Original Article

Benidipine, a Dihydropyridine-Ca^{2+} Channel Blocker, Increases the Endothelial Differentiation of Endothelial Progenitor Cells *In Vitro*

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Benidipine is a dihydropyridine-Ca^{2+} channel blocker used in the treatment of hypertension and angina pectoris. In the present study, we examined the effects of benidipine on the endothelial differentiation of circulating endothelial progenitor cells (EPCs) using an *in vitro* culture method. Peripheral blood derived mononuclear cells (PBMCs) containing EPCs were isolated from C57BL/6 mice, and then the cells were cultured on vitronectin/gelatin-coated slide glasses. After 7 days of culture, endothelial cells differentiated from EPCs were identified as adherent cells with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine–labeled acetylated low density lipoprotein (DiI-Ac-LDL) uptake and lectin binding under a fluorescent microscope. Incubation of PBMCs for 7 days with benidipine (0.01–1 μmol/l) significantly increased the number of DiI-Ac-LDL+/fluorescein isothiocyanate-lectin (FITC-Lectin)+ cells. Wortmannin, a phosphoinositide-3 kinase (PI3K) inhibitor, selectively attenuated the effect of benidipine on the endothelial differentiation. In addition, benidipine treatment augmented the phosphorylation of Akt, indicating that the PI3K/Akt pathway contributed, at least in part, to the endothelial differentiation induced by benidipine. These results suggest that the treatment with benidipine may increase the endothelial differentiation of circulating EPCs and contribute to endothelial protection, prevention of cardiovascular disease, and/or an improvement of the prognosis after ischemic damage. (*Hypertens Res* 2006; 29: 1047–1054)

***Key Words*: benidipine, Ca^{2+} channel blocker, endothelial progenitor cell, phosphoinositide-3 kinase, Akt**

Introduction

Improved neovascularization is a therapeutic goal to rescue tissues from critical ischemia (*1*). Vascularization of ischemic tissues in adults was once thought to be mediated by migration and proliferation of mature endothelial cells. However, a variety of evidence has suggested that circulating endothelial progenitor cells (EPCs) derived from bone marrow home to sites of ischemia and contribute to the formation of new blood vessels (*2*, *3*).

Endothelial dysfunction plays an important role in the pathogenesis of cardiovascular diseases. Recent studies have reported that the number of circulating EPCs was reduced in patients with multiple cardiovascular risk factors, and endothelial dysfunction was inversely correlated with the number of circulating EPCs (*4*, *5*). On the other hand, other studies showed that vascular trauma or acute myocardial infarction resulted in an increase in the number of circulating EPCs (*6*, *7*). These findings suggest that circulating EPCs may contribute to prevention of cardiovascular disease and/or to improvement of the prognosis after ischemic damage. In fact, transplantation of *ex vivo* expanded EPCs (*8*) or granulocyte colony–stimulating factor mobilized CD34+ cells (*9*) was

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shown to improve myocardial function and limit myocardial remodeling after myocardial ischemia.

Benidipine is a 1,4-hydropyridine Ca^{2+} channel blocker possessing slow-onset and long-lasting vasodilating effects and clinically useful in the treatment of hypertension and angina pectoris (*10*). It has been shown that the treatment of hypertensive patients with benidipine inhibits oxidative stress (*11*) and improves arterial stiffness (*12*), and that benidipine treatment led to an improved prognosis in patients with vasospastic angina (*13*). In experimental models of endothelial cell damages associated with hypertension (*14*, *15*), ischemia-reperfusion injury (*16*, *17*), and challenge with toxic chemicals such as sodium citrate (*18*) or vitamin D2 plus nicotine (*19*), benidipine prevented endothelial dysfunction. Previous studies have demonstrated that benidipine exhibits vascular endothelial protective effects such as stimulation of nitric oxide (NO) synthesis (*20–22*), anti-oxidant and anti-inflammation effects (*19*, *23*), which were independent of the antagonistic action of benidipine on L-type Ca^{2+} channels. These endothelial protective effects of benidipine may be at least partly responsible for the beneficial effects against cardiovascular diseases.

In the present study, to obtain further insight into the vasoprotective effects of benidipine, we examined the effect of benidipine on the endothelial differentiation of murine circulating EPCs using an *in vitro* culture method.

Methods

Murine Peripheral Blood Mononuclear Cells Culture

All procedures using mice were performed in conformity with institutional guidelines in compliance with national laws and policies. Murine peripheral blood was obtained from C57BL/6 male mice (CLEA Japan, Inc., Tokyo, Japan) at 6–8 weeks of age and separated by Histopaque-1083 (Sigma-Aldrich, St. Louis, USA) density gradient centrifugation. Light density peripheral blood mononuclear cells (PBMCs) were harvested and washed with Dulbecco's phosphate-buffered saline (PBS; no calcium or magnesium) (Invitrogen, Carlsbad, USA) supplemented with 2 mmol/l ethylenediamine tetraacetic acid (EDTA) (DPBS-E). Contaminated red blood cells were hemolyzed using ammonium chloride solution (0.8% NH_4Cl, 0.1% $KHCO_3$, 0.06% EDTA). After rinsing with DPBS-E, 1×10^6 cells/cm^2 PBMCs were cultured in EBM-2 (Clonetics, San Diego, USA) medium supplemented with 2% or 5% fetal bovine serum (FBS) and EGM-2-MV SingleQuots (except hydrocortisone; Clonetics) on 4- or 8-well glass slides coated with rat plasma vitronectin (Sigma-Aldrich) and bovine gelatin (Sigma-Aldrich). Benidipine hydrochloride ((±)-(R*)-3-((R*)-1-benzyl-3-piperidyl)methyl-1,4-dihydro-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate hydrochloride) (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) and wortmannin (Sigma-Aldrich) were dissolved in ethanol and dimethyl sulfoxide, respectively, and added to the culture. Half of the medium was changed for fresh medium with or without the compound after 3–4 days. After 7 days of culture, adherent cells were subjected to the following analysis.

Identification of the Differentiated Endothelial Cells

After 7 days of culture, adherent cells were incubated with 2 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine–labeled acetylated low density lipoprotein (DiI-Ac-LDL) (Biomedical Technologies, Stoughton, USA) for 3 h and then fixed with 2% paraformaldehyde for 10 min. After washing with PBS, the cells were reacted with 10 μg/ml fluorescein Griffonia simplicifolia lectin I, isolectin B4 (FITC-Lectin) (Vector Laboratories, Burlingame, USA) for 1 h. After nuclei staining with 4′,6-diamino-2-phenylindole (DAPI) (Vector Laboratories), the cells demonstrating DiI-Ac-LDL+/FITC-Lectin+ (double-positive cells) were identified as differentiated endothelial cells with a fluorescent microscope. The number of double-positive cells per well was evaluated by counting in 13 randomly selected high-magnification fields (×100)/cm^2.

Flow Cytometric Analysis

Adherent cells were detached with scratching after treatment with 0.25% trypsin. Cells were incubated for 45 min at 4°C with fluorescein isothiocyanate–conjugated (FITC-conjugated) anti-CD31 antibody or phycoerythrin-conjugated (PE-conjugated) anti-Flk-1 antibody (Becton Dickinson Japan, Tokyo, Japan). Isotype-matched antibodies served as controls. After washing, the cells were analyzed by a FACS Calibur flow cytometer (Becton Dickinson Japan).

Measurement of Adherent Viable Cells

Adherent viable cells were evaluated using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, USA). Cultured medium was removed and 100–200 μl of CellTiter-Glo reagent was added to the cells. After 10 min incubation at room temperature, luciferase activity as an index of ATP contents was measured using a Wallac 1420 ARVO fluoroscan (Perkin-Elmer Japan, Yokohama, Japan).

Western Blot Analysis

Cells were lysed in lysis buffer containing 20 mmol/l 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES) (pH 7.4), 0.2% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 5 μg/ml leupeptin. The protein concentration was determined using a *DC* Protein Assay kit (Bio-Rad, Hercules, USA). The lysate (30 μg) was solubilized in loading buffer consisting of 0.1 mol/l Tris-HCl (pH 6.0), 2% sodium dodecyl sulfate (SDS), and 5% 2-mercapto-

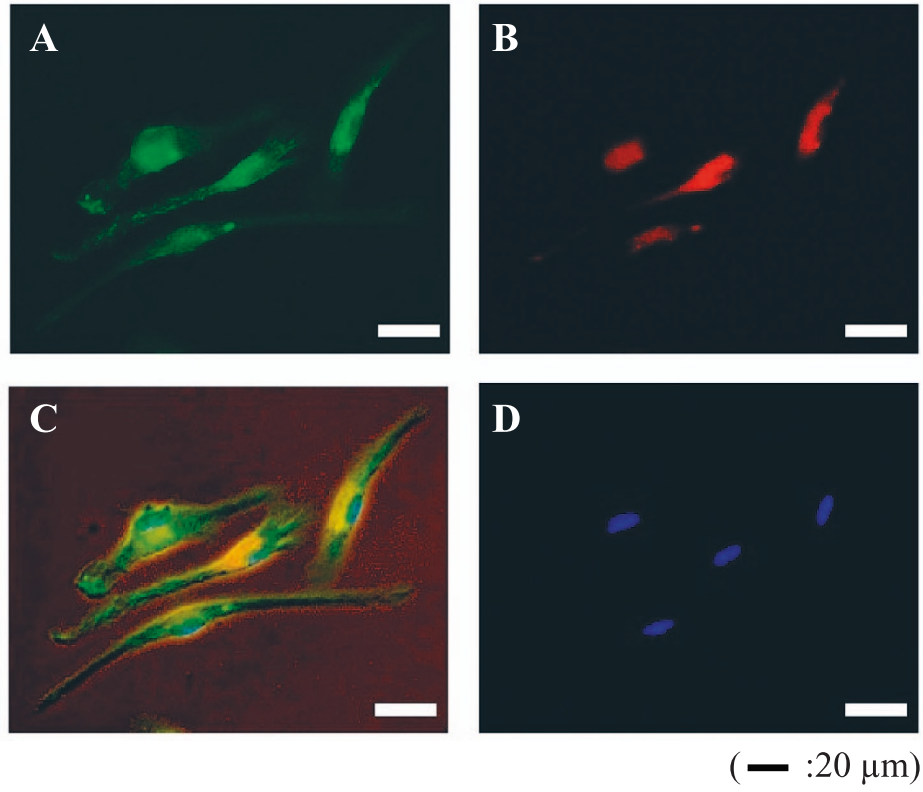

Fig. 1. *Murine endothelial cells differentiated from circulating endothelial progenitor cells (EPCs). Peripheral blood mononuclear cells (PBMCs) were harvested from C57BL/6 mice and cultured in 2% or 5% serum medium. After 7 days of culture, murine differentiated endothelial cells were recognized as cells with spindle-shaped morphology (C) and double-stained cells with FITC-Lectin binding (A, green) and DiI-Ac-LDL uptake (B, red) were identified under a fluorescent microscope. C: Merge. D: Nuclei were stained with DAPI.*

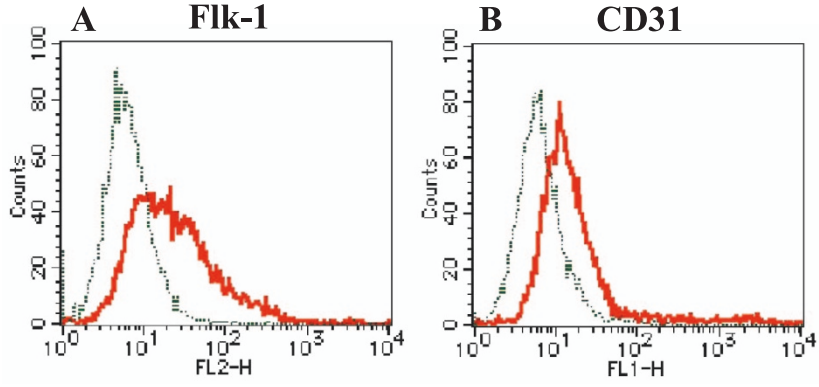

Fig. 2. *Flow cytometric analysis of adherent cells after 7 days of culture. Adherent cells after 7 days of culture were harvested and stained with PE-conjugated anti-Flk-1 antibody (A, red line) or FITC-conjugated anti-CD31 antibody (B, red line). Black dot-line: fluorescent-labeled control IgG.*

ethanol, boiled for 5 min, and separated by 8.5% SDS-polyacrylamide gel electrophoresis. The proteins were then electrophoretically transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% bovine serum albumin (BSA) and incubated with rabbit polyclonal anti-Akt antibody or anti-phosphorylated serine-473 residue of Akt antibody (Cell Signaling Technology Inc., Beverly, USA) in Tris-buffered saline containing 0.05% Tween 20 and

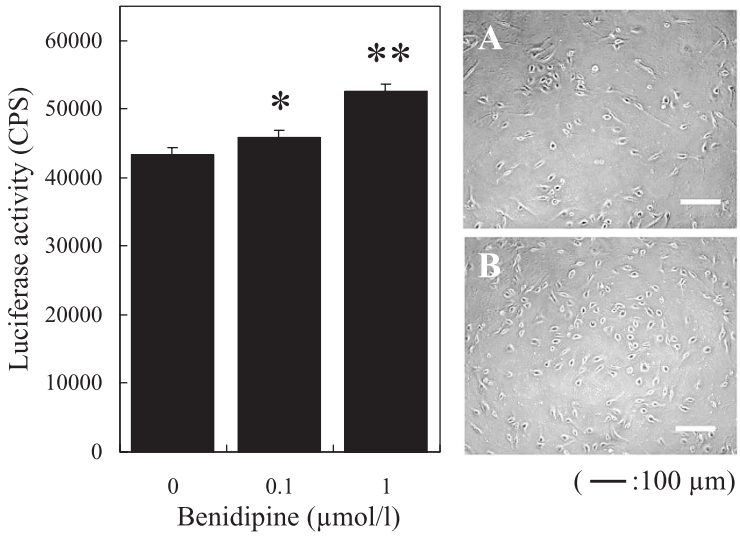

Fig. 3. *Benidipine increased the number of adherent viable cells after 7 days of culture. Murine PBMCs were incubated with benidipine at the indicated concentrations for 7 days in 5% serum medium, and then the number of adherent viable cells was evaluated by a CellTiter-Glo Luminescent Cell Viability Assay. Benidipine increased the number of adherent viable cells significantly. Data are the means±SD of experiments performed in quadruplicate. Reproducible results were observed in two independent experiments.* *p*<0.05, **p*<0.01. *A, B: Microscopic images of adherent cells with (B) or without (A) 1 μmol/l of benidipine.*

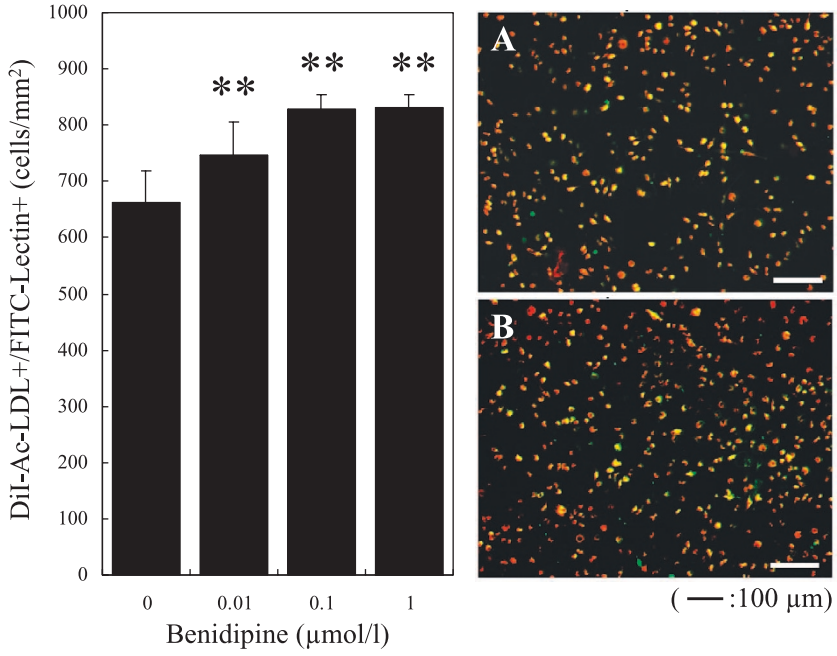

Fig. 4. *Benidipine increased the number of double-positive cells after 7 days of culture. Murine PBMCs were incubated with benidipine at the indicated concentrations for 7 days in 5% serum medium and the number of double-positive cells, which were stained with FITC-Lectin and DiI-Ac-LDL, was counted under a fluorescent microscope. Benidipine increased the number of double-positive cells significantly. Data are the means±SD of experiments performed in quadruplicate. Reproducible results were observed in two independent experiments.* **p*<0.01. A, B: Microscopic images of double-positive cells with (B) or without (A) 1 μmol/l of benidipine.*

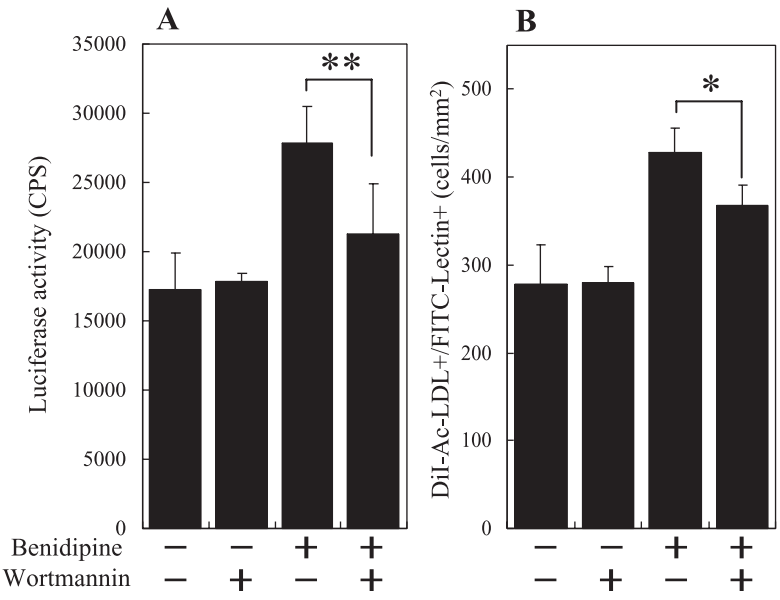

Fig. 5. *Inhibitory effect of wortmannin on the increase in double-positive cells in response to benidipine. Murine PBMCs were incubated with 1 μmol/l of benidipine and with or without 1 nmol/l of wortmannin in 2% serum medium. After 7 days of culture, the number of adherent viable cells and double-positive cells was evaluated by a CellTiter-Glo Luminescent Cell Viability Assay (A) and counting under a fluorescent microscope (B), respectively. Data are the means±SD of experiments performed in quadruplicate. Reproducible results were observed in two independent experiments.* *p<*0.05,* **p<*0.01.*

1% BSA. After washing, the membrane was reacted with horseradish peroxidase–conjugated anti-rabbit IgG (Cell Signaling Technology Inc.) and the bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, USA).

Statistical Analysis

All results are expressed as the means±SD. All statistical calculations were performed using a computer and statistical analysis software (SAS, version 8.2, SAS Institute, Inc., Cary, USA). Statistical analysis was performed using Student's *t*-test for comparisons between two groups, or using one-way ANOVA followed by the Dunnett's test, or the Kruskal-Wallis test followed by the Steel's test for multiple comparisons. A difference was considered to be statistically significant at $p<0.05$.

Results

Benidipine Increased the Endothelial Differentiation from Murine PBMCs *In Vitro*

PBMCs were isolated from C57BL/6 male mice and were cultured on vitronectin/gelatin-coated slide glasses in EBM-2 medium supplemented with FBS, vascular endothelial growth factor, fibroblast growth factor-B, epidermal growth factor and insulin-like growth factor. After 7 days of culture, differentiated endothelial cells were recognized as cells with endothelial-like spindle-shaped morphology and were identified as double-positive for DiI-Ac-LDL uptake and lectin binding with a fluorescent microscope (Fig. 1). PBMCs were cultured at a concentration of 1×10^6 cells/cm^2, and as a consequence, $3–7\times10^4$ cells/cm^2 were identified as double-positive. Flow cytometric analysis revealed that the cells expressed the endothelial-specific antigens Flk-1 and CD31 (Fig. 2).

To evaluate the effect of benidipine on the endothelial differentiation, we added benidipine to this culture. Incubation of isolated murine PBMCs with benidipine for 7 days significantly increased the number of adherent viable cells and double-positive cells (Figs. 3, 4). The morphology and surface markers of differentiated endothelial cells were not distinct between the culture with and without benidipine (data not shown). Benidipine (1 μmol/l) induced a 25% increase of double-positive cells (control *vs*. 1 μmol/l benidipine, 662.5±55.2 *vs*. 830.2±22.8; $p<0.01$) (Fig. 4).

Effect of Benidipine on Endothelial Differentiation Was Mediated *via* the Phosphoinositide-3 Kinese/Akt Pathway

We investigated the molecular mechanisms underlying the effects of benidipine on endothelial differentiation. We used the pharmacological phosphoinositide-3 kinase (PI3K) inhibitor wortmannin to assess whether the PI3K-pathway is involved. Wortmannin (1 nmol/l) was found to significantly

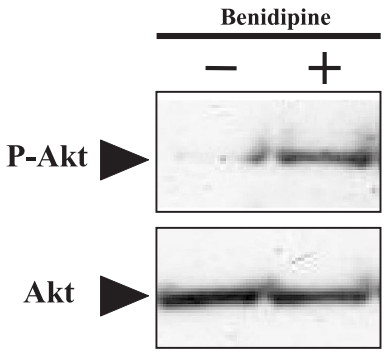

Fig. 6. *Augmentation of phosphorylated Akt with benidipine. Murine PBMCs were cultured with or without 1 μmol/l of benidipine for 7 days. Then, cells were collected, lysed and analyzed by Western blotting using anti-Akt antibody and anti-phosphorylated Akt antibody. The level of phosphorylated Akt was greater than that in the control.*

inhibit the benidipine-induced increases of viable cells and double-positive cells (Fig. 5). Next, to clarify whether Akt signaling was augmented after exposure to benidipine, isolated murine PBMCs were incubated with or without benidipine (1 μmol/l) for 7 days and subjected to Western blot analysis for detection of Akt phosphorylation using anti-phosphorylated serine-473 residue of Akt antibody. As a result, an increase in phosphorylated Akt on serine-473 was detected by benidipine treatment as compared with the control (Fig. 6).

Discussion

Ca^{2+} channel blockers are believed to elicit vasodilatation of coronary and peripheral arteries by inhibition of Ca^{2+} influx *via* L-type Ca^{2+} channels. However, recent studies have suggested that several Ca^{2+} channel blockers, including benidipine, also possess vasoprotective effects that are independent of the blood pressure–lowering actions (*24*). Although the mechanisms underlying the direct protective effects of Ca^{2+} channel blockers on endothelial cells are not fully understood, such vasoprotective effects as the amelioration of endothelial dysfunction (*25*, *26*), induction of angiogenesis (*27*), and decrease in the proliferation of vascular smooth muscle cells (*28*), oxidative stress and inflammation (*29*, *30*), may be at least partly responsible for the beneficial effects against cardiovascular diseases.

In our study, endothelial cells differentiated from circulating EPCs were identified as double-positive for DiI-Ac-LDL uptake and lectin binding, and the expression of Flk-1 and CD31 was detected by flow cytometric analysis. These phenotypes were very similar to endothelial cells and consistent with previously described endothelial cells differentiated from EPCs (*3*, *31*, *32*). In our culture method, $3–7 \times 10^4$ cells/cm^2 were identified as double-positive cells, which were derived from 1×10^6 cells/cm^2 PBMCs after 7 days of culture. This ratio (3–7%) is similar to the frequency of Sca-1 positive cells (about 10%), which were an EPCs-enriched population, in the circulation of C57BL/6 mice (*3*), suggesting that most of the double-positive cells were derived from circulating EPCs.

In the present study, we examined the direct effect of benidipine on the differentiation of endothelial cells from murine EPCs. The results showed that benidipine increased the number of differentiated endothelial cells *in vitro* and a PI3K inhibitor, wortmannin, inhibited the increase of endothelial differentiation by benidipine. In addition, benidipine treatment augmented the phosphorylation of Akt. These results showed that the PI3K/Akt pathway was at least partly involved in the increase in differentiated endothelial cells caused by benidipine. Although it remains to be clarified how benidipine stimulates the PI3K/Akt pathway, Umemoto *et al*. reported that amlodipine, which is another dihydropyridine Ca^{2+} channel blocker, also increased phosphorylation of Akt in rats aortas (*33*), and in our preliminary experiment, amlodipine induced a slight but not significant increase in the differentiation of endothelial cells from murine EPCs under the present culture conditions (data not shown). Moreover, Loomans *et al*. have reported that nifedipine causes a significant increase in the differentiation and proliferation of EPCs from hypertensive patients (*34*). These results suggest that dihydropyridine-Ca^{2+} channel blockers may have a similar pharmacological effect on EPCs.

The concentration of benidipine (0.01–1 μmol/l) used to increase the differentiation of endothelial cells from murine EPCs in the present study is higher than the plasma concentration of the drug. However, the lipophilic properties of dihydropyridine Ca^{2+} channel blockers, including benidipine, lead to intracellular accumulation, suggesting that the effective concentrations may be higher than those in plasma (*24*). Therefore, chronic administration of benidipine may enhance the endothelial differentiation of EPCs *in vivo*.

Previous studies have suggested that one of the vascular endothelial protective effects by benidipine is due to stimulation of NO synthesis *via* endothelial NO synthase (eNOS) on endothelial cells (*20–22*). The activation of eNOS and NO synthesis contributes not only to endothelial protection, but also to mobilization, proliferation, migration and differentiation of EPCs (*35–37*). The PI3K/Akt pathway stimulates NO synthesis *via* phosphorylation of eNOS on endothelial cells (*38*). Although further examinations are necessary to elucidate the underlying mechanisms of the effect of benidipine on EPCs, benidipine might enhance the differentiation of endothelial cells from EPCs through the eNOS/NO pathway *via* PI3K/Akt stimulation.

In the present study, we revealed that benidipine exhibited stimulatory effects on the endothelial differentiation of murine circulating EPCs by using an *in vitro* culture method. The effect of benidipine on EPCs may be one of the mechanisms underlying the endothelial protection and augmentation

of blood vessel repair, leading to the prevention of cardiovascular disease and/or an improvement in the prognosis after ischemic damage.

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