Possible Involvement of Endothelin-1 in Cardioprotective Effects of Benidipine


Benidipine hydrochloride has been developed as an antagonist for the L-type calcium channel and is used as an anti-hypertensive drug. But recent studies have reported that benidipine exerts not only anti-hypertensive actions but also anti-hypertrophic actions on cardiac muscles. Endothelin-1 (ET-1), one of the endogenous pathological humoral factors of cardiovascular diseases such as hypertension and heart failure, has a strong vasoconstrictive action and could induce hypertension and cardiac hypertrophy. So, it is a matter of great interest whether or not calcium antagonists can decrease cardiac hypertrophy induced by the pathological vasoactive substances such as ET-1. Thus, the present study was designed to elucidate the effects of benidipine on cardiac hypertrophy, and particularly on the interaction with ET-1, using neonatal rat cardiac myocytes (MCs) and cardiac non-myocytes (NMCs) culture systems. Cells were cultured with or without ET-1, benidipine, and nifedipine and the effects of calcium antagonists on cardiac hypertrophy were evaluated by incorporations of [3H]-leucine and [3H]-thymidine into MCs and/or NMCs. Benidipine significantly decreased the ET-1-induced increase of [3H]-leucine and [3H]-thymidine uptake into cardiac MCs and NMCs, whereas no significant effects of nifedipine were observed. Furthermore, benidipine (10^{-8}M) attenuated ET-1 secretions from NMCs. In summary, benidipine at least partially decreased the cardiac hypertrophy induced by paracrine mechanisms through its attenuation of ET-1 secretions from NMCs. Benidipine could thus be a useful tool for preventing cardiac hypertrophy due to hypertension. (Hypertens Res 2000; 23: 491-496)

Key Words: benidipine, hypertension, cardiac hypertrophy, endothelin-1

Introduction

Hypertension causes various cardiovascular diseases, such as cerebral infarction and hemorrhage, development of aortic aneurysm, progression of atherosclerosis, renal failure (nephrosclerosis), and cardiac hypertrophy leading to cardiac dysfunction and ischemia. Endothelin-1 (ET-1), which was originally discovered as a strong vasoconstrictor expressed in various tissues, including heart tissue (1, 2), is thought to be a causative agent of cardiac hypertrophy (3-7). In addition, ET-1 has also been reported to be elevated in the plasma of patients with hypertension and cardiac failure (8-11). Because it is important to prevent complications of hypertension, especially complications of the cardiovascular system, as previously reported by Levy et al. (12), it is necessary to evaluate the effects of antihypertensive drugs on cardiac hypertrophy. A recent study reported that nilvadipine, a calcium channel antagonist, attenuated the induction of ET-1 mRNA in var-
ious tissues (13), indicating that some calcium channel antagonists could exert anti-hypertrophic actions independent of blood-pressure reduction. In recent studies, (+) - (R*)-2,6-dimethyl-4-(m-nitrophenol)-1,4-dihydropyridine-3,5-dicarboxic acid (R*)-1-benzyl-3-piperidinyl ester, or methyl ester hydrochloride (benidipine hydrochloride), which is one of the dihydropyridine derivatives developed as an antagonist for the L-type calcium channel, was prescribed to treat hypertension (14–18), and was shown to exert not only anti-hypertensive actions but also anti-proliferative effects on cardiovascular cells (19, 20). Furthermore, benidipine has been reported to have various other useful cardiovascular actions (21–23). Based on these facts, it is a matter of great interest whether or not benidipine might interfere with hypertrophic effects, particularly those induced by ET-1. The present study was therefore designed to elucidate the anti-hypertrophic actions of benidipine. The effects of benidipine were compared with those of nifedipine, one of the widely-used calcium channel antagonists, using cultured neonatal rat cardiomyocytes.

Materials and Methods

Cell Culture

MCs and cardiac non-myocytes (NMCs), which consisted mainly of cardiac fibroblasts, were prepared from the cardiac ventricles of 2- to 4-day-old neonatal Wistar rats by the previously described Percoll gradient and adhesion method with slight modification (3–5). Briefly, ventricular cardiomyocytes were minced and dispersed in a balanced solution containing 0.04% collagenase IA (Sigma Chemical Co., St Louis, MO) and 0.06% pancreatin (GIBCO Laboratories, Grand Island, NY) with agitation for 20 min at 37°C and digested three times. The differentiation of MCs from NMCs was performed by the discontinuous Percoll gradient method. MCs selectively migrated to the interface of the discontinuous layers after centrifugation at 3,000 rpm for 30 min at 20°C, and NMCs migrated to the surface of the upper Percoll layer. NMCs were suspended in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO Laboratories) containing 10% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin G (PCG) and 10 μg/ml streptomycin (SM)) and plated in 10 cm dishes. The dishes were washed with phosphate-buffered saline (PBS) after 30-min incubation to remove endothelial cells (4), and NMCs were incubated in DMEM containing 10% FCS (the 1st generation). When NMCs reached sub-confluence, they were removed by trypsinization (0.25% trypsin in EDTA-2Na) and again incubated with DMEM containing 10% FCS (the 2nd generation). MCs were plated in 24-well plates at a density of 1.0×10⁵ cells/well with (MCs/NMCs) or without the 3rd generation of NMCs at a density of 1.0×10⁵ cells/well and incubated with DMEM containing 10% FCS. Third-generation NMCs were also plated in 24-well plates at a density of 1.0×10⁵ cells/well and incubated with DMEM containing 10% FCS.

Measurement of Incorporation of Tracers into Cultured Cells

After plating MCs, MCs/NMCs, and NMCs in 24-well plates, MCs and MCs/NMCs were incubated for 30 h and NMCs were incubated for 48 h. MCs and MCs/NMCs were further incubated for 10 h and NMCs were incubated for 48 h in serum-free DMEM. MCs and MCs/ NMCs were incubated for 24 h, and NMCs for 18 h, in DMEM containing 0.1% bovine serum albumin (BSA) with or without various compounds, such as synthetic ET-1 (10⁻⁸ M; Peptide Institute, Osaka, Japan), benidipine hydrochloride (the gift of Kyowa Hakko Kogyo Co., Ltd., Shizuoka, Japan), and nifedipine (Sigma Chemical Co.). Then, 0.5 μCi/well of [³H]-leucine was added to the MCs and MCs/NMCs and incubated for 24 h. 0.5 μCi/ well of [³H]-thymidine was added to the NMCs and incubated for 18 h (both tracers were purchased from Life Science Products, Inc., Boston, MA). Benidipine and nifedipine were dissolved in demethyl sulfoxide (DMSO) and diluted to suitable concentrations (final concentrations of DMSO in culture medium were less than 0.05%). Concentrations of benidipine and nifedipine in culture medium were adjusted 10⁻⁸ M, 5×10⁻⁹ M, and 10⁻⁹ M according to their clinically used dosages as previously reported (24, 25).

Measurement of ET-1 in Culture Medium

After NMCs were plated in 24-well plates, cells were incubated with DMEM containing 10% FCS for 48 h and further incubated with serum-free DMEM for 48 h at 37°C. Then, culture medium was changed for 500 μl of DMEM containing 0.1% BSA with or without stimulation agents (vehicle, DMSO: 0.005%, 0.0025%, and 0.0005%; nifedipine: 10⁻⁸ M, 5×10⁻⁹ M, and 10⁻⁹ M) and incubated at 37°C. Concentrations of ET-1 in culture medium were measured using a specific radioimmunoassay (RIA) kit (Life Science Products, Inc.) after 24-h incubation with benidipine. The cross reactivities with ET-2, ET-3, and big endothelin were 53.3%, 3.8%, and 69.6% on a molar basis, respectively, and the minimum detectable quantity was 38 pg/ml.

Statistical Analysis

Statistical analysis was performed by Student’s t-test and analysis of variance (Stat View 4.5J, Abacus Concept, Berkeley, CA). P values less than 0.05 were considered to indicate statistical significance, and all data are ex-
pressed as the means ± SD.

**Ethics**

All experiments were performed in accordance with the Guidelines on Animal Experimentation of Jikei University.

**Results**

In the experiments on the effects of benidipine and nifedipine on MCs, both benidipine and nifedipine by themselves failed to decrease [3H]-leucine uptake into MCs. When benidipine was co-incubated with ET-1, benidipine (10⁻⁸ M) significantly decreased the ET-1 induced increase of [3H]-leucine uptake (10⁻⁸ M ET-1: 176.8 ± 9.7% of control; 10⁻⁸ M ET-1 + 10⁻⁸ M benidipine: 161.0 ± 2.2% of control; p < 0.05, respectively; Fig. 1A), although nifedipine did not (Fig. 1B). In the MCs/NMCs coculture system, not only did benidipine (10⁻⁸ M, 5 × 10⁻⁹ M, and 10⁻⁹ M) dose-dependently decrease [3H]-leucine uptake into MCs/NMCs when used alone (56.2 ± 3.0%, 64.7 ± 2.3%, and 80.8 ± 9.6% of control; p < 0.01, p < 0.05, respectively; Fig. 2A) but, when co-incu-
bated with ET-1, benidipine also dose-dependently decreased the ET-1-induced increase of [3H]-leucine uptake into MCs/NMCs (10^{-8} M ET-1 + 10^{-8} M, 5 \times 10^{-9} M, and 10^{-9} M benidipine; 101.7\pm 6.0\%, 107.4\pm 1.9\%, and 117.2\pm 0.8\% of control; p<0.01, p<0.01, and p<0.05 vs. 10^{-8} M ET-1; 133.6\pm 6.0\% of control, respectively; Fig. 2A). Nifedipine, on the other hand, did not decrease [3H]-leucine uptake into MCs/NMCs (Fig. 2B). In addition, although benidipine (10^{-8} M and 10^{-9} M) alone did not decrease [3H]-thymidine uptake into NMCs, benidipine (10^{-8} M and 10^{-9} M) decreased the ET-1 (10^{-8} M)-induced increase of [3H]-thymidine uptake into NMCs (10^{-8} M ET-1 + 10^{-8} M and 10^{-9} M benidipine; 83.9\pm 23.4\%, and 94.0\pm 0.6\% of control; p<0.01, p<0.01 vs. 10^{-8} M ET-1; 122.1\pm 9.6\% of control, respectively; Fig. 3). Nifedipine did not decrease [3H]-thymidine uptake into NMCs (data not shown).

Finally, as shown in Fig. 4, benidipine (10^{-8} M) decreased ET-1 secretions from NMCs (control: 57.2\pm 4.5 pg/well vs. 10^{-8} M benidipine: 43.9\pm 7.4 pg/well; p<0.01).

DMSO alone had no significant effect on either the incorporations of tracers into cultured cells or ET-1 secretions from NMCs (data not shown) at the dose used in the present study.

**Discussion**

Hypertension is a common disease, particularly in elderly populations, and causes cardiovascular diseases such as cerebral infarction and hemorrhage, development of aortic aneurysm, progression of atherosclerosis, renal failure due to nephrosclerosis, and cardiac hypertrophy leading to cardiac dysfunction and ischemia. Recently, the National Health Institute placed cardiac hypertrophy under the category of target organ damages/clinical cardiovascular disease by hypertension (26). In addition, a recent study reported that left ventricular mass as estimated by echocardiography was related to prognosis of hypertension (12). The prevention of cardiac hypertrophy is thus highly important in therapeutic strategies of hypertension.

Cardiac hypertrophy is not only caused by the increased afterload of an elevated systemic pressure (27), but also might be caused by various agents that induce cardiac hypertrophy. ET-1, which has been suggested to be elevated in the plasma of hypertensive patients (8), might be one of the strong endogenous hypertrophic factors for MCs, since ET-1 has been shown to exert a direct hypertrophic effect in several *in vivo* and *in vitro* studies (3-7). ET-1 had a direct hypertrophic effect on cardiac myocytes (3-5), and Nakamura et al. (7) demonstrated the involvement of ET-1 in cardiac hypertrophy associated with NO-nitro-L-arginine methyl ester (L-NAME)-induced hypertension. As for the mechanism of ET-1-induced cardiac hypertrophy, Yamazaki et al. (6) recently showed that ET-1 plays an important role in mechanical stress-induced cardiac hypertrophy through the protein kinase-C (PKC)-dependent but src and ras-independent pathways. Angiotensin II (Ang II), which is one of the key hormones in hypertension, and transforming growth factor-β1 (TGF-β1) have also been suggested to stimulate
ET-1 secretions from NMCs, and ET-1 secreted from NMCs could affect both MCs and NMCs in an autocrine/paracrine fashion (4). In fact, BQ-123, an antagonist for ETA receptor, inhibited secretions of atrial and brain natriuretic peptides, which were indicators of cardiac hypertrophy, from MCs stimulated by exogenous ET-1 and MCs/NMCs incubated with or without Ang II or TGF-β1. In addition, Naruse et al. (13) reported that nilvadipine, a calcium channel antagonist, attenuated the expression of ET-1 mRNA in various organs, including the heart, indicating that some of the calcium channel antagonists can modulate synthesis and excretion of humoral factors. These facts indicated that ET-1 could be one of the candidate agents involved in cardiac hypertrophy in hypertensive patients, although other vasoactive substances might also be involved in cardiac hypertrophy.

The present study strongly implied that, in addition to the decrease of elevated afterload by lowering blood pressure, inhibitory effects on ET-1 secretion could be involved in the anti-hypertensive mechanisms of benidipine. This hypothesis was also supported not only by the facts that anti-hypertrophic actions of benidipine were demonstrated in MCs/NMCs and NMCs (Figs. 2A and 3), whereas benidipine failed to decrease [3H]-leucine uptake into MCs without ET-1 (Fig. 1A), but also by the fact that benidipine (10⁻⁸ M) decreased ET-1 secretions from NMCs.

Because the ET-1 pathway in MCs involves the PKC pathway (6, 28) and the sarcolemma T-type calcium channel, in addition to the L-type calcium channel (28), benidipine was unlikely to have completely inhibited. Tanaka and Deguchi reported that benidipine showed a cardioprotective effect against hypoxia by decreasing the activity of MCs (21), and other studies have reported that benidipine stimulates nitric oxide (NO) synthetase (29, 30). The fact that chronic inhibition of NO by L-NAME caused cardiac hypertrophy (7) implies that stimulation of NO synthesis could result in reduction of cardiac hypertrophy. Therefore, the decrease of [3H]-leucine uptake into MCs co-incubated with ET-1 (10⁻⁸ M) and benidipine (10⁻⁸ M) suggests that benidipine might decrease the activity of MCs by blocking the L-type calcium channel and/or that benidipine might stimulate NO production (29, 30). On the other hand, the benidipine-induced decreases of [3H]-leucine uptake into MCs/NMCs incubated with or without ET-1 were more prominent than those into MCs. Based on these facts, it could be speculated that, at least in part, the benidipine-induced inhibition of ET-1 secretions via a paracrine mechanism might be more associated with attenuation of cardiac hypertrophy, although a possible role of other known or unknown growth factors in the attenuation of cardiac hypertrophy by benidipine cannot be ruled out.

In contrast to benidipine, nifedipine did not significantly decrease the anti-hypertrophic actions in either MCs or NMCs. Although previous studies have reported that nifedipine decreased left ventricular mass (31, 32), it has been speculated that the mechanism by which nifedipine decreased left ventricular hypertrophy might be associated with the reduction of blood pressure (33). In addition, our present results, as well as the previously reported failure of nifedipine to decrease clenbuterol-simulated muscle hypertrophy (34), would seem to support the above hypothesis. Therefore, nifedipine does not seem to play a major role in inhibition of cardiac hypertrophy via a paracrine mechanism. Benidipine might thus be a useful agent for preventing cardiac hypertrophy, since it has previously shown such useful effects as inhibition of the insulin-induced increase of [3H]-thymidine uptake into rat aortic smooth muscle cells (20) and cardioprotection against hypoxic myocardial injury (21), and since, in the present study, it showed anti-hypertrophic effects on cardiomyocytes by attenuating ET-1 secretions from NMCs.

In conclusion, the present study suggested that benidipine hydrochloride can at least partially attenuate the cardiac hypertrophy induced by paracrine mechanisms through its attenuation of ET-1 production in the heart. These findings suggest that benidipine could be a useful therapeutic tool in the prevention of cardiac hypertrophy due to hypertension.

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References


