JPP Journal of Pharmacy and Pharmacology

Royal Pharmaceutical Society of Great Britain

JPP Journal of Pharmacy and Pharmacology

JPP 2010, 62: 1740–1745

Received September 26, 2009
Accepted June 29, 2010
DOI
10.1111/j.2042-7158.2010.01164.x
ISSN 0022-3573

Research Paper

Amlodipine suppressed cardiac gene expression of brain natriuretic peptide, transforming growth factor-β_1 and fibronectin mediated by aldosterone in male stroke-prone spontaneously hypertensive rats

Shinsuke Harasawa[a], Yuji Otsuka[a], Katsuaki Okubo[a], Miyuki Koike[a], Hirotaka Fujita[a], Toshio Kushiro[a], Ken Nagao[a] and Atsushi Hirayama[b]

[a]Department of Cardiology, Nihon University Surugadai Hospital and [b]Division of Cardiology, Department of Medicine, Nihon University School of medicine, Tokyo, Japan

Abstract

Objectives Amlodipine, a calcium channel blocker (CCB), is one of the most common antihypertensive medicines in Japan. We evaluated whether the calcium channel blocker confers cardiac protection through the renin–angiotensin–aldosterone system in male stroke-prone spontaneously hypertensive rats (SHR-SP).

Methods Fifteen week-old rats were divided into 2 groups: amlodipine group (3 mg/kg/day, $n = 5$) and control group ($n = 5$).

Key findings The CCB lowered systolic blood pressure significantly ($P < 0.05$). Plasma aldosterone concentration in the amlodipine group was remarkably lower than in the control group ($P < 0.05$), but plasma renin activity and plasma angiotensin II concentration were not different between the two groups. The CCB also suppressed the mRNA expression of brain natriuretic peptide, transforminggrowth factor-β_1, and fibronectin extracted from the left ventricle.

Conclusions These results suggest that amlodipine attenuates cardiac damage by lowering plasma aldosterone concentration in hypertensive rats with developing arteriosclerosis.

Keywords aldosterone; amlodipine; cardiac gene expression; fibronectin; SHR-SP

Introduction

In recent years interest has focused on the importance of the cardio-renal continuum. Antihypertensive agents not only lower blood pressure, but have also come to be used on the basis of their protection of the brain, heart and kidney. The renin–angiotensin–aldosterone system (RAAS) plays an indispensable role in this mechanism. Aldosterone is a unique hormone secreted by the adrenal cortex. It works primarily on epithelial-like cells in the kidney to promote retention of water and sodium, and thereby induces a rise in blood pressure. Its effects on non-epithelial tissues, such as the heart and brain, have also recently aroused interest. In stroke-prone spontaneously hypertensive rats (SHR-SP) and some other animal models, aldosterone has been reported to induce myocardial hypertrophy, fibrosis, necrosis[1–4] and perivascular inflammation,[3,5] irrespective of its blood pressure-increasing effect. It is suggested that aldosterone is generated in the cardiac ventricle and increased synthesis is related to elevated ventricular aldosterone in myocardial infarction and cardiac failure.[6,7]

For cardiovascular protection, it thus appears useful to block the RAAS with angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers.[4] This has been demonstrated in recent large clinical studies such as RALES (Randomized Aldactone Evaluation Study)[8] and EPHESUS (Eplerenone Heart Failure Efficacy and Survival Study).[9]

On the other hand, Staessen *et al*. reported increased plasma aldosterone concentration after transient decrease during treatment with ACE inhibitors,[10] and this phenomenon is called 'aldosterone breakthrough'. This raised concern that re-increased aldosterone may attenuate the beneficial effects of the RAAS blocker particularly in long-term treatment.[11] According to the ALLHAT (Antihypertensive and Lipid-Lowering Treatment to Prevent

Correspondence: Shinsuke Harasawa, Department of Cardiology, Nihon University Surugadai Hospital, 1-8-13, Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8309, Japan.
E-mail: ii28181@yb3.so-net.ne.jp

Table 1 Oligonucleotide primer sequences and cycling conditions of GAPDH, brain natriuretic peptide, transforming growth factor-β_1 and fibronectin for the quantitative real time polymerase chain reaction

mRNA	Primer sequence	Product size (bp)	Denaturation temp. (°C)/time (s)	Annealing temp. (°C)/time (s)	Extension temp. (°C)/time (s)	Cycle number
GAPDH	F: 5′-AGATCCACAACGGATACATT-3′ R: 5′-TCCCTCAAGATTGTCAGCAA-3′	309	94/30	65/30	72/60	32
BNP	F: 5′-CTGGGAAGTCCTAGCCAGTCTCCA-3′ R: 5′-GCGACTGACTGCGCCGATCCGGTC-3′	250	93/30	53/30	73/60	32
TGF-β_1	F: 5′-TATAGCAACAATTCCTGGCGTTACCT-3′ R: 5′-AAGGTCGGTTCATGTCATGGATG-3′	250	94/60	60/60	72/60	32
Fibronectin	F: 5′-AGACTGCAGTGACCACCATTC-3′ R: 5′-CAATGTGTCCTTGAGAGCATAGAC-3′	251	94/60	58/90	73/120	32

BNP, brain natriuretic peptide; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; TGF-β_1, transforming growth factor-β_1; F and R, forward and reverse primers, respectively, in 5′→3′ orientation: bp, base pairs, temp, temperature.

Heart Attack Trial) study results,[12] calcium channel blockers were not as useful for cardiac or renal protection as ACE inhibitors. This was also confirmed by the JLIGHT (Japanese Losartan Therapy Intended for Global Renal Protection in Hypertensive Patients) study.[13] On the other hand, amlodipine, a calcium channel blocker, significantly decreased mortality in patients with severe chronic heart failure caused by non-ischaemic heart disease in the PRAISE (Prospective Randomized Amlodipine Survival Evaluation) study.[14] Amlodipine, a calcium channel blocker, is one of the most common antihypertensive medicines in Japan. There are many recent reports that combined therapy including amlodipine has cardioprotective effects.[15,16] Also, some studies have shown that amlodipine suppresses cardiac fibrosis.[17] However, it is unclear whether the beneficial effects of amlodipine in cardiac protection are related to its influence on the RAAS.

We studied the effects of amlodipine on cardiovascular protection and its relationship to plasma aldosterone concentration in male SHR-SP, a hypertensive model rat with developing atherosclerosis, by measuring brain natriuretic peptide (BNP) mRNA, as a marker for heart failure, mRNA for transforming growth factor-β_1 (TGF-β_1), as a mediator for cardiac fibrosis, and mRNA for fibronectin as a marker for myocardial fibrosis.

Methods

Materials and experimental design

Fifteen-week-old male stroke-prone spontaneously hypertensive rats (SHR-SP; Funabashi Farm, Chiba, Japan) were exposed to a 12-h environmental light cycle, 24°C ambient temperature and 40% humidity, with free access to standard rat chow (Oriental Yeast Co., Ltd, Tokyo, Japan) in individual cages for the 4-week experimental period.[18]

Amlodipine (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was mixed with drinking water. The drug concentration for each rat was calculated based on the mean intake of water and mean body weight over two days, so as to achieve the target dose.

Ten male rats were divided into two groups, as follows: the amlodipine was administered at a dose of 3 mg/kg/day mixed with purified water for four weeks (amlodipine group: $n = 5$) as described previously;[19] the control group received purified water only ($n = 5$).

This study was approved by the Ethics Committee and we followed the Guidelines for Animal Experimentation of the Nihon University School of Medicine.

Measurement of systolic blood pressure, heart rate and body weight

Systolic blood pressure (SBP) and heart rate (HR) were measured in the morning once a week by the tail-cuff method with a Rat Tail Manometer Tachometer System (KN-210-1; Natsume Seisakusho, Tokyo, Japan). Body weight was measured in the morning throughout the four-week administration period.

Measurement of plasma renin activity, plasma angiotensin II concentration and plasma aldosterone concentration

Blood was collected via the tail vein to measure the plasma renin activity, plasma angiotensin II concentration and plasma aldosterone concentration at the 4th week, and these samples were measured by standard radioimmunoassay (SRL, Inc., Tokyo, Japan).

Measurement of brain natriuretic peptide, transforming growth factor-β_1 and fibronectin mRNA expression from the left ventricle by quantitative real-time polymerase chain reaction

The rats were sacrificed at end of the 4th week and hearts were prepared for histological determination as described previously.[18] The left ventricle was cut at the papillary muscle level four weeks later, and its apex was frozen with liquid nitrogen. Total RNA was extracted, and left ventricular BNP, TGF-β_1 and fibronectin mRNA expression was examined by quantitative real-time polymerase chain reaction (PCR) assay with SYBR Green Reagents (Applied Biosystems, Foster City, USA). Table 1 shows the primer sequences and PCR conditions of each gene used in this study.

Extraction of RNA

A 100-mg sample of myocardial tissue was homogenized in 1 ml of Trizol Reagent (Molecular Research Center Inc.,

Table 2 Systolic blood pressure, heart rate and body weight of stroke-prone spontaneously hypertensive rats

	0 week	1 week	2 week	3 week	4 week
Amlodipine group					
Systolic blood pressure (mmHg)	215 ± 11	201 ± 10	229 ± 9	213 ± 9	218 ± 10
Heart rate (beats/min)	464 ± 5	371 ± 16	405 ± 18	398 ± 18	395 ± 27
Body weight (g)	290 ± 2	293 ± 5	296 ± 2	299 ± 2	306 ± 2
Control group					
Systolic blood pressure (mmHg)	223 ± 5	215 ± 7	229 ± 2	244 ± 3	247 ± 4
Heart rate (beats/min)	423 ± 13	398 ± 14	450 ± 14	432 ± 15	439 ± 18
Body weight (g)	288 ± 6	289 ± 7	285 ± 9	290 ± 1	291 ± 13
P-value					
Systolic blood pressure		0.26	1.00	0.01*	0.02**
Heart rate		0.24	0.77	0.18	0.22
Body weight		0.65	0.27	0.37	0.29

*$P < 0.01$ and **$P < 0.05$, compared with control.

Cincinnati, USA). RNA was extracted and purified by an RQ1 RNase-Free DNase Kit (Promega, Madison, USA). The total RNA was identified by a bioanalyser (Agilent Technologies, Palo Alto, USA) and kept at −80°C.[18]

cDNA synthesis

cDNA was synthesized from 3 μg of the stored total RNA with a First-strand cDNA Synthesis Kit (Amersham Biosciences Co., Piscataway, USA) and stored at −80°C.[18]

Preparation of standard cDNA

PCR amplification was performed for each gene under the conditions shown in Table 1, using a Gene Amp PCR System 9700, Amplitaq Gold 10 × PCR buffer and a dNTP Kit (all Applied Biosystems). After electrophoretic identification, the target band of cDNA was extracted and purified with a GENECLEAN Kit (Q Biogene, Irvine, USA). The second PCR was performed using the PCR product. The PCR product was purified with a QIA Quick PCR Purification Kit (QIAGEN, Hilden, Germany) and identified by electrophoresis. The concentration of the PCR product was assessed at 260 nm with a spectroscope (Biotech Ultrospec 2000; Amersham Biosciences Co.), after which the copy number of standard cDNA per μl was calculated, and the product was stored at −80°C. Before use, RNase-free water was used to prepare dilutions of 10^6 copies down to 10^3 copies of each standard cDNA.[18]

Quantitative real-time polymerase chain reaction

Quantitative real-time PCR analysis was carried out using a Gene Amp 5700 Sequence Detection System (Applied Biosystems) with SYBR Green Reagents (Applied Biosystems). Amplifications were performed in a 50-μl volume containing either the unknown cDNA sample or standard cDNA diluted at each concentration according to the PCR conditions shown in Table 1. An RNA sample not subjected to the reverse transcription reaction was used as a negative control. A non-template control was run with each assay and all determinations were performed at least twice independently so as to achieve reproducibility. The absence of dimer primer was verified by the dissociation curve.

At the end of the PCR, the Gene Amp 5700 Sequence Detector System software saved the results of analysis. The amount of each mRNA was standardized with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as the internal standard.[18]

Statistical analysis

Results are expressed as the mean ± SEM. Clinical and laboratory data were analysed by Mann–Whitney *U*-test for paired samples. $P < 0.05$ was considered statistically significant.

Results

Systolic blood pressure, heart rate and body weight

As shown in Table 2, the value of systolic blood pressure was significantly lowered by approximately 15% at weeks 3 and 4 in the amlodipine group compared with the control group ($P < 0.01$ and $P < 0.05$, respectively).

Heart rate showed no significant difference between the two groups.

Body weight tended to be lower in the control group compared with the amlodipine group.

Plasma renin activity, plasma angiotensin II concentration and plasma aldosterone concentration

Plasma renin activity and angiotensin II concentration in the amlodipine group and control groups did not differ (Figure 1a and 1b).

Plasma aldosterone concentration was decreased by approximately 60% in the amlodipine group compared with the control group ($P < 0.05$) (Figure 1c).

Brain natriuretic peptide, transforming growth factor-β_1 and fibronectin expression in the heart

As shown in Figure 2, the BNP mRNA level in rat left ventricle was markedly lower by approximately 40% in the amlodipine group compared with the control group ($P < 0.01$).

The TGF-β_1 mRNA level in the left ventricle was lower by approximately 40% in the amlodipine group compared with the control group ($P < 0.01$) (Figure 3).

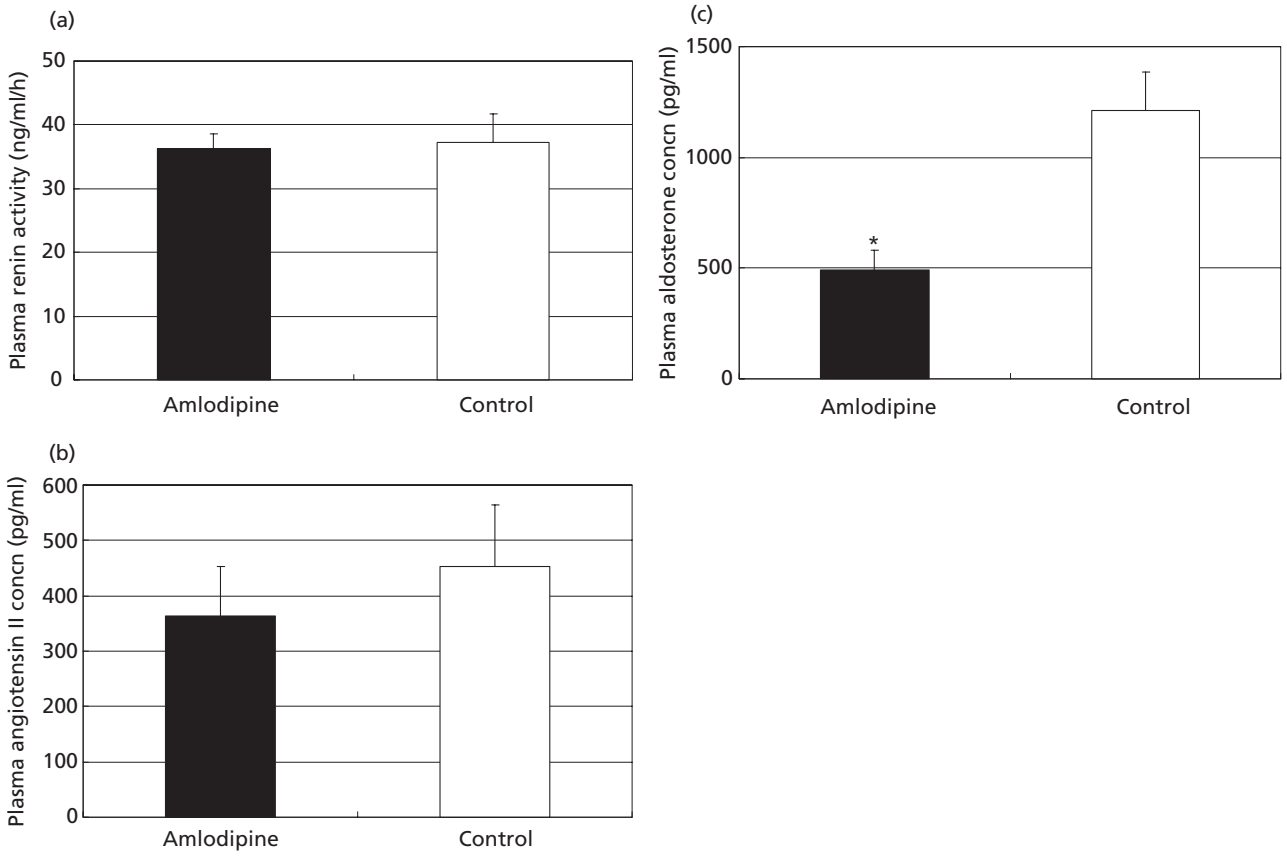

Figure 1 (a) Plasma renin activity, (b) plasma angiotensin II concentration and (c) plasma aldosterone concentration in stroke-prone spontaneously hypertensive rats.

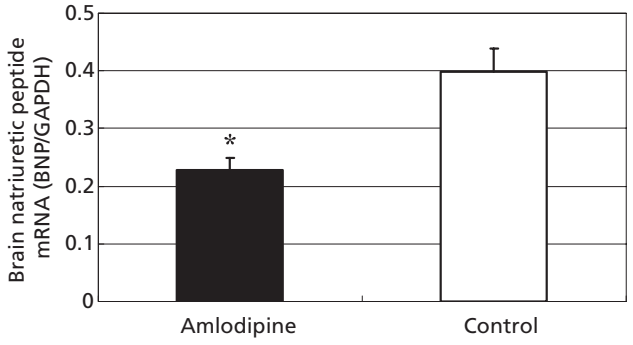

Figure 2 Brain natriuretic peptide mRNA in stroke-prone spontaneously hypertensive rats.

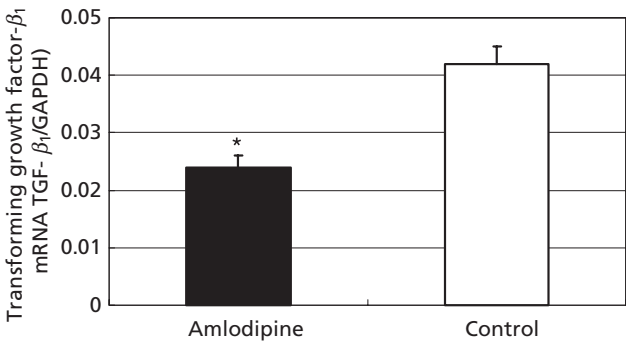

Figure 3 Transforming growth factor-β_1 mRNA in stroke-prone spontaneously hypertensive rats.

Figure 4 shows that the fibronectin mRNA level in the left ventricle was remarkably lower, by approximately 60%, in the amlodipine group (0.002 ± 0.0003) compared with the control group (0.005 ± 0.001) ($P < 0.05$).

Discussion

It is not clear whether amlodipine exerts cardiac protection in SHR-SP in the setting of mild blood pressure reduction, and if it does whether the effect is related to suppression of the RAAS. According to previous studies,[17,19] we chose a low dose of amlodipine (i.e. its effect on blood pressure is mild). Our results showed that systolic blood pressure slightly decreased in the amlodipine group compared with the control group. However the heart rate was not significantly different between these groups. These results suggest that the reduction of blood pressure with amlodipine was not associated with subsequent enhancement of sympathetic nerve activity.

In the amlodipine group, gene expression of rat BNP, TGF-β_1 and fibronectin was significantly suppressed. Our data

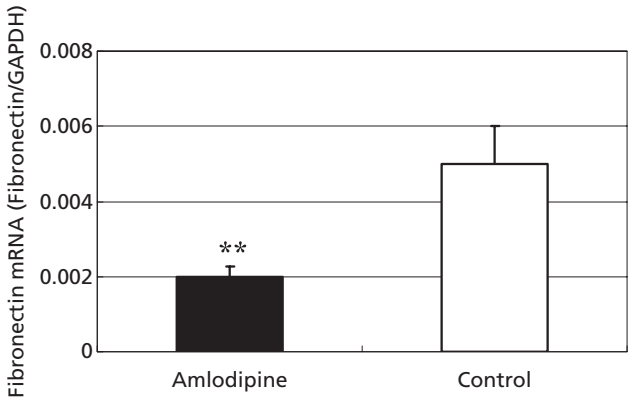

Figure 4 Fibronectin mRNA in stroke-prone spontaneously hypertensive rats.

indicate that amlodipine exerts cardiac protection even in the setting of mild blood pressure reduction.

Aldosterone is synthesized in the zona glomerulosa of the adrenal cortex through the L- or T-type voltage-dependent calcium channel, following stimulation by angiotensin II or potassium. There have been inconsistent reports regarding the effects of calcium channel blockers on plasma aldosterone concentrations. It seems that synthesis and release of aldosterone works through various regulatory mechanism, such as permeation of the skin being changed by drug concentration.[20] While Favre *et al*.[21] and Okayama *et al*.[22] reported that nifedipine and nilvadipine increased plasma aldosterone concentration, Imagawa *et al*.[23] and Uebele *et al*.[24] showed that nifedipine, mibefradil and efonidipine decreased it. Ishimitsu *et al*.[25] indicated that amlodipine had no effect.

Favre *et al*.[21] suggested that increased plasma aldosterone may result from activated synthesis of aldosterone due to elevated angiotensin II, whereas Okayama *et al*.[22] concluded that efonidipine, a T-type calcium channel blocker, directly decreased plasma aldosterone since it concurrently increased plasma renin activity and angiotensin II.

Imagawa *et al*.[23] reported that nifedipine also decreased plasma aldosterone concentration, although not as potently as efonidipine or mibefradil, and attributed this difference to the fact that the latter two T-type calcium channel blockers inhibited aldosterone synthetase whereas the L-type calcium channel blockers (e.g. nifedipine) inhibited only potassium-mediated actions.

In this study, the low dose of amlodipine decreased plasma aldosterone concentration, but did not change plasma renin activity or angiotensin II concentration. The data suggest that amlodipine inhibits aldosterone synthesis without direct action on renin and angiotensin II.

It is also possible that amlodipine-induced vasodilation and resultant diuresis might decrease the extracellular potassium concentration and thus aldosterone synthesis; especially, amlodipine might inhibit cytochrome P450 aldosterone synthase (CYP11B2).[6] However, there was no difference between the two groups in terms of the urine volume or biochemical abnormalities (data not shown).

In this study, the heart rate of the rats tended to be reduced, although not significantly; since it has been reported that noradrenaline promotes aldosterone synthesis,[26] amlodipine may have decreased plasma aldosterone concentration by inhibiting the sympathetic nervous system, as shown by Hirooka *et al*.[19]

Consistent with many reports that show myocardial fibrosis and hypertrophy to be adversely affected by aldosterone,[1,2,27] a low dose of amlodipine lowered the blood aldosterone concentration as well as the expression of mRNA for BNP, TGF-β_1 and fibronectin in this study. This decrease in expression may be attributed partly to decreased blood pressure by amlodipine. In the study, blood pressure decreased approximately 15% in the amlodipine groups as compared with the control group. However, approximately 40%, 40% and 60% decreases in BNP, TGF-β_1 and fibronectin, respectively, were disproportionately large, suggesting that the decrease in blood pressure is not the only mechanism. We assume that aldosterone is at least partly involved in this mechanism. In addition, oxidative stress[5,28] and activation of the local RAAS[29] have been proposed as a mechanism of aldosterone-induced organ damage. This suggests that amlodipine reduced aldosterone-induced organ damage by decreasing oxidative stress and inhibiting the local RAAS in our study. Calcium channel blocker-induced inhibition of oxidative stress,[30] especially amlodipine-induced inhibition of oxidative stress,[19,31–33] and angiotensin type 1 (AT_1) receptor expression[34] have been reported.

There is limitation and perspective in our study. It is still unclear what degree of blood pressure lowering affects decreased plasma aldosterone concentration and attenuation of cardiac damage. It is necessary to examine other antihypertensive drugs matched with degree of blood pressure lowering. The mechanism by which aldosterone suppresses the expression of BNP, TGF-β_1 and fibronectin still remains unclear. We will evaluate not only surrogate markers such as BNP, but also directly measure organ damage (e.g. by pathological methods and left ventricular mass index). We did not perform protein assays this time because many reports[35] showed that expression of mRNA using the real-time PCR and protein assay by Western blot did not have a paradoxical result in similar experiments.

Conclusions

A low dose of amlodipine suppressed plasma aldosterone concentration and cardiac gene expression of brain natriuretic peptide, transforming growth factor-β_1 and fibronectin in the setting of mild blood pressure reduction in SHR-SP, suggesting that the calcium channel blocker exerts cardiac protection and that suppression of aldosterone synthesis contributes to its mechanism.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Acknowledgements

We thank Ms Mika Watanabe and Ms Fumie Anzai for their expert technical assistance. We are also grateful to Dr Takae Asai in Scotland.

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