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Effects of cilnidipine on nitric oxide and endothelin-1 expression and extracellular signal-regulated kinase in hypertensive rats

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Abstract

We evaluated the effects of cilnidipine, a long-acting Ca^{2+} channel antagonist, on endothelial nitric oxide synthase (eNOS), preproendothelin-1 and endothelin ETA receptor expression in the left ventricle, and evaluated the relations between these effects and coronary microvascular remodeling and extracellular signal-regulated kinases belonging to one subfamily of mitogen-activated protein kinases in deoxycorticosterone acetate (DOCA)-salt hypertensive rats. Cilnidipine (DOCA-cilnidipine, 1 mg/kg/day, subdepressor dose) or vehicle (DOCA-vehicle) was given after induction of DOCA-salt hypertension for 5 weeks. The eNOS mRNA and protein expression in the left ventricle was significantly lower in DOCA-vehicle than in control rats and significantly higher in DOCA-cilnidipine than in DOCA-vehicle rats. Preproendothelin-1 and endothelin ETA receptor expression levels and phospho-p42/p44 extracellular signal-regulated kinase activities were significantly increased in DOCA-vehicle compared with control rats and significantly suppressed in DOCA-cilnidipine compared with DOCA-vehicle rats. DOCA-vehicle rats showed a significant increase in the wall-to-lumen ratio, perivascular fibrosis and myocardial fibrosis, with all these parameters being significantly improved by cilnidipine. These results led us to conclude that phospho-p42/p44 extracellular signal-regulated kinase activities may contribute to the coronary microvascular remodeling of DOCA rats and that protective effects of cilnidipine on cardiovascular remodeling may be at least in part mediated by an increased eNOS expression and a decreased endothelin-1 and endothelin ETA receptor expression in the left ventricle.

Keywords: Ca^{2+} channel antagonist; Endothelin-1; Extracellular signal-regulated kinase; Nitric oxide (NO); (Rat)

1. Introduction

Left ventricular hypertrophy is a strong predictor, independent of blood pressure or other risk factors, of cardiac and cerebrovascular morbidity and mortality in patients with hypertension (Verdecchia et al., 1994). In hypertensive heart disease, myocytes hypertrophy and interstitial components undergo hyperplasia, hypertrophy and remodeling (Weber and Brilla, 1991). Excess collagen production by fibroblasts increases total interstitial and perivascular fibrosis. In addition, vascular smooth muscle cells undergo hyperplasia and hypertrophy, resulting in media hypertrophy, coronary artery wall remodeling and increased coronary wall to lumen ratio (Schwarzkopff et al., 1993). These structural changes decrease vasodilator capacity and may be associated with the endothelial dysfunction. Endothelial dysfunction has been demonstrated in conditions associated with premature development of atherosclerosis and hypertension. Mitogen-activated protein kinases are an ubiquitous group of protein serine/threonine kinases and are important mediators of the signal transduction pathway, which is responsible for cellular proliferation. Extracellular signal-regulated kinases (ERKs) are a subgroup of the mitogen-activated protein kinase family and are composed of p42ERK and p44ERK (Davis, 1993). Recent evidence indicates that ERKs in cultured neonatal rat cardiac myocytes are rapidly activated by various extracellular stimuli, such as growth factors and other mitogens, and play a key role in cell growth and the regulation of various gene expressions. Recent reports on

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cultured cardiac myocytes support the idea that ERKs participate in the mechanism of cardiac hypertrophy and remodeling (Kojima et al., 1994; Bogoyevitch et al., 1996).

The potential importance of endothelin-1 in cardiovascular disease was suggested by its production in endothelial cells and its potent vasoconstricting and growth-promoting properties (Yamazaki et al., 1999). Infusion of exogenous endothelin-1 has been reported to produce significant coronary vasoconstriction in humans, supporting a role for endothelin-1 as an important potential mediator of myocardial ischemia in states of endothelin-1 activation, as in atherosclerosis (Lerman et al., 1991). The vasoconstrictor actions of endothelin-1 are mediated by at least two receptor subtypes, including the endothelin ETA and ETB receptors. Endothelin-1 has direct effects on contractile function, protein synthesis and electrophysiological events in cardiac myocytes, and these effects are mediated primarily by the endothelin ETA receptor (Ishikawa et al., 1988). Indeed, aortic tissue endothelin-1 content acts as a local mediator of vascular dysfunction and aortic hypertrophy, and endothelin ETA receptor antagonism may have a therapeutic potential for lowering vascular endothelin-1 content, improving endothelial function and preventing structural changes in the cardiovascular hypertrophy (Barton et al., 1998).

Endothelium-derived relaxing factor, nitric oxide (NO), reduces monocyte and leukocyte adhesion to endothelial cells and is an important inhibitor of platelet aggregability and platelet and vessel wall interaction. Furthermore, NO decreases endothelial permeability and thus diminishes the transport of lipoproteins into the vessel wall and suppresses vascular smooth muscle proliferation and migration both in vivo and in vitro (Cooke and Dzau, 1997). The observation that induction of hypertension in animal models results in impaired endothelial-dependent vasodilation suggested that effective antihypertensive therapy may normalize, or at least improve, endothelial vasodilator function (Lüscher et al., 1987). Indeed, long-term treatment with an angiotensin-converting enzyme inhibitor or a Ca^{2+} channel antagonist improved endothelial dysfunction in a rat model of NO-deficient hypertension (Takase et al., 1996). Cilnidipine, a novel dihydropyridine Ca^{2+} channel antagonist, has been shown to act as a slow-onset and long-lasting antihypertensive drug in clinical and experimental studies (Tominaga et al., 1997), and to possess a potent inhibitory action on the N-type as well as on the L-type voltage-dependent Ca^{2+} channel in rats (Uneyama et al., 1997). In addition, cilnidipine is effective as a once-daily antihypertensive agent and causes reflex tachycardia less than does nisoldipine (Minami et al., 1998). Recently, we evaluated the effects of chronic treatment with the L-type dihydropyridine Ca^{2+} channel antagonists, benidipine (Kobayashi et al., 1999b) and amlodipine (Kobayashi et al., 1999c) on endothelial NO synthase (eNOS) mRNA expression in two-kidney, one-clip Goldblatt rats (Kobayashi et al., 1999b) and in rats with prolonged nitric oxide blockade-induced hypertension (Kobayashi et al., 1999c). Downregulation of eNOS mRNA expression in the left ventricle of these models was significantly increased by a subdepressor dose of these agents. However, very few studies have evaluated whether the beneficial effects of cilnidipine on coronary microvascular remodeling is associated with direct local gene expression of eNOS and endothelin-1 mRNA in the left ventricle. The purpose of the present study was to evaluate the effects of long-term treatment with a subdepressor dose of cilnidipine on gene expression of eNOS, preproendothelin-1 and endothelin ETA receptor mRNA in the left ventricle, and the relation of these effects to coronary microvascular remodeling and phospho-p42/p44 ERK activity in deoxycorticosterone acetate (DOCA)-salt hypertensive rats.

2. Methods

2.1. Animal models and experimental designs

All procedures were in accordance with institutional guidelines for animal research. Twenty-four male normotensive Wistar rats (Oriental Bioservice Kanto Ibaragi, Japan) aged 6 weeks were used, and DOCA-salt hypertension was induced in 16 rats as described previously (Takanohashi et al., 1996). Eight rats received weekly subcutaneous injections of DOCA (30 mg/kg) after right nephrectomy and were given 1% saline as drinking water (DOCA-vehicle, $n = 8$). The remaining eight DOCA-salt rats were treated with cilnidipine (UCB Japan, Tokyo, Japan) dissolved in peanut oil and administered subcutaneously every other day, with 1% saline as the drinking water for 5 weeks (DOCA-cilnidipine, $n = 8$) (Kobayashi et al., 1997, 1999b,c). The 1 mg/kg/day of cilnidipine was not expected to influence blood pressure in rats, according to our preliminary data (data not shown). Age-matched sham-operated rats ($n = 8$) served as a control group. Systolic blood pressure was measured using the tail-cuff method (Muromachi Kikai, model MK-1100, Tokyo, Japan) before the operation and at 1-week intervals thereafter (protocol 1). The experimental designs and numbers of animals used in protocols 2 and 3 were identical to those described above for protocol 1. Rats were housed at a constant temperature (25 ± 1 °C) and fed a standard laboratory rat chow (0.4% sodium content).

2.2. Protocol 1

2.2.1. Reverse transcription-polymerase chain reaction (RT-PCR) for eNOS, preproendothelin-1 and endothelin ETA receptor mRNA expression in the left ventricle

After 5 weeks of treatment, the rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and decapitated, and the heart was immediately excised. The left

ventricle was carefully separated from the atria and right ventricle, weighed, immediately frozen in liquid nitrogen, and stored at −80 °C until extraction of total RNA. Total RNA was prepared as previously described (Chomczynski and Sacchi, 1987). RT-PCR was performed by standard methods with 1 μg of total RNA. First-strand cDNA was synthesized with random primers and Molony murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). PCR amplification was then performed with synthetic gene-specific primers for eNOS (upstream primer, 5′-TCCAGTAACACAGACAGTGCA-3′; downstream primer, 5′-CAGGAAG TAAGTGAGAGC-3′; product length, 693 bp) (Seki et al., 1997), preproendothelin-1 (upstream primer, 5′-CTAGGTCTAAGCGATCCTTG-3′; downstream primer, 5′-TTCTGGTCTCTGTAGAGTTC-3′; product length, 319 bp) (Sakurai et al., 1991) and endothelin ETA receptor (upstream primer, 5′-GTGTT-TAAGCTGTTGGCGGG-3′; downstream primer, 5′-CGAGGTCATGAGGCTTTTGG-3′; product length, 780 bp) (Terada et al., 1992a), using a DNA PCR kit (Perkin Elmer, Norwalk, CT, USA) for 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min. Parallel amplification of rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was performed for reference with primers as described (Terada et al., 1992b). Reaction conditions were optimized to obtain reproducible and reliable amplification within the logarithmic phase of the reaction, as determined in preliminary experiments. The reaction was linear to 35 cycles with the ethidium bromide detection method. PCR products were separated by electrophoresis on a 2% agarose gel containing ethidium bromide and were visualized by ultraviolet-induced fluorescence. The intensity of each band was quantified using a densitometer. The resulting densities of the eNOS, preproendothelin-1 and endothelin ETA receptor bands were expressed relative to the corresponding densities of the GAPDH bands from the same RNA sample (Kobayashi et al., 1999a,b,c, 2000a,b; Higashi et al., 2000).

2.3. Protocol 2

2.3.1. Western blot analysis for eNOS and endothelin ETA receptor protein expression in the left ventricle

The left ventricles were homogenized (25% w/v) in 10 mmol/l HEPES buffer, pH 7.4, containing 320 mmol/l sucrose, 1 mmol/l EDTA, 1 mmol/l dithiothreital (DTT), 10 μg/ml leupeptin, and 2 μg/ml aprotinin at 0–4 °C with a Polytron homogenizer. The homogenate was centrifuged at $1000 \times g$ for 5 min at 4 °C, and the resulting supernatant was used as a post-nuclear fraction. Protein concentrations were determined with bovine serum albumin as a standard protein (Bradford, 1976). The post-nuclear fraction (eNOS, endothelin ETA receptor: 50 μg of protein) of samples was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10–13% gels (Laemmli, 1970). The proteins in the gels were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Amersham Life Science, Arlington Heights, IL, USA) for 1 h at 2 mA/cm^2 as described (Towbin et al., 1979). The membranes were immunoblotted with an anti-eNOS and anti-endothelin ETA receptor antibody (Transduction Laboratories, Immuno-Biological Lab) in a buffer containing 10 mmol/l Tris–HCl, pH 7.5, 100 mmol/l NaCl, 0.1% Tween 20, and 5% skim milk followed by peroxidase-conjugated goat anti-mouse immunoglobulin (Amersham Life Science) (Towbin et al., 1979). The eNOS and endothelin ETA receptor proteins transferred to the sheets were detected using the enhanced chemiluminescence (ECL) immunoblotting detection system (Amersham Life Science). The amount of protein was quantified using a densitometer in a linear range and expressed as percentage relative to that in non-treated rats (Kobayashi et al., 1999a; Higashi et al., 2000).

2.3.2. Western blot analysis for phospho-p42/p44ERK activity in the left ventricle

Left ventricular ERK activity was measured as described in detail previously (Kim et al. 1998). Briefly, by using rabbit polyclonal phospho-specific ERK antibody (New England Biolabs) and anti-total ERK antibody (Santa Cruz) recognizing tyrosine-phosphorylated forms (active forms) of p44ERK and p42ERK, we measured left ventricular phosphorylated ERK proteins by Western blot analysis. Left ventricular protein extracts were boiled for 5 min in Laemmli sample buffer, then electrophoresed on an SDS-PAGE using 13% gels, and the separated proteins were electrophoretically transferred to Hybond-PVDF membranes. Complete protein transfer to the membrane was verified by staining the gels with Coomassie blue. The membrane was incubated with phospho-specific ERK antibody for 1 h at room temperature, washed four times with Tris-buffered saline containing 0.1% Tween 20 (TBS–T), and then incubated with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham Life Sciences).

2.4. Protocol 3

2.4.1. Histologic examination and evaluation of coronary microvascular remodeling

Histological examination was as described in detail previously (Kobayashi et al. 1997, 1999a,b,c, 2000a,b; Higashi et al., 2000). Briefly, the excised hearts were perfused with physiological saline solution containing adenosine 10 μg/kg and nitroglycerin 10 μg/kg and then with 6% formaldehyde solution via retrograde infusion into the ascending aorta at a pressure of 90 mm Hg. The left ventricle was separated from the right ventricle, the

atria, and the great vessels, and cut into five pieces perpendicular to the long axis. For light microscopy, 1.5-μm-thick sections were cut (microtome, type ROM-380, Yamato Kohki, Saitama, Japan). Paraffin slices from each heart were mounted on glass slides and stained with hematoxylin-eosin and Masson's trichrome stains. All histopathological sections of each animal were examined using a 3CCD color video camera (Sony, model DXC-930, Tokyo, Japan) mounted on a standard microscope (Olympus, BHS-F, Tokyo, Japan). Drawings of the limits of the vessels were made on the screen of a multiscan color computer display (Sony, model CPD-17SF7) and then digitized with a two-dimensional analysis system (Mac SCOPE, Mitani, Fukui, Japan) connected with a Macintosh computer system (Power Macintosh G3, Apple Computer, Cupertino, CA, USA). Histopathological findings of the myocardium and coronary arterioles were examined. We always measured the capillary density and cross-sectional surface area in the endocardium of the posterior portion of the left ventricular free wall. In this part of the heart, shrinkage was minimal and orientation of the myocardial fibres was similar from one heart to another. We analyzed five sites from each ventricle in all rats. To assess thickening of the coronary arterial wall and perivascular fibrosis, the transsectional images of the area of the total small arteriolar lumen $\leq 10^4$ μm^2 were studied. The inner border of the lumen and the outer border of the tunica media were traced in each arterial image with hematoxylin-eosin staining at $\times 100$ to $\times 400$ magnification, and the areas encircled by the tracings were calculated. On quantification, non-round vessels resulting from oblique transsection or branching were excluded, and only round vessels were studied. The wall-to-lumen ratio (the area of the vessel wall divided by the area of the total blood vessel lumen) was determined. The area of fibrosis immediately surrounding blood vessels was calculated, and perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total area of the vessel. To assess the area of myocardial fibrosis, the area of pathological collagen deposition was measured in the microscopic field of each Masson's trichrome-stained section. The ratio of the total area of fibrosis within the left ventricular myocardium to the total area of the left ventricular myocardium in each heart was calculated and was used for analysis. Histopathology on the sections from each rat was carried out by an operator who was blinded to the treatment groups.

2.5. Statistical analysis

All results are expressed as means ± S.E.M. The mean values were compared among the three groups using analysis of variance (ANOVA) followed by the Bonferroni test. Differences with $P < 0.05$ were considered statistically significant. Calculations, including those of derived values, and statistical tests were performed using the appropriate software (Stat View-J 4.5, Abacus Concepts, Berkeley, CA, USA) and a Power Macintosh computer system (G4, Apple Computer).

3. Results

3.1. Systemic hemodynamics, body weight and left ventricular weight

Systolic blood pressure in DOCA-vehicle and DOCA-cilnidipine rats was similar and significantly higher than that in sham-operated rats (186 ± 6 and 183 ± 5 vs. 129 ± 3 mm Hg, sham-operated rats, $P < 0.01$, respectively). Heart rate was similar in sham-operated rats and DOCA-vehicle rats (396 ± 15, 389 ± 17 bpm) and was not changed by the administration of cilnidipine (387 ± 17 bpm). Body weight was also similar among the three groups. The left ventricular mass of the DOCA-vehicle rats was significantly increased compared to that of sham-operated rats using body weight-corrected values (2.80 ± 0.06 vs. 1.94 ± 0.04 mg/g, $P < 0.01$) and significantly decreased in DOCA-cilnidipine (2.44 ± 0.07 mg/g, $P < 0.01$) compared with the DOCA-vehicle rats after 5 weeks treatment with cilnidipine.

3.2. eNOS, preproendothelin-1 and endothelin ETA receptor mRNA expression in the left ventricle

The level of eNOS mRNA in the left ventricle was significantly decreased in DOCA-vehicle compared with that of sham-operated rats and significantly increased in DOCA-cilnidipine compared with that of sham-operated rats and DOCA-vehicle (Figs. 1 and 2A). Preproendothelin-1 mRNA levels were significantly increased in DOCA-vehicle compared with sham-operated rats and sig-

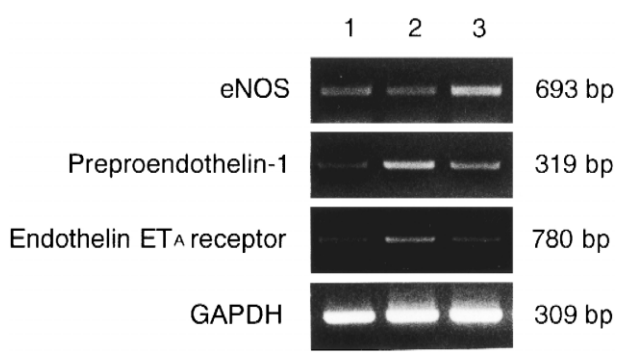

Fig. 1. Typical gel electrophoresis of RT-PCR of left ventricular eNOS mRNA, preproendothelin-1 mRNA, endothelin ETA receptor mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Lane 1 refers to sham-operated rats, lane 2 refers to deoxycorticosterone acetate (DOCA)-salt hypertensive rats (DOCA-vehicle), and lane 3 refers to DOCA-salt hypertensive rats treated with cilnidipine (DOCA-cilnidipine).

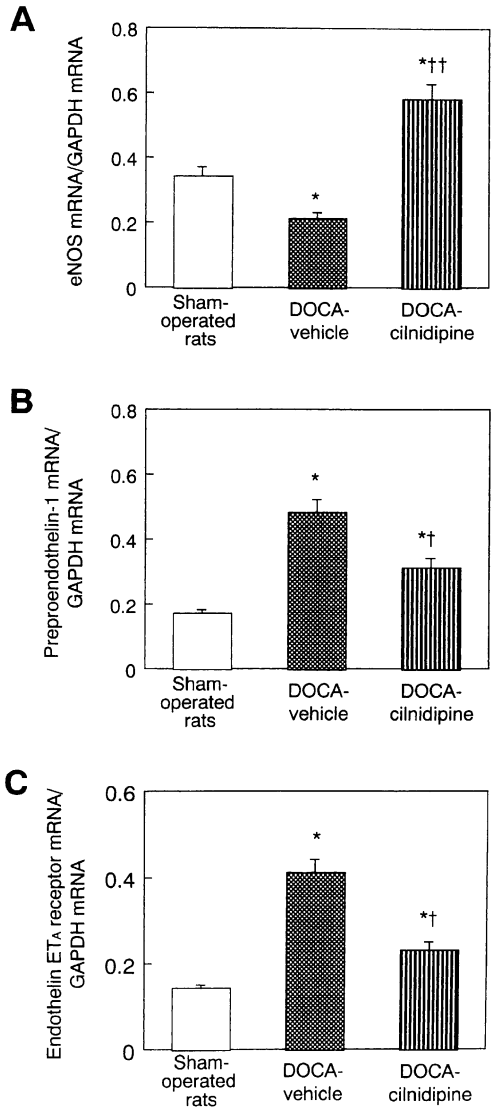

Fig. 2. Effect of chronic cilnidipine treatment on eNOS (A), preproendothelin-1 (B) and endothelin ETA receptor (C) mRNA expression. Total RNA was assayed by RT-PCR with gene-specific primers for eNOS, preproendothelin-1, endothelin ETA receptor and GAPDH. Each panel represents the mean densities of the eNOS (A), preproendothelin-1 (B) and endothelin ETA receptor (C) in relation to the bands obtained for GAPDH. Values are expressed as means ± S.E.M. $n = 8$ per group. $^{*}P < 0.05$ vs. sham-operated rats. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ vs. DOCA-vehicle.

nificantly decreased in DOCA-cilnidipine compared with DOCA-vehicle rats (Figs. 1 and 2B). Endothelin ETA receptor mRNA levels were significantly greater in DOCA-vehicle than in sham-operated rats and were significantly less in DOCA-cilnidipine than in DOCA-vehicle rats (Figs. 1 and 2C).

3.3. eNOS and endothelin ETA receptor protein expression in the left ventricle

The data are depicted in Figs. 3 and 4. The eNOS protein mass in the left ventricle was significantly decreased in DOCA-vehicle compared with that of sham-operated rats and significantly increased in DOCA-cilnidipine compared with DOCA-vehicle rats (Fig. 3A,B). The endothelin ETA receptor protein mass of the left ventricle was significantly increased in DOCA-vehicle compared with that of sham-operated rats and significantly decreased in DOCA-cilnidipine compared with DOCA-vehicle rats (Fig. 4A,B).

3.4. Left ventricular phospho-p42 / p44 ERK activity in the left ventricle

As shown by Western blot analysis in Fig. 5, left ventricular phospho-p44ERK and phospho-p42ERK levels were significantly higher in DOCA-vehicle than in sham-operated rats and were significantly lower in DOCA-cilnidipine than in DOCA-vehicle. However, total p44ERK and total p42ERK levels were not changed by cilnidipine.

3.5. Coronary microvascular remodeling

The wall-to-lumen ratio was significantly increased in DOCA-vehicle compared with that of sham-operated rats and was significantly decreased by cilnidipine treatment (Fig. 6A). The perivascular fibrosis was significantly greater in DOCA-vehicle than in sham-operated rats and

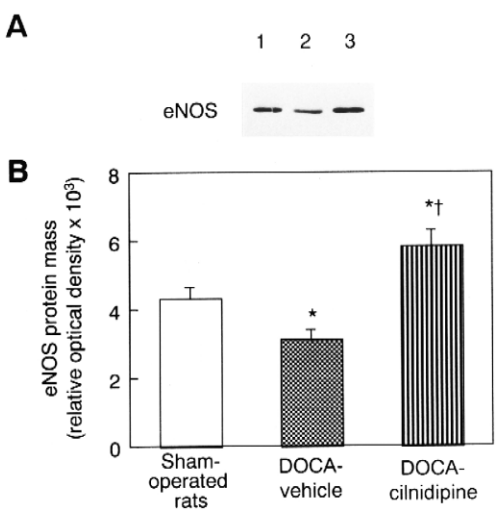

Fig. 3. Effect of chronic cilnidipine treatment on eNOS protein expression levels. (A) A typical Western blot of eNOS in the LV of a rat treated with cilnidipine. Western blot analysis using anti-eNOS antibodies was performed. Lane 1 refers to the sham-operated rats, lane 2, to deoxycorticosterone acetate (DOCA)-salt hypertensive rats (DOCA-vehicle), and lane 3, to DOCA-salt hypertensive rats treated with cilnidipine (DOCA-cilnidipine). (B) Group data depicting relative optical densities of left ventricular eNOS protein bands in the study groups. Values are expressed as means ± S.E.M. $n = 8$ per group. $^{*}P < 0.05$ vs. sham-operated rats. $\dagger P < 0.05$ vs. DOCA-vehicle.

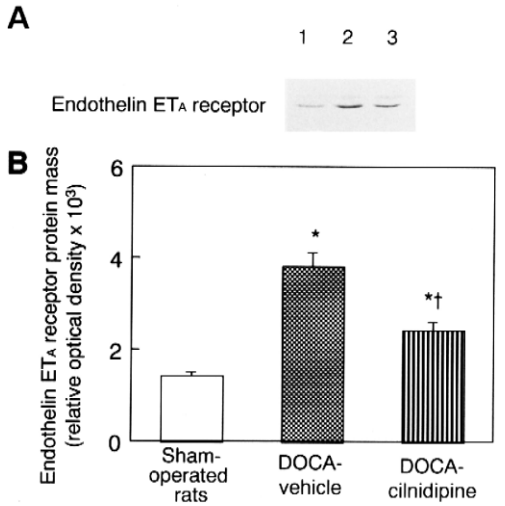

Fig. 4. Effect of chronic cilnidipine treatment on endothelin ETA receptor protein expression levels. (A) A typical Western blot of endothelin ETA receptor in the left ventricle of a rat treated with cilnidipine. Western blot analysis using anti-endothelin ETA receptor antibodies was performed. Lane 1 refers to the sham-operated rats, lane 2, to deoxycorticosterone acetate (DOCA)-salt hypertensive rats (DOCA-vehicle), and lane 3, to DOCA-salt hypertensive rats treated with cilnidipine (DOCA-cilnidipine). (B) Group data depicting relative optical densities of left ventricular endothelin ETA receptor protein bands in the study groups. Values are expressed as means ± S.E.M. $n = 8$ per group. $^{*}P < 0.05$ vs. sham-operated rats. †$P < 0.05$ vs. DOCA-vehicle.

was also significantly decreased by cilnidipine treatment (Fig. 6B). Compared with that in sham-operated rats, myocardial fibrosis was significantly greater in DOCA-vehicle rats and was significantly less in DOCA-cilnidipine than in DOCA-vehicle rats (Fig. 6C).

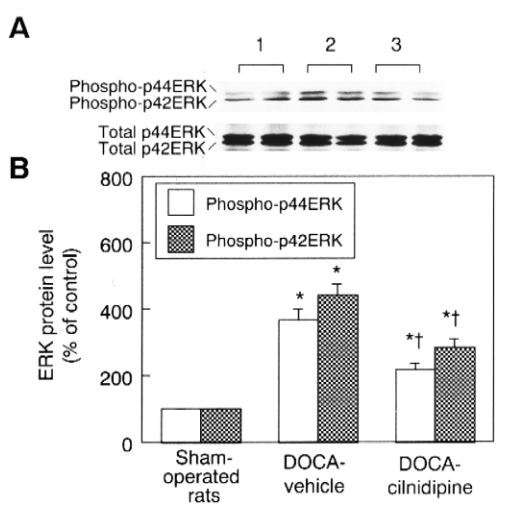

Fig. 5. Effect of chronic cilnidipine treatment on phospho-p42/p44ERK protein expression levels. (A) A typical Western blot of phospho-p42/p44ERK and total-p42/p44ERK in the left ventricle. Lane 1 refers to the sham-operated rats, lane 2, to deoxycorticosterone acetate (DOCA)-salt hypertensive rats (DOCA-vehicle), and lane 3, to DOCA-salt hypertensive rats treated with cilnidipine (DOCA-cilnidipine). (B) Group data depicting relative optical densities of left ventricular phospho-p42/p44ERK protein bands in the study groups. Values are expressed as means ± S.E.M. $n = 8$ per group. $^{*}P < 0.05$ vs. sham-operated rats. †$P < 0.05$ vs. DOCA-vehicle.

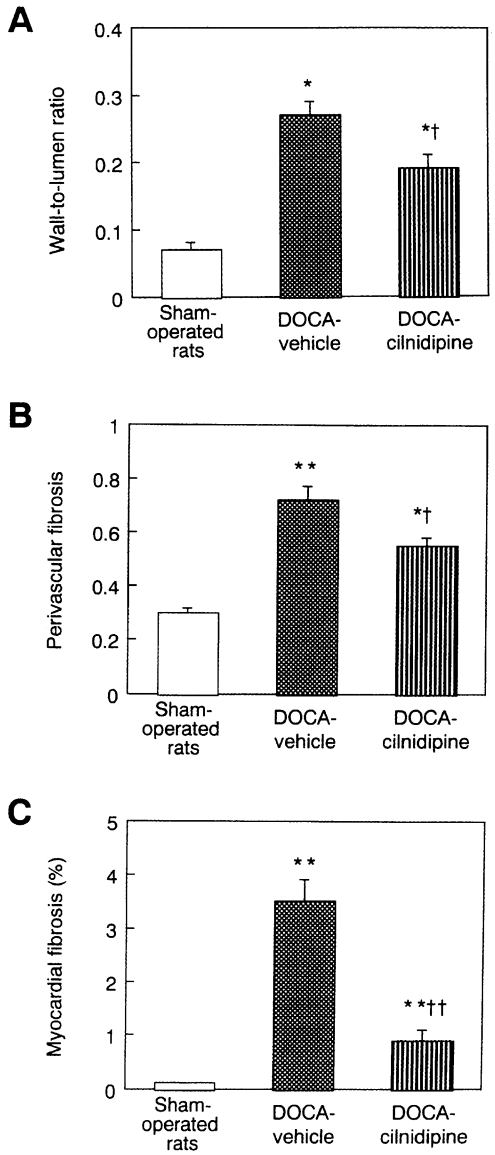

Fig. 6. Effect of cilnidipine on (A) wall-to-lumen ratio, (B) perivascular fibrosis, and (C) myocardial fibrosis in deoxycorticosterone acetate (DOCA)-salt hypertensive rats. DOCA-vehicle, deoxycorticosterone acetate (DOCA)-salt hypertensive rats; DOCA-cilnidipine, DOCA-salt hypertensive rats treated with cilnidipine. Values are expressed as means ± S.E.M. $n = 8$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. sham-operated rats. †$P < 0.05$, ††$P < 0.01$ vs. DOCA-vehicle.

4. Discussion

The present study demonstrated that the production of eNOS mRNA and protein expression is downregulated, and preproendothelin-1 and endothelin ETA receptor expression and phospho-p42/p44ERK activity are upregulated in the left ventricle of DOCA-salt hypertensive rats. The chronic administration of a long-acting dihydro-

pyridine Ca^{2+} channel antagonist, cilnidipine, increased eNOS mRNA and protein expression, and decreased preproendothelin-1 and endothelin ETA receptor expression. In addition, after 5 weeks of treatment, cilnidipine effectively improved left ventricular hypertrophy and coronary microvascular remodeling, and inhibited left ventricular phospho-p42/p44ERK activity. Therefore, endogenous increased eNOS, and decreased preproendothelin-1 and endothelin ETA receptor expression may play an important role in the amelioration of coronary microvascular and myocardial remodeling in DOCA-salt hypertensive rats.

In the present study, chronic administration of a subdepressor dose of a Ca^{2+} channel antagonist, cilnidipine, increased eNOS mRNA and protein expression and improved left ventricular hypertrophy and myocardial remodeling. With regard to Ca^{2+} channel antagonists and eNOS, several in vivo and in vitro experimental studies have been reported. Ding and Vaziri (1998) examined the effect of felodipine, a dihydropyridine Ca^{2+} channel antagonist, on eNOS expression in cultured rat aorta endothelial cells to distinguish whether the effects seen were due to a direct or indirect action of the drug on endothelial cells. The results showed a marked upregulation of eNOS expression after a 24-h incubation period with felodipine in cultured endothelial cells. In addition, authors also demonstrated that nifedipine, dihydropyridine and diltiazem, benzothiazepine Ca^{2+} channel antagonists, upregulated eNOS expression in cultured human coronary artery endothelial cells. However, no such effects were observed with verapamil, which belongs to the phenylalkylamine class of L-type Ca^{2+} channel antagonists (Ding and Vaziri, 2000). Moreover, Wang and Vaziri (1999) showed that erythropoietin downregulates basal and acetylcholine-stimulated NO production, depresses eNOS expression, and stimulates proliferation in isolated human coronary artery endothelial cells. The suppressive effects of erythropoietin on NO production and on eNOS expression are reversed by the Ca^{2+} channel antagonist, felodipine. We also evaluated the effects of long-term treatment with the L-type dihydropyridine Ca^{2+} channel antagonists, benidipine (Kobayashi et al., 1999b) and amlodipine (Kobayashi et al., 1999c), on gene expression of eNOS mRNA in the left ventricle and examined these effects in relation to coronary microvascular and myocardial remodeling in two-kidney, one-clip Goldblatt rats (Kobayashi et al., 1999b) and in rats made hypertensive by long-term blockade of NO synthesis by the chronic administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME) (Kobayashi et al., 1999c). Downregulation of eNOS mRNA expression in the left ventricle of these models was significantly increased, and myocardial remodeling was significantly ameliorated by a subdepressor dose of Ca^{2+} channel antagonists. These results indicate that eNOS expression may have an important role in the beneficial cardioprotective effect of myocardial remodeling. Additional studies are needed to elucidate the mechanism by which dihydropyridine Ca^{2+} channel antagonists upregulate eNOS mRNA and protein expression. Recently, however, some interesting reports have been shown that amlodipine releases NO from blood vessels, unlike nifedipine and diltiazem, and the mechanism responsible for the release of NO by amlodipine is similar to that of angiotensin-converting enzyme inhibitors, that is, modulation of the actions or formation of kinins (Zhang and Hintze, 1998). Furthermore, Zhang et al. (1999) indicated that perfusion with amlodipine increases NO release from the coronary microvasculature of failing human cardiac explants. This phenomenon was blockable by concurrent use of a bradykinin B_2 antagonist or a kallikrein inhibitor, suggesting a kinin-mediated mechanism. This may partly contribute to the beneficial therapeutic actions of this drug in the treatment of DOCA-salt hypertensive rats.

In the present study, we demonstrated that augmentation of preproendothelin-1 and endothelin ETA receptor expression in the left ventricle of DOCA-salt hypertensive rats is significantly decreased by the subdepressor dose of cilnidipine. Karam et al. (1996) indicated that DOCA-salt hypertensive rats exhibited a marked cardiac hypertrophy associated with a decrease of maximal coronary blood flow and interstitial and perivascular fibrosis, and that endothelin might play an important, independent role in regulating this hypertrophy as well as collagen accumulation within the left ventricle. They concluded that mibefradil, a T-type Ca^{2+} channel antagonist, was able to prevent it, and that, in DOCA-salt hypertension, endothelin-1 might play a role apart from increasing high blood pressure in cardiac remodeling. In addition, Li et al. (1996) indicated that DOCA-salt hypertensive rats exhibit a very severe degree of cardiovascular hypertrophy, which may in part be mediated by overexpression of the endothelin-1 gene. Treatment with the Ca^{2+} channel antagonist, mibefradil, resulted in lower blood pressure, reduced cardiac hypertrophy, a near-normal structure of conduit and small arteries and a lower endothelin-1 mRNA expression. Authors demonstrated that mibefradil might interfere with mechanisms underlying vascular hypertrophy in DOCA-salt hypertensive rats via blockade of Ca^{2+} entry or by reducing vascular endothelin-1 gene expression when the blood pressure is lowered. With regard to the effect of Ca^{2+} channel antagonists on endothelin-1 expression in other experimental hypertensive rats, Feron et al. (1996) showed that lacidipine, a dihydropyridine-type Ca^{2+} channel antagonist, exerted a dose-related inhibition of ventricular hypertrophy and preproendothelin-1 mRNA expression in stroke-prone spontaneously hypertensive rats, and that this effect was unrelated to systolic blood pressure changes. The dose dependence of this inhibition suggested that salt-induced cardiac hypertrophy could be related to endothelin-1 gene overexpression. Moreover, endothelin release from cultured coronary smooth muscle and endothelial cells is decreased by diltiazem and verapamil, and the anti-atherosclerotic effect of Ca^{2+} channel antagonists might be mediated in part by a reduction in endothelin

secretion (Haug et al., 1998). These results suggest that inhibition of preproendothelin-1 and endothelin ETA receptor expression may contribute to the beneficial actions of the Ca^{2+} channel antagonist, cilnidipine, in DOCA-salt hypertensive rats.

In the DOCA-salt hypertensive rat model, Kubo et al. (2000) showed that endothelium removal-induced ERK activation is enhanced in aorta strips and therefore suggested that the vascular structural remodeling function may be enhanced. In addition, because the endothelin receptor antagonist, BQ123, inhibited ERK activation, they concluded that the enhancement of ERK activation results at least in part from enhanced function of the vascular endothelin system in this model. In the present study, preproendothelin-1 and endothelin ETA receptor expression is upregulated in the left ventricle. Therefore, these results suggested that the enhanced reactivity of the endothelin system might contribute to the enhanced left ventricular phospho-p42/p44 ERK activity in DOCA-salt hypertensive rats. Whereas, with regard to the Ca^{2+} channel antagonist and p42/p44 ERK activity, Katoch et al. (1999) has reported that intracellular calcium could dose dependently activate p42/p44 ERK in vascular smooth muscle, and that ERK activity could be decreased by inhibiting calcium and calmodulin kinase II, suggesting that calcium plays a role in upregulated ERK activation. In addition, Zhang et al. (2000) demonstrated that amlodipine inhibited basic fibroblast growth factor-induced p42/p44 ERK activation in a dose-dependent manner, and suggested that basic fibroblast growth factor-induced vascular smooth muscle cell proliferation may be related to p42/p44 ERK activation, and that the antiproliferative effect of amlodipine may be related to its inhibition of ERK activation. The present study has demonstrated that left ventricular phospho-p42/p44ERK activity is upregulated in DOCA-salt hypertensive rats, and that chronic administration of cilnidipine effectively improved left ventricular hypertrophy and coronary microvascular remodeling, and inhibited phospho-p42/p44ERK activity. These results suggested that the protective effect of cilnidipine on coronary microvascular remodeling might be at least in part related to inhibition of phospho-p42/p44 ERK activation.

In summary and conclusion, we have evaluated the effects of long-term treatment with cilnidipine on expression of eNOS, preproendothelin-1 and endothelin ETA receptor in the left ventricle, and the relation of these effects to coronary microvascular remodeling and phospho-p42/p44 ERK activity in DOCA-salt hypertensive rats. The results showed that downregulation of eNOS mRNA and protein expression in DOCA-vehicle was significantly increased, and the upregulated preproendothelin-1 and endothelin ETA receptor expression and phospho-p42/p44 ERK activities in DOCA-vehicle rats was significantly decreased in the left ventricle by cilnidipine treatment. These results led us to conclude that phospho-p42/p44 ERK activities may contribute to the coronary microvascular remodeling of DOCA rats, and that coronary microvascular remodeling of DOCA-salt hypertensive rats was significantly ameliorated by a subdepressor dose of cilnidipine, which may be due to an increase in eNOS mRNA and protein expression and a decrease in endothelin-1 and endothelin ETA receptor expressions in the left ventricle.

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References

Barton, M., d'Uscio, L.V., Shaw, S., Meyer, P., Moreau, P., Lüscher, T.F., 1998. ETA receptor blockade prevents increased tissue endothelin-1, vascular hypertrophy, and endothelial dysfunction in salt-sensitive hypertension. Hypertension 31, 499–504 [Part 2].

Bogoyevitch, M.A., Anderson, M.B., Gillespie-Brown, J., Clerk, A., Glennon, P.E., Fuller, S.J., Sugden, P.H., 1996. Adrenergic receptor stimulation of the mitogen-activated protein kinase cascade and cardiac hypertrophy. Biochem. J. 314, 115–121.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Cooke, J.P., Dzau, V.J., 1997. Nitric oxide synthase: role in the genesis of vascular disease. Annu. Rev. Med. 48, 489–509.

Davis, R.J., 1993. The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 268, 14553–14556.

Ding, Y., Vaziri, N.D., 1998. Calcium channel blockade enhances nitric oxide synthase expression by cultured endothelial cells. Hypertension 32, 718–723.

Ding, Y., Vaziri, N.D., 2000. Nifedipine and diltiazem but not verapamil up-regulate endothelial nitric-oxide synthase expression. J. Pharmacol. Exp. Ther. 292, 606–609.

Feron, O., Salomone, S., Godfraind, T., 1996. Action of the calcium channel blocker lacidipine on cardiac hypertrophy and endothelin-1 gene expression in stroke-prone hypertensive rats. Br. J. Pharmacol. 118, 659–664.

Haug, C., Voisard, R., Baur, R., Hannekum, A., Hombach, V., Gruenert, A., 1998. Effect of diltiazem and verapamil on endothelin release by cultured human coronary smooth-muscle cells and endothelial cells. J. Cardiovasc. Pharmacol. 31 (Suppl. 1), S388–S391.

Higashi, T., Kobayashi, N., Hara, K., Shirataki, H., Matsuoka, H., 2000. Effects of angiotensin II type 1 receptor antagonist on nitric oxide synthase expression and myocardial remodeling in Goldblatt hypertensive rats. J. Cardiovasc. Pharmacol. 35, 564–571.

Ishikawa, T., Yanagisawa, M., Kimura, S., Goto, K., Masaki, T., 1988. Positive inotropic action of novel vasoconstrictor peptide endothelin on guinea pig atria. Am. J. Physiol. 255, H970–H973.

Karam, H., Heudes, D., Hess, P., Gonzales, M.F., Löffler, B.M., Clozel,

M., Clozel, J.P., 1996. Respective role of humoral factors and blood pressure in cardiac remodeling of DOCA hypertensive rats. Cardiovasc. Res. 31, 287–295.

Katoch, S.S., Su, X., Moreland, R.S., 1999. Ca^{2+}- and protein kinase C-dependent stimulation of mitogen activated protein kinase in detergent–skin vascular smooth muscle. J. Cell. Physiol. 179, 208–217.

Kim, S., Izumi, Y., Yano, M., Hamaguchi, A., Miura, K., Yamanaka, S., Miyazaki, H., Iwao, H., 1998. Angiotensin blockade inhibits activation of mitogen-activated protein kinases in rat balloon-injured artery. Circulation 97, 1731–1737.

Kobayashi, N., Kobayashi, K., Kouno, K., Yagi, S., Matsuoka, H., 1997. Effect of benidipine on microvascular remodeling and coronary flow reserve in two-kidney one clip Goldblatt hypertension. J. Hypertens. 15, 1285–1294.

Kobayashi, N., Higashi, T., Hara, K., Shirataki, H., Matsuoka, H., 1999a. Effects of imidapril on NOS expression and myocardial remodelling in failing heart of Dahl salt-sensitive hypertensive rats. Cardiovasc. Res. 44, 518–526.

Kobayashi, N., Kobayashi, K., Hara, K., Higashi, T., Yanaka, H., Yagi, S., Matsuoka, H., 1999b. Benidipine stimulates nitric oxide synthase and improves coronary circulation in hypertensive rats. Am. J. Hypertens. 12, 483–491.

Kobayashi, N., Yanaka, H., Tojo, A., Kobayashi, K., Matsuoka, H., 1999c. Effects of amlodipine on nitric oxide synthase mRNA expression and coronary microcirculation in prolonged nitric oxide blockade-induced hypertensive rats. J. Cardiovasc. Pharmacol. 34, 173–181.

Kobayashi, N., Hara, K., Higashi, T., Matsuoka, H., 2000a. Effects of imidapril on endothelin-1 and ACE gene expression in failing heart of salt-sensitive hypertensive rats. Am. J. Hypertens. 13, 1088–1096.

Kobayashi, N., Hara, K., Watanabe, S., Higashi, T., Matsuoka, H., 2000b. Effect of imidapril on myocardial remodeling in L-NAME-induced hypertensive rats is associated with gene expression of NOS and ACE mRNA. Am. J. Hypertens. 13, 199–207.

Kojima, M., Shiojima, I., Yamazaki, T., Komuro, I., Zou, Z., Wang, Y., Mizuno, T., Ueki, K., Tobe, K., Kadowaki, T., Nagai, R., Yazaki, Y., 1994. Angiotensin II receptor antagonist TCV-116 induces regression of hypertensive left ventricular hypertrophy in vivo and inhibits the intracellular signaling pathway of stretch-mediated cardiomyocyte hypertrophy in vitro. Circulation 89, 2204–2211.

Kubo, T., Ibusuki, T., Saito, E., Kambe, T., Hagiwara, Y., 2000. Different activation of vascular mitogen-activated protein kinases in spontaneously and DOCA-salt hypertensive rats. Eur. J. Pharmacol. 400, 231–237.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lerman, A., Edwards, B.S., Hallett, J.W., Heublein, D.M., Sandberg, S.M., Burnett, J.C., 1991. Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. N. Engl. J. Med. 325, 997–1011.

Li, J.S., Sventek, P., Schiffrin, E.L., 1996. Effect of antihypertensive treatment and N^{ω}-nitro-L-arginine methyl ester on cardiovascular structure in deoxycorticosterone acetate-salt hypertensive rats. J. Hypertens. 14, 1331–1339.

Lüscher, T.F., Vanhoutte, P.M., Raij, L., 1987. Antihypertensive treatment normalizes decreased endothelium-dependent relaxations in rats with salt-induced hypertension. Hypertension 9, III193–III197.

Minami, J., Ishimitsu, T., Higashi, T., Numabe, A., Matsuoka, H., 1998. Comparison between cilnidipine and nisoldipine with respect to effects on blood pressure and heart rate in hypertensive patients. Hypertens. Res. 21, 215–219.

Sakurai, T., Yanagisawa, M., Inoue, A., Ryan, U.S., Kimura, S., Mitsui, Y., Goto, K., Masaki, T., 1991. cDNA cloning, sequence analysis and tissue distribution of rat preproendothelin-1 mRNA. Biochem. Biophys. Res. Commun. 175, 44–47.

Schwarzkopff, B., Motz, W., Frenzel, H., Vogt, M., Knauer, S., Strauer, B.E., 1993. Structural and functional alterations of the intramyocardial coronary arterioles in patients with arterial hypertension. Circulation 88, 993–1003.

Seki, T., Naruse, M., Naruse, K., Katafuchi, T., Lodhi, K.M., Yoshimoto, T., Hagiwara, H., Demura, H., Hirose, S., 1997. Gene expression of endothelial type isoform of nitric oxide synthase in various tissues of stroke-prone spontaneously hypertensive rats. Hypertens. Res. 20, 43–49.

Takanohashi, A., Tojo, A., Kobayashi, N., Yagi, S., Matsuoka, H., 1996. Effect of trichlormethiazide and captopril on nitric oxide synthase activity in the kidney of deoxycorticosterone acetate-salt hypertensive rats. Jpn. Heart J. 37, 251–259.

Takase, H., Moreau, P., Kung, C.F., Nava, E., Lüscher, T.F., 1996. Antihypertensive therapy prevents endothelial dysfunction in chronic nitric oxide deficiency. Effect of verapamil and trandolapril. Hypertension 27, 25–31.

Terada, Y., Tomita, K., Nonoguchi, H., Marumo, F., 1992a. Different localization of two types of endothelin receptor mRNA in microdissected rat nephron segments using reverse transcription and polymerase chain reaction assay. J. Clin. Invest. 90, 107–112.

Terada, Y., Tomita, K., Nonoguchi, H., Marumo, F., 1992b. Polymerase chain reaction localization of constitutive nitric oxide synthase and soluble guanylate cyclase messenger RNAs in microdissected rat nephron segments. J. Clin. Invest. 90, 659–665.

Tominaga, M., Ohya, Y., Tsukashima, A., Kobayashi, K., Takata, Y., Koga, T., Yamashita, Y., Fujishima, Y., Abe, I., Fujishima, M., 1997. Ambulatory blood pressure monitoring in patients with essential hypertension treated with a new calcium antagonist, cilnidipine. Cardiovasc. Drugs Ther. 11, 43–48.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354.

Uneyama, H., Takahara, A., Dohmoto, H., Yoshimoto, R., Inoue, K., Akaike, N., 1997. Blockade of N-type Ca current by cilnidipine (FRC8653) in acutely dissociated rat sympathetic neurones. Br. J. Pharmacol. 122, 37–42.

Verdecchia, P., Porcellati, C., Schillaci, G., Borgioni, C., Ciucci, A., Battistelli, M., Guerrieri, M., Gatteschi, C., Zampi, I., Santucci, A., Santucci, C., Reboldi, G., 1994. Ambulatory blood pressure: an independent predictor of prognosis in hypertension. Hypertension 24, 793–801.

Wang, X.Q., Vaziri, N.D., 1999. Erythropoietin depresses nitric oxide synthase expression by human endothelial cells. Hypertension 33, 894–899.

Weber, K.T., Brilla, C.G., 1991. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin–angiotensin–aldosterone system: Pathological hypertrophy and cardiac interstitium. Circulation 83, 1849–1865.

Yamazaki, T., Komuro, I., Zou, Y., Yazaki, Y., 1999. Hypertrophic responses of cardiomyocytes induced by endothelin-1 through the protein kinase C-dependent but Src and Ras-independent pathways. Hypertens. Res. 22, 113–119.

Zhang, X., Hintze, T., 1998. Amlodipine releases nitric oxide from canine coronary microvessels: an unexpected mechanism of action of a calcium channel-blocking agent. Circulation 97, 576–580.

Zhang, X., Kichuk, M.R., Mital, S., Oz, M., Michler, R., Nasjletti, A., Kaley, G., Hintze, T.H., 1999. Amlodipine promotes kinin-mediated nitric oxide production in coronary microvessels of failing human hearts. Am. J. Cardiol. 84, 27L–33L.

Zhang, Y.Z., Gao, P.J., Wang, X.Y., Stepien, O., Marche, P., Zhang, Z.L., Zhu, D.L., 2000. The inhibitory mechanisms of amlodipine in human vascular smooth muscle cell proliferation. Hypertens. Res. 23, 403–406.