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ORIGINAL ARTICLE

Comparative effects of valsartan in combination with cilnidipine or amlodipine on cardiac remodeling and diastolic dysfunction in Dahl salt-sensitive rats

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Angiotensin receptor blockers (ARBs) are often supplemented with calcium channel blockers (CCBs) for treatment of hypertension. We recently showed that the L/N-type CCB cilnidipine has superior cardioprotective effects compared with the L-type CCB amlodipine in Dahl salt-sensitive (DS) rats. We have now compared the effects of the ARB valsartan combined with cilnidipine or amlodipine on cardiac pathophysiology in DS rats. DS rats fed a high-salt diet from 6 weeks of age were treated with vehicle, valsartan alone (10 mg kg^{-1} per day), or valsartan combined with either cilnidipine (1 mg kg^{-1} per day) or amlodipine (1 mg kg^{-1} per day) from 7 to 11 weeks. The salt-induced increase in systolic blood pressure apparent in the vehicle group was attenuated similarly in the three drug treatment groups. Valsartan–cilnidipine attenuated left ventricular (LV) fibrosis and diastolic dysfunction as well as cardiac oxidative stress and inflammation to a greater extent than did valsartan alone or valsartan–amlodipine. In addition, the increases in urinary excretion of dopamine and epinephrine as well as in cardiac renin-angiotensin-aldosterone-system (RAAS) gene expression apparent in vehicle-treated rats were attenuated to a greater extent by valsartan–cilnidipine than by the other two treatments. Valsartan–cilnidipine thus attenuated LV remodeling and diastolic dysfunction more effectively than did valsartan or valsartan–amlodipine in rats with salt-sensitive hypertension, and this superior cardioprotective action of valsartan–cilnidipine compared with valsartan–amlodipine is likely attributable, at least in part, to the greater antioxidant and antiinflammatory effects associated with both greater inhibition of cardiac RAAS gene expression and N-type calcium channel blockade.
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INTRODUCTION

Hypertension is a major risk factor for cardiovascular disease and the most common risk factor for death from any cause.[1,2] For hypertensive patients who do not respond to monotherapy, combinations of two or more antihypertensive drugs are recommended.[3] Combination therapies with an angiotensin receptor blocker (ARB) and either a thiazide diuretic or a calcium channel blocker (CCB), or both, are commonly administered to achieve strict blood pressure control and to prevent target organ damage.[4] However, the organ-protective action has been found to vary depending on CCB type.[5] Amlodipine is a dihydropyridine-based CCB and blocks L-type calcium channels that are expressed predominantly in vessels and regulate vascular tone. Cilnidipine is also a dihydropyridine-based CCB but blocks both N- and L-type calcium channels.[6,7] N-type calcium channels are expressed in, and are implicated in the release of norepinephrine from, sympathetic nerve terminals.[8]

Several actions of cilnidipine, including suppression of sympathetic nerve activity,[9,10] renal protection[11,12] and cardiovascular protection,[13,14] are thought to be mediated by its inhibitory effect on N-type calcium channels. We recently showed that cilnidipine ameliorated left ventricular (LV) concentricity, fibrosis and diastolic dysfunction to a greater extent than did amlodipine in rats with salt-sensitive hypertension.[15] The Cilnidipine vs. Amlodipine Randomised Trial for Evaluation in Renal Disease study also revealed that cilnidipine is superior to amlodipine in preventing the progression of proteinuria in hypertensive patients also treated with an inhibitor of the renin-angiotensin-aldosterone system (RAAS).[16] The mechanism underlying the superior cardioprotective action of an N/L-type CCB added to an ARB has remained unclear, however.

We have now investigated the effects of the combination of cilnidipine and the ARB valsartan, in comparison with amlodipine

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plus valsartan as well as with valsartan alone, on cardiac pathophysiology in rats with salt-sensitive hypertension.

MATERIALS AND METHODS

Animals and experimental protocols

Five-week-old male inbred Dahl salt-sensitive (DS) rats were obtained from Japan SLC (Hamamatsu, Japan), were handled in accordance with the guidelines of Nagoya University Graduate School of Medicine as well as with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996), and were fed laboratory chow containing 0.36% NaCl until 6 weeks of age. Rats fed an 8% NaCl diet after 6 weeks of age were treated with valsartan alone (10 mg kg^{-1}; Livzon Group Changzhou Kony Pharmaceutical, Changzhou, China) (Val group, $n=11$), valsartan (10 mg kg^{-1}) plus amlodipine (1 mg kg^{-1}; Lek, Kolodvorska, Slovenia) (VAml group, $n=11$), valsartan (10 mg kg^{-1}) plus cilnidipine (1 mg kg^{-1}; Ajinomoto, Tokyo, Japan) (VCil group, $n=11$) or vehicle (LVH group, $n=6$) from 7 weeks of age. The doses of valsartan, amlodipine and cilnidipine were determined on the basis of the results of a previous study.[17] The rats treated with vehicle gradually developed pronounced hypertension and manifested hypertension-induced compensated LV hypertrophy (LVH) at 11 weeks of age. DS rats maintained on a 0.36% NaCl diet after 6 weeks of age remain normotensive, and such animals served as age-matched controls (CONT group, $n=6$). Each diet and tap water were provided ad libitum throughout the experimental period. At 11 weeks of age, all rats were anesthetized by intraperitoneal injection of ketamine (50 mg kg^{-1}) and xylazine (10 mg kg^{-1}) and were subjected to echocardiographic and hemodynamic analyses. The animals were then killed, the heart was excised and LV tissue was separated for analysis.

Blood pressure measurement, echocardiography and hemodynamics

Systolic blood pressure (SBP) was measured weekly in conscious animals by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan). At 11 weeks of age, rats were subjected to transthoracic echocardiography as previously described.[15] In brief, M-mode echocardiography was performed with a 12.5-MHz transducer (Xario SSA-660A; Toshiba Medical Systems, Tochigi, Japan). In addition, the pulsed Doppler echocardiographic data were analyzed for assessment of LV diastolic function.[15] After echocardiography, a 2F micromanometer-tipped catheter (SPR-320; Millar Instruments, Houston, TX, USA) that had been calibrated relative to atmospheric pressure was inserted through the right carotid artery into the left ventricle.[18] Tracings of LV pressure and the electrocardiogram were digitized to determine LV end-diastolic pressure (LVEDP). The time constant of isovolumic relaxation (tau) was calculated by the derivative method of Raff and Glantz, as described previously.[19]

Histology and immunohistochemistry

LV tissue was fixed with ice-cold 4% paraformaldehyde for 48 h, embedded in paraffin, and processed for histology as described previously.[15] Transverse sections were stained either with hematoxylin-eosin for routine histological examination or with Azan-Mallory solution for evaluation of fibrosis. To evaluate macrophage infiltration into the myocardium, we performed immunostaining for the monocyte-macrophage marker CD68 with the paraffin-embedded sections.[20] All image analysis was performed with NIH Scion Image software (Scion, Frederick, MD, USA).

Measurement of biochemical parameters

A blood sample was collected from the right carotid artery of rats deprived of food overnight under anesthesia by intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}) and was centrifuged at 1400 g for 10 min at room temperature. The resultant serum or plasma supernatants were stored at $-80\,^\circ$C until analysis. Plasma renin activity was determined by radioimmunoassay with the use of renin RIA beads (Abbott Japan, Tokyo, Japan). Plasma angiotensin II concentration was determined by radioimmunoassay as described previously,[21] and serum aldosterone concentration was measured by radioimmunoassay with the use of a DPC aldosterone kit (Mitsubishi Chemical Medience, Tokyo, Japan). Urinary catecholamine concentrations

were measured by high-performance liquid chromatography, and urinary catecholamine excretion over 24 h was calculated as described previously.[22]

Assay of superoxide production

Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent superoxide production by homogenates of freshly frozen LV tissue was measured with an assay based on lucigenin-enhanced chemiluminescence as described previously.[15] The average of dihydroethidium fluorescence intensity values was calculated with the use of NIH Image software (ImageJ).[23]

Quantitative RT-PCR analysis

Total RNA was extracted from LV tissue and treated with deoxyribonuclease with the use of a spin-vacuum total RNA isolation kit (Promega, Madison, WI, USA). Portions of the RNA (2 µg) were subjected to reverse transcription (RT) with the use of a PrimerScript RT Reagent Kit (Takara, Shiga, Japan). Quantitative PCR analysis was performed with the use of SYBR Mix Ex Taq II (Takara), a Thermal Cycler Dice Real Time System II (Takara) and specific primers for cDNAs encoding atrial natriuretic peptide,[20] brain natriuretic peptide,[20] collagen type I or III,[20] transforming growth factor (TGF)-β1,[20] connective tissue growth factor,[20] monocyte chemoattractant protein (MCP)-1,[20] osteopontin,[15] angiotensin-converting enzyme,[20] the AT_{1A} receptor (type 1A receptor for angiotensin II),[20] the mineralocorticoid receptor,[20] serum/glucocorticoid-regulated kinase 1 (Sgk1),[20] or the p22^{phox} or gp91^{phox} subunits of NADPH oxidase.[20] Reagents for detection of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Applied Biosystems, Foster City, CA, USA) were used to quantify rat GAPDH mRNA as an internal standard.

Statistical analysis

Data are presented as means ± s.e.m. Differences among groups of rats at 11 weeks of age were assessed by one-way factorial analysis of variance; if a significant difference was detected, intergroup comparisons were performed with Fisher's multiple-comparison test. The time course of SBP was compared among groups by two-way repeated-measures analysis of variance. A P-value of <0.05 was considered as statistically significant.

RESULTS

LV geometry and function

SBP was similar in all groups of rats at 6 weeks of age, but it was significantly higher in the LVH group than in the CONT group at 7 weeks of age and thereafter (Figure 1; Table 1). At 9 weeks and thereafter, SBP was significantly reduced in the Val, VAml and VCil groups compared with the LVH group. Although the overall time course of SBP did not differ among the three treatment groups, SBP was significantly lower in the VAml and VCil groups than in the Val group at 11 weeks. The ratios of heart or LV weight to tibial length, indices of cardiac and LVH, respectively, were significantly increased in the LVH group compared with the CONT group at 11 weeks, and these effects were attenuated in all treatment groups (Table 1).

Echocardiography revealed that both LV end-diastolic dimension (LVDd) and LV end-systolic dimension were decreased, and that interventricular septum thickness, LV posterior wall thickness, LV fractional shortening, LV ejection fraction, LV mass and relative wall thickness (RWT) were increased in the LVH group compared with the CONT group (Table 2). All of these changes were attenuated in all treatment groups, and that in RWT was inhibited to a greater extent in the VCil group than in the Val and VAml groups. The isovolumic relaxation time, deceleration time and tau, all of which are indices of LV relaxation, as well as the ratio of LVEDP to LVDd, an index of LV diastolic stiffness, were increased in the LVH group compared with the CONT group. All of these effects were attenuated to a greater extent in the VCil group than in the Val or VAml groups.

Cardiomyocyte hypertrophy and cardiac fibrosis

The cross-sectional area of LV cardiomyocytes was increased in the LVH group compared with the CONT group, and this increase was attenuated to a greater extent in the VAml and VCil groups than in the Val group (Figures 2a and b). Hemodynamic overload also resulted in marked upregulation of atrial natriuretic peptide and brain natriuretic peptide gene expression in the left ventricle, and the increased expression of these fetal-type cardiac genes was similarly attenuated in both VAml and VCil groups (Figures 2c and d).

Azan-Mallory staining revealed that fibrosis in the perivascular and interstitial regions of the LV myocardium was increased in the LVH group compared with the CONT group, and these increases were partially attenuated in the Val and VAml groups and completely inhibited in the VCil group (Figures 3a–c). The abundance of collagen types I and III mRNAs was increased in the left ventricle of rats in the LVH group, and these effects were attenuated to a greater extent in the VCil group than in the Val or VAml groups (Figures 3d and e). The amounts of TGF-β1 and connective tissue growth factor mRNAs in the left ventricle were also significantly increased in the LVH group compared with the CONT group, and these effects were attenuated in the VAml group and to a greater extent in the VCil group (Figures 3f and g).
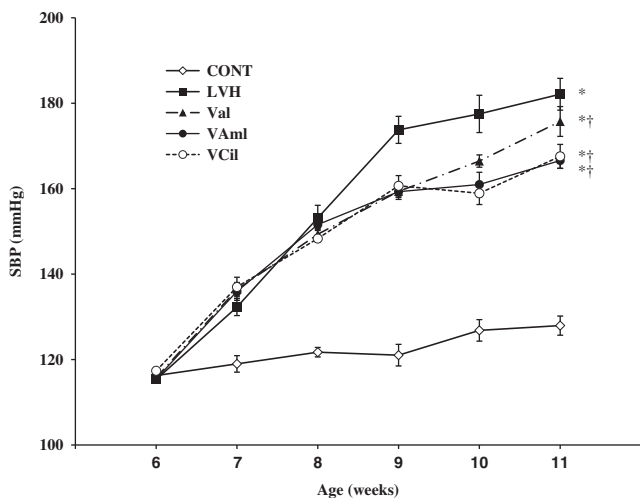

Figure 1 Time course of SBP in rats of the five experimental groups. Data are means ± s.e.m. ($n = 6$, 6, 11, 11 and 11 rats for the CONT, LVH, Val, VAml and VCil groups, respectively). *$P < 0.05$ vs. CONT group; †$P < 0.05$ vs. LVH group.

Cardiac oxidative stress

Both superoxide production in myocardial tissue sections as revealed by staining with dihydroethidium as well as the activity of NADPH oxidase in LV homogenates were markedly increased in the LVH group compared with the CONT group, and these effects were inhibited to a greater extent in the VCil group than in the Val and VAml groups (Figures 4a–c). The expression of genes for the p22phox and gp91phox membrane components of NADPH oxidase was also upregulated in the left ventricle of rats in the LVH group, and these effects were inhibited to a greater extent in the VCil group than in the Val or VAml groups (Figures 4d and e).

Cardiac inflammation

Immunostaining for the monocyte-macrophage marker CD68 revealed that macrophage infiltration in the LV myocardium was increased in the LVH group compared with the CONT group and that this effect of salt loading was attenuated to a greater extent in the VCil group than in the Val and VAml groups (Figures 5a and b). The expression of MCP-1 and osteopontin genes in the left ventricle was also increased in the LVH group, and these effects were inhibited to a greater extent in the VCil group than in the Val or VAml groups (Figures 5c and d).

RAAS and sympathetic activity

Salt loading reduced plasma renin activity as well as plasma angiotensin II and serum aldosterone concentrations, and these effects were not affected by any of the antihypertensive drugs (Table 1). Expression of the angiotensin-converting enzyme gene was significantly upregulated in the left ventricle of rats in the LVH group compared with the CONT group, and this effect was inhibited to a greater extent in the VCil group than in the Val and VAml groups (Figure 6a). Expression of the AT_{1A} receptor gene was similar in CONT and LVH groups and was inhibited to a greater extent in the VCil group than in the Val and VAml groups (Figure 6b). The abundance of mineralocorticoid receptor mRNA was significantly increased in the LVH group compared with the CONT group, and this effect was attenuated in the Val and VAml groups and to a greater extent in the VCil group (Figure 6c). The upregulation of Sgk1 gene expression apparent in the LVH group was attenuated in the VCil group but not in the Val or VAml groups (Figure 6d).

Urinary excretion of norepinephrine, dopamine and epinephrine was markedly increased in the LVH group compared with the CONT group (Figures 6e–g). Whereas the increase in urinary excretion of norepinephrine was similarly attenuated in all treatment groups, given

Table 1 Anatomic, metabolic and hormonal parameters in rats of the five experimental groups at 11 weeks of age

Parameter	CONT	LVH	Val	VAml	VCil
Body weight (g)	344.7 ± 4.9	342.0 ± 7.5	338.6 ± 6.3	325.9 ± 3.8a	333.7 ± 3.9
SBP (mm Hg)	128.0 ± 1.7	182.1 ± 3.6a	175.7 ± 2.5a	166.6 ± 3.0a,b,c	167.6 ± 1.7a,b,c
Heart rate (beats min⁻¹)	387.9 ± 6.9	403.6 ± 11.4	396.1 ± 8.1	387.4 ± 2.6	410.6 ± 13.1
Heart weight/tibial length (mg mm⁻¹)	27.73 ± 0.75	38.14 ± 0.63a	33.31 ± 0.95a,b	32.08 ± 0.62a,b	32.37 ± 0.76a,b
LV weight/tibial length (mg mm⁻¹)	20.76 ± 0.58	28.69 ± 0.50a	25.51 ± 0.56a,b	24.61 ± 0.45a,b	24.78 ± 0.55a,b
Plasma renin activity (ng ml⁻¹ h⁻¹)	5.76 ± 0.38	0.30 ± 0.18a	0.52 ± 0.12a	0.52 ± 0.13a	0.46 ± 0.12a
Plasma angiotensin II (pg ml⁻¹)	134.0 ± 10.3	15.2 ± 2.0a	20.0 ± 2.2a	20.3 ± 2.4a	20.8 ± 2.9a
Serum aldosterone (pg ml⁻¹)	404.8 ± 53.5	239.8 ± 13.5a	207.7 ± 11.1a	229.3 ± 26.5a	229.3 ± 7.77a

Abbreviations: LV, left ventricular; LVH, LV hypertrophy; SBP, systolic blood pressure. Data are means ± s.e.m. ($n = 6$, 6, 11, 11 and 11 rats for the CONT, LVH, Val, VAml and VCil groups, respectively).
a$P < 0.05$ vs. CONT group.
b$P < 0.05$ vs. LVH group.
c$P < 0.05$ vs. Val group.

Table 2 Cardiac morphological and functional parameters in rats of the five experimental groups at 11 weeks of age

Parameter	CONT	LVH	Val	VAml	VCil
IVST (mm)	1.67 ± 0.05	2.53 ± 0.08[a]	2.05 ± 0.02[a,b]	2.05 ± 0.02[a,b]	2.01 ± 0.02[a,b]
LVDd (mm)	7.96 ± 0.10	6.26 ± 0.22[a]	7.33 ± 0.08[a,b]	7.17 ± 0.10[a,b]	7.27 ± 0.25[a,b]
LVPWT (mm)	1.65 ± 0.04	2.52 ± 0.09[a]	2.02 ± 0.05[a,b]	2.04 ± 0.02[a,b]	1.98 ± 0.08[a,b]
LVDs (mm)	4.28 ± 0.33	2.30 ± 0.44[a]	3.25 ± 0.19[a,b]	3.18 ± 0.19[a,b]	3.32 ± 0.30[a,b]
LVFS (%)	45.05 ± 2.90	70.03 ± 3.06[a]	55.24 ± 1.83[a,b]	55.97 ± 6.33[a,b]	56.38 ± 2.68[a,b]
LVEF (%)	82.6 ± 2.2	96.7 ± 0.9[a]	90.9 ± 1.0[a,b]	91.7 ± 1.4[a,b]	92.0 ± 1.6[a,b]
LV mass (mg)	920 ± 29	1333 ± 49[a]	1155 ± 38[a,b]	1079 ± 26[a,b]	1091 ± 56[a,b]
RWT	0.41 ± 0.01	0.79 ± 0.06[a]	0.62 ± 0.01[a,b]	0.60 ± 0.02[a,b]	0.50 ± 0.02[a,b,c,d]
IRT (ms)	33.8 ± 0.9	47.8 ± 0.5[a]	41.0 ± 2.0[a,b]	38.4 ± 1.4[b]	35.4 ± 1.6[b,c]
DcT (ms)	46.7 ± 1.0	65.5 ± 1.4[a]	56.9 ± 1.4[a,b]	55.0 ± 2.5[a,b]	49.3 ± 1.4[b,c,d]
Tei index	0.48 ± 0.01	0.66 ± 0.05[a]	0.59 ± 0.02	0.55 ± 0.01[b]	0.49 ± 0.02[b,c]
tau (ms)	22.1 ± 1.5	38.75 ± 2.3[a]	32.5 ± 4.8[a]	29.07 ± 1.5[a]	24.4 ± 0.8[b,c]
LVEDP (mm Hg)	4.2 ± 0.2	8.0 ± 0.3[a]	8.0 ± 0.3[a]	7.0 ± 1.0[a]	5.7 ± 0.9[b,c]
LVEDP/LVDd (mm Hg per mm)	0.48 ± 0.02	0.99 ± 0.01[a]	1.05 ± 0.03[a]	0.93 ± 0.11[a]	0.71 ± 0.12[b,c]

Abbreviations: DcT, deceleration time; IVST, interventricular septum thickness; LV, left ventricular; LVDd, LV end-diastolic dimension; LVDs, LV end-systolic dimension; LVEDP, LV end-diastolic pressure; LVFS, LV fractional shortening; LVEF, LV ejection fraction; LVPWT, LV posterior wall thickness; IRT, isovolumic relaxation time; RWT, relative wall thickness; tau, time constant of isovolumic relaxation. Data are means ± s.e.m. ($n = 6, 6, 11, 11$ and 11 for the CONT, LVH, Val, VAml and VCil groups, respectively.
[a]$P < 0.05$ vs. CONT group.
[b]$P < 0.05$ vs. LVH group.
[c]$P < 0.05$ vs. Val group.
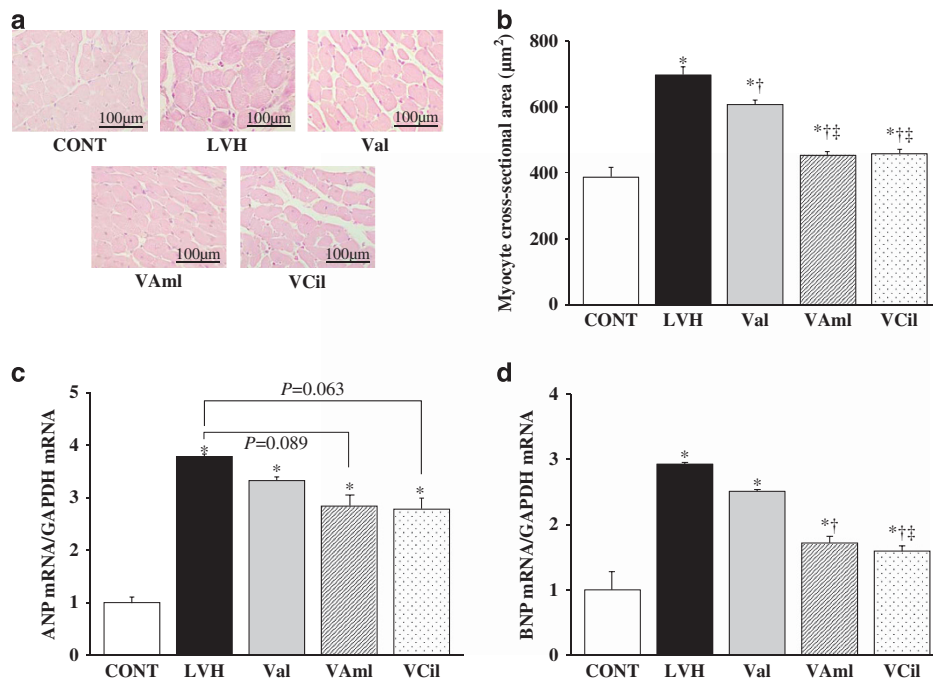[d]$P < 0.05$ vs. VAml group.

Figure 2 Myocyte cross-sectional area and expression of fetal-type cardiac genes in the left ventricle of rats in the five experimental groups at 11 weeks of age. (**a**) Hematoxylin-eosin staining of transverse sections of the LV myocardium. Scale bars, 100 μm. (**b**) Cross-sectional area of cardiac myocytes determined from sections similar to those in (**a**). (**c**, **d**) Quantitative RT-PCR analysis of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) mRNAs, respectively. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the mean value for the CONT group. Data in (**b**) through (**d**) are means ± s.e.m. ($n = 6, 6, 11, 11$ and 11 rats for the CONT, LVH, Val, VAml and VCil groups, respectively). *$P < 0.05$ vs. CONT group; [†]$P < 0.05$ vs. LVH group; [‡]$P < 0.05$ vs. Val group.

that in dopamine or epinephrine excretion was attenuated to a greater extent in the VCil group than in the Val and VAml groups.

DISCUSSION

We have shown here that valsartan monotherapy as well as combination therapy with valsartan and either amlodipine or cilnidipine similarly attenuated hypertension and LVH in DS hypertensive rats, whereas the combination of valsartan and cilnidipine ameliorated LV concentricity, fibrosis and diastolic dysfunction to a greater extent than did valsartan alone or the combination of valsartan and amlodipine. The superior cardioprotective effects of the combination of valsartan and cilnidipine were associated with more effective inhibition of cardiac oxidative stress, inflammation and RAAS gene expression. The sympatholytic action of cilnidipine, as revealed by its
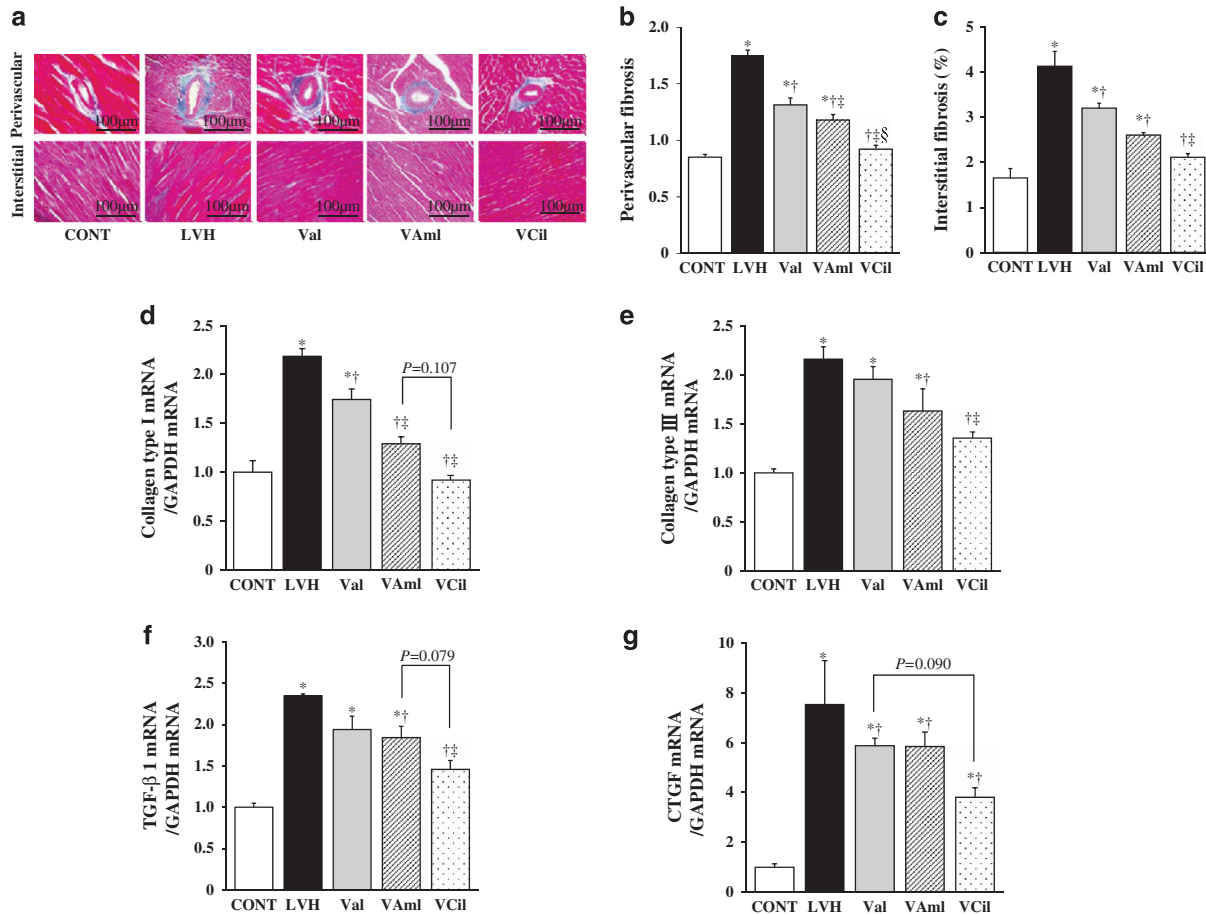
Figure 3 Cardiac fibrosis in the left ventricle of rats in the five experimental groups at 11 weeks of age. (**a**) Collagen deposition as revealed by Azan-Mallory staining in perivascular or interstitial regions of the myocardium. Scale bars, 100 μm. (**b**, **c**) Relative extents of perivascular and interstitial fibrosis, respectively, in the LV myocardium as determined from sections similar to those in (**a**). (**d–g**) Quantitative RT-PCR analysis of collagen type I, collagen type III, TGF-β1 and connective tissue growth factor (CTGF) mRNAs, respectively. The abundance of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the mean value for the CONT group. Data in (**b**) through (**g**) are means ± s.e.m. ($n = 6, 6, 11, 11$ and 11 rats for the CONT, LVH, Val, VAml and VCil groups, respectively). *$P < 0.05$ *vs.* CONT group; [†]$P < 0.05$ *vs.* LVH group; [‡]$P < 0.05$ *vs.* Val group; [§]$P < 0.05$ *vs.* VAml group.

effect on urinary catecholamine (dopamine and epinephrine) excretion, might also have contributed to the more effective amelioration of cardiac remodeling and diastolic dysfunction by the combination of valsartan and cilnidipine in comparison with the other drug treatment groups.

All of the treatment groups manifested attenuation of cardiac hypertrophy, which did not differ significantly between the Val group and the combination therapy groups. This latter finding might be due to the fact that the combination therapies reduced SBP to a greater extent than did valsartan alone only at 11 weeks of age. In a previous study of the same animal model with equivalent doses of drugs and similar experimental protocols, both the Val group and the combination therapy groups manifested significant attenuation of the increase in SBP within 1 week of treatment onset.[17] Although the reason for this discrepancy remains unclear, it is possible that differences in the time to measure blood pressure or breeding conditions may have affected the time course of SBP.

Sympathetic hyperactivity results in activation of the RAAS[24,25] and is considered as a hallmark of hypertensive cardiovascular disease morbidity and mortality,[26] given that chronic activation of the sympathetic nervous system has been shown to have adverse effects on the myocardium and peripheral circulation. These effects are

thought to contribute to cardiac and vascular structural alterations that may advance disease progression.[27] Many studies have shown that cilnidipine suppresses sympathetic nerve activity and the RAAS,[28,29] likely as a result of its inhibition of N-type calcium channels. In the present study, urinary norepinephrine excretion was suppressed to a similar extent in all three treatment groups. However, the increases in the urinary excretion of dopamine and epinephrine apparent in the LVH group were suppressed to a greater extent in the VCil group than in the Val and VAml groups, suggesting that valsartan suppressed sympathetic hyperactivity but that cilnidipine also contributed to such suppression. The increase in RWT was attenuated to a greater extent in the VCil group than in the Val and VAml groups. Furthermore, although the suppression of the systemic RAAS by salt loading was not affected by any of the antihypertensive drug treatments, the expression levels of RAAS-related genes in the heart tended to be lower in the VCil group than in the Val and VAml groups, suggesting that the cardiac RAAS may have been inhibited to a greater extent by the combination of valsartan and cilnidipine. It is thus possible that LVH *per se* is primarily load dependent,[30] whereas LV concentricity is affected by cardiac sympathetic activity and the RAAS as well as by load.[31]
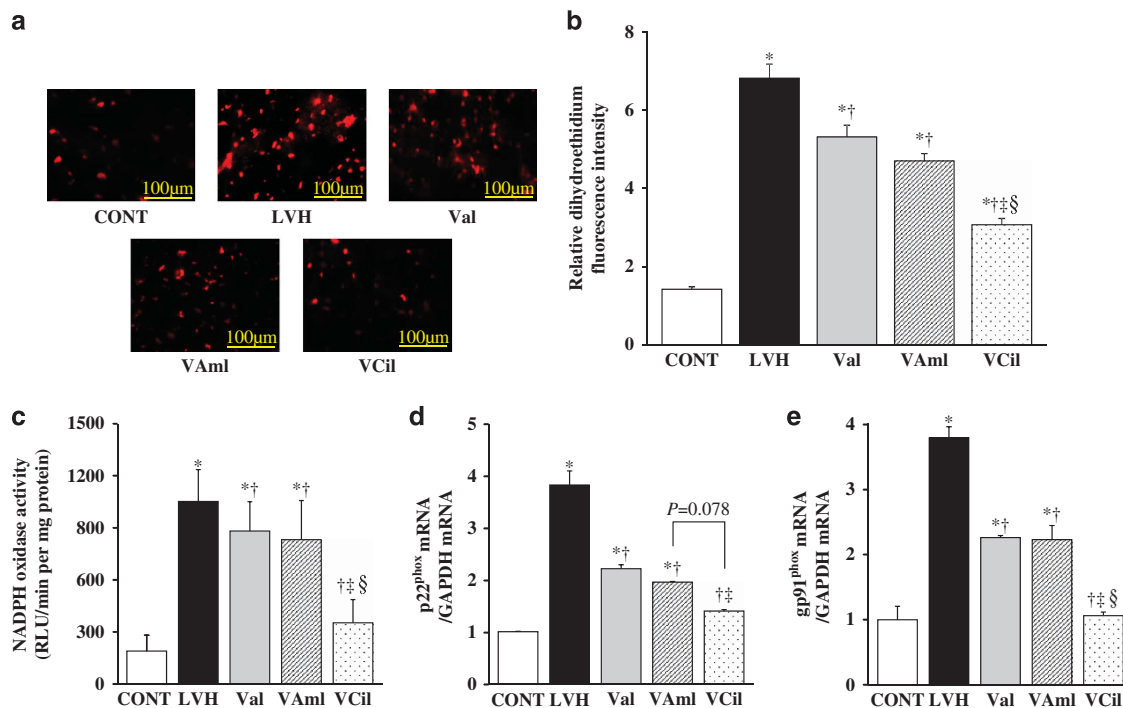
Figure 4 Superoxide production as well as NADPH oxidase activity and gene expression in the left ventricle of rats in the five experimental groups at 11 weeks of age. (**a**) Superoxide production as revealed by dihydroethidium staining in sections of the LV myocardium. Scale bars, 100 μm. (**b**) Quantitation of dihydroethidium fluorescence intensity in sections similar to those in (**a**). (**c**) NADPH-dependent superoxide production in LV homogenates. Results are expressed as relative light units (RLUs) per minute per milligram of protein. (**d**, **e**) Quantitative RT-PCR analysis of p22^{phox} and gp91^{phox} mRNAs, respectively. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the mean value for the CONT group. Data in (**b**) through (**e**) are means ± s.e.m. ($n = 6$, 6, 11, 11 and 11 rats for the CONT, LVH, Val, VAml and VCil groups, respectively). *$P < 0.05$ vs. CONT group; †$P < 0.05$ vs. LVH group; ‡$P < 0.05$ vs. Val group; §$P < 0.05$ vs. VAml group.

Although we did not investigate cardiac sympathetic nerve activity, ^{123}I-metaiodobenzylguanidine cardiac imaging previously suggested that cilnidipine suppresses cardiac sympathetic overactivity in individuals with hypertension, whereas amlodipine is largely without such an effect.[32] These findings suggest that the differential effects of cilnidipine and amlodipine on cardiac sympathetic nerve activity are related to the difference in their cardioprotective effects. Our results indicate that, when combined with valsartan, cilnidipine also may suppress cardiac RAAS activity and exhibit synergistic or additive cardioprotective effects as a result of its inhibition of sympathetic neurotransmitter release mediated by N-type calcium channel blockade.

Excess reactive oxygen species may also contribute to impairment of LV diastolic function through inhibition of Ca^{2+}-handling proteins.[33] Dihydropyridine derivatives act as lipophilic chain-breaking antioxidants. However, these drugs exhibit large differences in their lipophilicity, with cilnidipine being more lipophilic than amlodipine.[34] Our present findings regarding cardiac oxidative stress are consistent with previous results showing that expression of the p22^{phox} gene is regulated in a redox-sensitive manner in endothelial cells.[35] The superior cardioprotective effects of valsartan plus cilnidipine in comparison with valsartan either alone or together with amlodipine may thus be attributable, at least in part, to the greater inhibition of cardiac oxidative stress by the former treatment. Loss of redox homeostasis results in activation of proinflammatory and profibrotic pathways in the heart, which in turn leads to diastolic dysfunction, likely as a result of increased LV diastolic stiffness. Collagen type I is thought to be a major determinant of myocardial stiffness.[36] We have now shown that the upregulation of both collagen type I and type III genes apparent in the LVH group were attenuated to a greater extent in the VCil group than in the Val and VAml groups. Together, these data are consistent with previous results showing that the synthesis of collagen type I in cardiac fibroblasts is inhibited by antioxidants.[37]

Macrophages have been implicated in fibrosis associated with various pathological conditions. LV fibrosis, macrophage infiltration into the interstitial space of the myocardium and cardiac expression of the MCP-1 and osteopontin genes were attenuated to a greater extent in the VCil group than in the Val and VAml groups despite the similar lowering of SBP apparent in all three groups. The greater anti-inflammatory and antifibrotic effects in the myocardium of the combination of valsartan plus cilnidipine are therefore likely, at least in part, independent of its antihypertensive effect. The upregulation of TGF-β1 and connective tissue growth factor gene expression in the heart was also attenuated to a greater extent in the VCil group than in the Val and VAml groups. TGF-β-mediated diastolic dysfunction and fibrosis in pressure-overloaded hearts were previously associated with MCP-1-dependent macrophage accumulation, and these processes were suggested to be related to the RAAS.[38,39]

Given that cilnidipine exerts greater renoprotective effects than amlodipine when administered together with valsartan,[17] combination therapy with cilnidipine and valsartan is a potential approach to the treatment of hypertensive patients. Several dihydropyridine CCBs have been found to possess mineralocorticoid receptor antagonist activity.[40] Further studies are therefore warranted to elucidate the mechanisms underlying the superior effects of combination therapy with cilnidipine and valsartan.
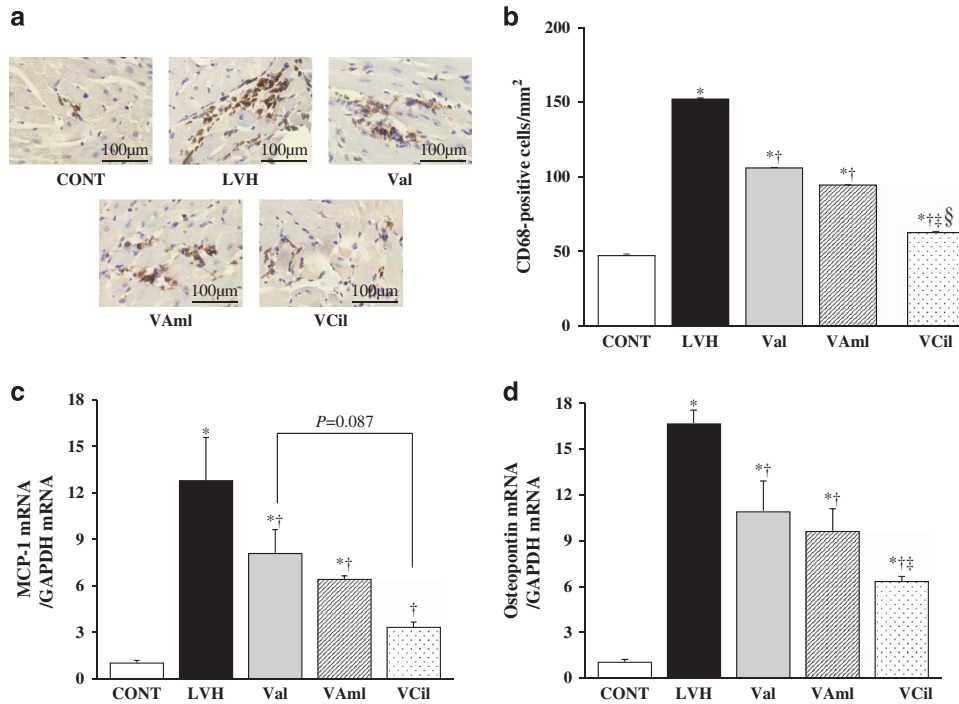
Figure 5 Macrophage infiltration as well as expression of MCP-1 and osteopontin genes in the left ventricle of rats in the five experimental groups at 11 weeks of age. (**a**) Immunohistochemical staining for the monocyte-macrophage marker CD68. Scale bars, 100 μm. (**b**) Density of CD68-positive cells in the LV myocardium as determined from sections similar to those in (**a**). (**c**, **d**) Quantitative RT-PCR analysis of MCP-1 and osteopontin mRNAs, respectively. The amount of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the mean value for the CONT group. Data in (**b**) through (**d**) are means ± s.e.m. ($n = 6, 6, 11, 11$ and 11 rats for the CONT, LVH, Val, VAml and VCil groups, respectively). $*P < 0.05$ vs. CONT group; $^{†}P < 0.05$ vs. LVH group; $^{‡}P < 0.05$ vs. Val group; $^{§}P < 0.05$ vs. VAml group.
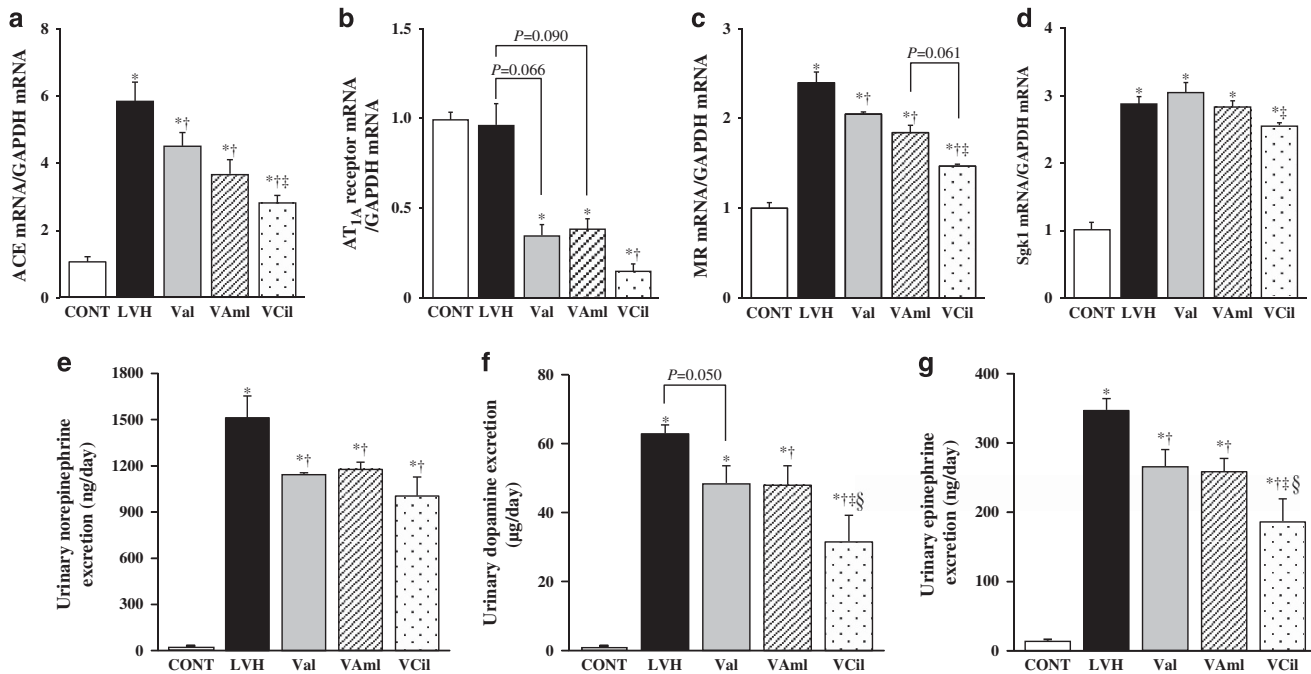

Figure 6 Expression of cardiac RAAS-related genes in the left ventricle as well as urinary excretion of catecholamines in rats of the five experimental groups at 11 weeks of age. (**a–d**) Quantitative RT-PCR analysis of angiotensin-converting enzyme (ACE), AT_{1A} receptor, mineralocorticoid receptor (MR), and Sgk1 mRNAs, respectively. The abundance of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the mean value for the CONT group. (**e–g**) Urinary excretion of norepinephrine, dopamine and epinephrine, respectively. All data are means ± s.e.m. ($n = 6, 6, 11, 11$ and 11 rats for the CONT, LVH, Val, VAml and VCil groups, respectively). $*P < 0.05$ vs. CONT group; $^{†}P < 0.05$ vs. LVH group; $^{‡}P < 0.05$ vs. Val group; $^{§}P < 0.05$ vs. VAml group.

With regard to limitations of the present study, we collected a blood sample for measurements of renin activity and angiotensin II concentration in plasma under anesthesia by intraperitoneal injection of sodium pentobarbital because of our experiment protocols for multiple purposes. It is recommended to collect blood promptly by killing animals with rapid decapitation (unanesthetized) to prepare high quality blood samples, because anesthesia can influence sympathetic nerve activity to alter renin release from the kidney, leading to changes in plasma angiotensin II levels. Furthermore, plasma-derived or locally produced renin could be activated during the process for sample preparation, therefore increasing *in vitro* production of angiotensin II.

CONCLUSIONS

We have shown that valsartan monotherapy as well as combination therapy with valsartan and either amlodipine or cilnidipine similarly attenuated hypertension and LVH in DS hypertensive rats, and that the combination of valsartan and cilnidipine ameliorated LV concentricity, fibrosis and diastolic dysfunction to a greater extent than did the other two treatments. The superior cardioprotective action of valsartan plus cilnidipine compared with valsartan plus amlodipine is likely attributable, at least in part, to the greater antioxidant and anti-inflammatory effects associated with both greater inhibition of cardiac RAAS gene expression and N-type calcium channel blockade.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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