatment Effects on Organ Damages in SHR

Figure 1 shows the organ-damage parameters studied: LVW/BW (reflecting left ventricular hypertrophy), AW/length (reflecting aortic hypertrophy) and GSS (reflecting renal damage). It was found that long-term hydrochlorothiazide, nifedipine or both all significantly decreased LVW/BW (by −9% [$p<0.01$], −9% [$p<0.01$], and −19% [$p<0.01$], respectively), AW/length (by −16% [$p<0.01$], −8% ([$p<0.01$], and −23% [$p<0.01$], respectively) and GSS (by −17% [$p<0.01$], −9% [$p<0.05$], and −28% [$p<0.01$], respectively) in SHR. The combination therapy clearly had a more pronounced effect on organ protection than monotherapy.

Synergism of Hydrochlorothiazide and Nifedipine in SHR

Table 2 shows the results of the probability sum test using data from SHR treated with hydrochlorothiazide, nifedipine or both. It was found that the q values for BPV, BPV-CV, BRS and the organ-damage parameters were all larger than 1.15, whereas the q values for SBP and DBP were less than 1.15. These results imply that the combination of hydrochlorothiazide and nifedipine has a significant synergistic effect on BPV and BPV-CV reduction, BRS enhancement and organ protection in SHR, but exhibits no synergism on BP reduction.

Relationships between BP, BPV, BRS and Organ Damages in SHR

When all the SHR employed in the present study were pooled ($n=48$) for linear regression analysis, the following relationships between BP, BPV, BRS and organ damages were observed (Table 3). BP, BPV, BPV-CV and BRS were significantly correlated with all three of the pathological parameters studied, with the exception of DBP, which was not markedly related to GSS. Furthermore, compared with BP level, BPV, BPV-CV, and BRS values showed a much closer relationship with organ-damage parameters in SHR. Neither HP nor HPV was related with any organ damage parameter studied (data not shown). The most important of these correlations are shown in Fig. 2.

The relative dependencies of organ damage on hemodynamic parameters were assessed by stepwise multiple-regression analysis. When BPV was expressed by the standard deviation of BP, both LVW/BW and AW/length were independently associated with higher SBPV ($\beta=0.619$ [$p<0.01$]

and β=0.503 [p<0.01], respectively) and lower BRS (β=−0.250 [p<0.05] and β=−0.352 [p<0.01], respectively); and the glomerulosclerosis score was independently associated with lower BRS (β=−0.671 [p<0.01]). When BPV was expressed by the coefficient of variation of BP, LVW/BW was independently associated with higher SBPV-CV, SBP, and DBPV-CV (β=0.406 [p<0.01], β=0.419 [p<0.01], and β=0.296 [p<0.05], respectively); AW/length was independently associated with higher SBP and DBPV-CV (β=0.368 [p<0.01] and β=0.349 [p<0.01], respectively) and lower BRS (β=−0.388 [p<0.01]); and the glomerulosclerosis score was independently associated with lower BRS (β=−0.671 [p<0.01]).

Discussion

In the present work, long-term treatment with the combination of low-dose hydrochlorothiazide (10 mg/kg/d) and nifedipine (10 mg/kg/d) produced a mildly greater reduction in BP levels than monotherapy in SHR, whereas a significant synergism on BP reduction was not found by this combination therapy (q=1.04 and 0.91 for SBP and DBP, respectively). However, the combination therapy had a marked synergistic effect on BPV reduction (q=1.68, 1.88, 2.34 and 2.07 for SBPV, DBPV, SBPV-CV and DBPV-CV, respectively) and BRS restoration (q=1.21), and more importantly, a significant synergistic effect on organ protection (q=2.73, 2.14 and 1.39 for LVW/BW, AW/length and GSS, respectively).

Complications associated with hypertension, including stroke, heart failure and renal failure, are often lethal. End-organ damages, including cardiac hypertrophy, arteriosclerosis and renal lesions, occur during the early phase of these complications. Therefore, it is important to prevent or reduce end-organ damage in the treatment of hypertension (*7*, *8*). Based on this consideration, the synergistic effects of the hydrochlorothiazide-nifedipine combination on the organ protection may implications for the clinical treatment of hypertension, even though there is disagreement about the effectiveness of diuretic-calcium antagonist combinations on BP reduction (*3–6*).

Clinical observations have suggested that BPV is related to organ damages in hypertensive patients (*8*, *23–25*). Numerous animal studies have been carried out to investigate this relation, and have shown the following. 1) BPV is related to end-organ damage in aged SHR (*10*); 2) increased BPV alone, without hypertension, can also induce organ damage in Sprague-Dawley normotensive rats (*8*, *10*, *18*); 3) a decrease in BPV makes an important contribution to the organ protection induced by long-term treatment with nitrendipine (*26*). Accordingly, it seems very important to emphasize the role of BPV reduction in antihypertensive therapy. In the present work, compared with the BP level, BPV showed a much closer relationship with organ-damage parameters in SHR, and multiple-regression analysis showed that the decrease in LVW/BW was independently associated with the reduction in SBPV, and the decrease in AW/length was independently associated with the decrease in DBPV. Therefore, the reduction in BPV may play an important role in the organ protection conferred by drugs in SHR.

Despite these facts, only limited information is available about methods for improving BPV control in the treatment of hypertension. In the present work, chronic treatment with a combination of low-dose hydrochlorothiazide and nifedipine markedly decreased BPV in SHR, exhibiting a clear synergistic effect. These results suggested that a combination therapy might be more effective than monotherapy in controlling BPV in patients with hypertension. It has been demonstrated that the standard deviation of BP provides an index of overall BPV that is concentrated at low frequencies when studied in the frequency domain, and one major source of slow (or low frequency) hemodynamic perturbations is the myogenic response of vascular smooth muscle cells, which can be attenuated by dihydropyridine calcium channel blockers (*27*, *28*). In addition, a recent study proposed that thiazide-like diuretics could inhibit agonist-induced vasoconstriction by calcium desensitization in smooth muscle cells (*29*). Accordingly, the capacity of nifedipine (a dihydropyridine calcium channel blocker) and hydrochlorothiazide (a thiazide-like diuretic) to attenuate myogenic responses of regional circulations may contribute to their synergistic effects on BPV reduction.

Arterial baroreflex dysfunction is another feature of hypertension. It has been well recognized that BRS is impaired in hypertensive humans and animals (*30–32*). Our previous studies proposed that BRS was one of the independent variables related to end-organ damage score in hypertension, and the restoration of BRS may be a new strategy for the prevention of stroke (*33*, *34*). In the present work, compared with the BP level, BRS showed a much closer relationship with organ-damage parameters in SHR, and multiple-regression analysis showed that the decrease in any organ-damage parameter studied was independently associated with the enhancement of BRS. Accordingly, the restoration of baroreflex may be one of the major mechanisms by which these drugs confer organ protection in SHR. Although the exact mechanisms underlying such an effect are still unclear, there are two possibilities. 1) Reduction of BPV: An enhanced baroreflex function could reduce BPV, and BPV reduction could contribute to organ protection as mentioned above. 2) Inhibition of inflammation: It has been suggested that impaired baroreflex function may initiate an inflammation reaction—expressed as an increase in plasma tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β)—in sinoaortic denervated rats (*35*). The relation between inflammation and organ damage is certain.

In conclusion, long-term treatment with a combination of hydrochlorothiazide and nifedipine had a clear synergistic effect on BPV reduction, BRS restoration and organ protection in SHR. In addition to BP reduction, the decrease in BPV and the enhancement of BRS may make important contribu-

tions to this organ protection.

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