Nifedipine Inhibits Vascular Smooth Muscle Cell Dedifferentiation via Downregulation of Akt Signaling

Taeko Kaimoto, Osamu Yasuda, Mitsuru Ohishi, Masaki Mogi, Yukihiro Takemura, Toshimitsu Suhara, Toshio Ogihara, Keisuke Fukuo, Hiromi Rakugi

Abstract—Calcium is an essential signaling molecule that controls vascular smooth muscle cell (VSMC) contraction, proliferation, and differentiation. Here, we show that the calcium antagonist nifedipine inhibits VSMC dedifferentiation in vitro and in vivo. Differentiated VSMCs cultured on laminin-coated dishes were transferred to laminin-free dishes to induce dedifferentiation. Induction of dedifferentiation resulted in the upregulation of nonmuscle myosin heavy chain expression, a marker of dedifferentiation, and the downregulation of smooth muscle myosin heavy chain expression, a marker of differentiation. Nifedipine significantly inhibited both the induction of these phenotypic changes and upregulation of Akt signaling in these cells. Administration of nifedipine at a low concentration that did not affect blood pressure could inhibit the increase in nonmuscle myosin heavy chain expression and decrease in smooth muscle myosin heavy chain expression in a rat balloon-injury model. Furthermore, nifedipine suppressed neointimal hyperplasia and upregulation of Akt signaling. However, phospho-Akt expression was not suppressed in the regenerating arterial endothelium of the nifedipine-treated rats. The inhibitory effect of the downregulation of Akt signaling by dominant-negative Akt on the induction of VSMC dedifferentiation in the intima was identical to that of nifedipine. In contrast, upregulation of Akt signaling by transfection of the cells with a constitutively active Akt reversed the nifedipine-induced inhibition of VSMC dedifferentiation. In conclusion, nifedipine inhibits VSMC dedifferentiation by suppressing Akt signaling, thereby preventing neointimal thickening. (Hypertension. 2010;56:247-252.)

Key Words: calcium antagonist ■ hypertension ■ vascular smooth muscle cell ■ Akt ■ dedifferentiation

Excessive proliferation of vascular smooth muscle cells (VSMCs) plays a major role in the pathogenesis of vascular diseases. Unlike skeletal or cardiac muscle cells that have undergone terminal differentiation, VSMCs of adult animals retain plasticity and can shuttle between a quiescent, contractile phenotype and a proliferative, synthetic phenotype. Animals retain plasticity and can shuttle between a quiescent, contractile phenotype and a proliferative, synthetic phenotype. Here, we show that the calcium antagonist nifedipine inhibits VSMC dedifferentiation in vitro and in vivo. Differentiated VSMCs cultured on laminin-coated dishes were transferred to laminin-free dishes to induce dedifferentiation. Induction of dedifferentiation resulted in the upregulation of nonmuscle myosin heavy chain expression, a marker of dedifferentiation, and the downregulation of smooth muscle myosin heavy chain expression, a marker of differentiation. Nifedipine significantly inhibited both the induction of these phenotypic changes and upregulation of Akt signaling in these cells. Administration of nifedipine at a low concentration that did not affect blood pressure could inhibit the increase in nonmuscle myosin heavy chain expression and decrease in smooth muscle myosin heavy chain expression in a rat balloon-injury model. Furthermore, nifedipine suppressed neointimal hyperplasia and upregulation of Akt signaling. However, phospho-Akt expression was not suppressed in the regenerating arterial endothelium of the nifedipine-treated rats. The inhibitory effect of the downregulation of Akt signaling by dominant-negative Akt on the induction of VSMC dedifferentiation in the intima was identical to that of nifedipine. In contrast, upregulation of Akt signaling by transfection of the cells with a constitutively active Akt reversed the nifedipine-induced inhibition of VSMC dedifferentiation. In conclusion, nifedipine inhibits VSMC dedifferentiation by suppressing Akt signaling, thereby preventing neointimal thickening. (Hypertension. 2010;56:247-252.)

Key Words: calcium antagonist ■ hypertension ■ vascular smooth muscle cell ■ Akt ■ dedifferentiation

Calcium antagonists are widely used to treat angina pectoris and hypertension. There is evidence that calcium antagonist therapy reduces cardiovascular morbidity and mortality and the progression of atherosclerosis in hypertensive patients, partly because of an antioxidant effect and amelioration of free radical damage. One of the most widely used calcium antagonists, nifedipine, suppresses the development of atherosclerosis in cholesterol-fed rabbits without reducing hypercholesterolemia. Nifedipine dose-dependently reduces the expression of proliferative cell nuclear antigen in the thoracic aorta after balloon injury and inhibits neointimal thickening. However, the influence of calcium antagonists on the phenotypic changes of VSMCs remains unclear. We hypothesized that calcium antagonists may inhibit dedifferentiation of VSMCs by modulating the phosphatidylinositol 3-kinase/Akt signaling pathway.

Methods

More information on Materials and Methods can be found in the online Data Supplement (please see http://hyper.ahajournals.org).
Animals
Male Sprague-Dawley rats were obtained from SLC Japan (Shizuoka, Japan). Animals were maintained at room temperature on a 12-hour light/dark cycle and given access to food and water ad libitum. The experimental protocols were approved by the Osaka University Medical School Animal Care and Use Committee and were performed according to the Osaka University Medical School Guidelines for the Care and Use of Laboratory Animals.

Cell Culture
Human aortic smooth muscle cells were purchased from Kurabo and cultured in HuMedia-SG2 medium (Kurabo) supplemented with 5% FBS, 50 μg/mL of gentamicin, and 50 ng/mL of amphotericin B at 37°C in a humidified atmosphere containing 5% CO₂.

Immunohistochemistry
Differentiation marker was visualized by the streptavidin-biotin method using a Labeled Streptavidin Biotin kit (Dako Cytochemistry). Mounted sections were preincubated with 0.1 mol/L of PBS containing 1% albumin for 30 minutes at room temperature and then incubated overnight at 4°C with mouse anti-smooth muscle actin antibody (Clone1A4) or mouse anti-SMemb, mouse anti-SM2, or rabbit antiphospho-Akt (Ser473) antisera diluted with PBS containing 1% albumin. Then the samples were washed in PBS and incubated for an additional 1 hour at room temperature with biotinylated goat antimouse IgG or antirabbit IgG, followed by further incubation with horsedarish peroxidase-labeled streptavidin for 1 hour at room temperature. After washing in PBS, the reaction products were visualized by incubation with 0.020% (weight/volume) 3,3′-diaminobenzidine and 0.005% (volume/volume) H₂O₂ in 50 mmol/L of Tris-HCl buffer for 5 to 15 minutes. Finally, the samples were dehydrated and cover slips were applied with Permount (Entellan Neu, Merck).

Western Blot Analysis for α-Actin, SMemb, SM2, and Phospho-Akt
VSMCs were washed twice in PBS, harvested by scraping, and were adjusted to 10⁶ cells per 10 μL of lysis buffer (1% SDS, 100 mmol/L of NaCl, 50 mmol/L of Tris-HCl [pH 8.0], and 20 mmol of EDTA). The protein concentration of the lysate was determined with a Bio-Rad protein assay kit (Bio-Rad). Samples containing 20 μg of protein were run on a 7% to 10% SDS-PAGE gel and electrobotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Blots were blocked for 1 hour with 3% skim milk in PBS containing 0.1% Tween 20; incubated overnight at 4°C with antibodies for α-smooth muscle actin (Clone1A4), SMemb, SM2, or phospho-Akt (Ser473); and washed 3 times with PBS containing 0.1% Tween 20. Then, blocking with 3% skim milk in PBS containing 0.1% Tween 20 was done for 1 hour, followed by incubation with the peroxidase-conjugated secondary antibodies (antimouse or antirabbit IgG, Promega) for 2 hours at room temperature and washing 3 times with PBS containing 0.1% Tween 20. Immunoblots were developed with an ECL Western blotting detection system (Amersham International plc), and the blots were then reprobed with α-tubulin to confirm equal loading of protein into each well.

Immunocytochemistry
VSMCs grown in 2-chamber culture dishes (Laboratory-Tek, Nunc, Inc) were fixed for 5 minutes with 4% paraformaldehyde in PBS at room temperature, washed twice for 5 minutes each with PBS, and then preincubated with PBS containing 1% albumin for 30 minutes. The labeled streptavidin biotin kit (Dako) was used for immunostaining.

Adenoviral Constructs
To modulate Akt activity, we used 2 adenoviral constructs tagged with the hemagglutinin epitope, as described previously.15,16 In the dominant-negative Akt construct (DN-Akt), 2 phosphorylation sites (serine 473 and threonine 308) were both mutated to alanine, resulting in a form of Akt that could not be phosphorylated. A replication-defective adenovirus vector expressed the constitutively active form of murine Akt (CA-Akt) under the control of the cytomegalovirus promoter, whereas Adenovirus–β-galactosidase expressed the LacZ gene under the cytomegalovirus promoter as a control vector.17 All of the viral constructs were grown in 293 cells and purified by CsCl gradient ultracentrifugation. Viral titers were determined by the plaque assay.

Statistical Analysis
Statistical analysis was performed by 1-way ANOVA. Results are expressed as the mean±SEM, and P<0.05 was considered significant.

Results
Nifedipine Inhibits VSMC Dedifferentiation In Vitro
To investigate the influence of nifedipine on the VSMC phenotype, we examined whether nifedipine modulates dedifferentiation of VSMCs in culture. Differentiated VSMCs were obtained by culture on laminin-coated dishes, as reported previously.18,19 Then, dedifferentiation of these cells was induced by transfer to uncoated culture dishes, with the cells being incubated in the presence or absence of nifedipine. In the absence of nifedipine, the percentage of SMemb-positive cells increased from 6 to 72 hours (Figure 1A and 1B), whereas that of SM2-positive cells decreased (Figure 1C and 1D). These phenotypic changes were significantly inhibited by nifedipine. Western blot analysis also showed that exposure to nifedipine significantly suppressed both upregulation of SMemb expression (Figure S1) and downregulation of SM2 expression (Figure S2) induced by dedifferentiation of VSMCs. These findings indicate that nifedipine inhibits dedifferentiation of VSMCs in culture.

Nifedipine Inhibits the Akt Signaling in VSMCs In Vitro
We next examined whether nifedipine influences Akt signaling during dedifferentiation of VSMCs in vitro. Western blot analysis showed that transfer of differentiated VSMCs to uncoated dishes resulted in elevated phospho-Akt expression, which peaked at 6 hours after the transfer. Nifedipine treatment significantly suppressed this elevation induced by dedifferentiation for as long as 3 to 24 hours after induction of dedifferentiation (Figure 2). Nifedipine treatment also significantly suppressed the increase in phospho-Akt expression induced by exposure of cultured VSMCs to PDGF-BB (Figure S3). Furthermore, a different calcium antagonist, amiodipine, significantly suppressed the insulin-like growth factor 1–induced increase in phospho-Akt expression (Figure S4), indicating that both nifedipine and amiodipine inhibit Akt signaling.

Downregulation of Akt Inhibits VSMC Dedifferentiation In Vitro
To examine the direct role of the Akt signaling in dedifferentiation of VSMCs in culture, the adenoviral construct expressing DN-Akt was transfected into differentiated VSMCs cultured on laminin-coated dishes. Then, these cells were transferred to uncoated dishes to induce dedifferentiation. Suppression of the Akt signaling by DN-Akt signifi-
cantly inhibited both upregulation of SMemb (Figure 3A and 3B) expression and downregulation of SM2 (Figure 3C and 3D) expression induced by dedifferentiation. These findings indicate that the Akt signaling is involved in dedifferentiation of VSMCs in culture.

Nifedipine Inhibits the Akt Signaling in the Intimal VSMCs In Vivo

Next, we examined whether nifedipine inhibits the Akt signaling during VSMC dedifferentiation in vivo. As shown in Figure 4, phospho-Akt was highly expressed by actin-positive VSMCs in the intima. Nifedipine treatment significantly inhibited the Akt signaling in intimal VSMCs. In contrast, phospho-Akt–positive endothelial cells were present in the regenerating endothelium of nifedipine-treated arteries, suggesting that the Akt signaling in the endothelium is not affected by nifedipine.

Nifedipine and Downregulation of Akt Inhibit VSMC Dedifferentiation In Vivo

To test whether nifedipine inhibits VSMC dedifferentiation through downregulation of the Akt signaling in vivo, we evaluated the effect of nifedipine treatment and Akt gene transfer on VSMC dedifferentiation in the balloon injury model. Nifedipine treatment significantly suppressed the intimal hyperplasia (Figure 5A and 5B) and inhibited upregulation of SMemb expression and downregulation of SM2 expression in the intima (Figure 5C). Downregulation of Akt by transfer with DN-Akt was as effective as nifedipine at inhibiting hyperplasia and VSMC dedifferentiation in the intima. Conversely, upregulation of Akt by transfer with CA-Akt reversed the inhibition of VSMC dedifferentiation. These findings may provide causal evidence that the modulation of the Akt signaling is responsible for inhibiting VSMC dedifferentiation by nifedipine treatment.

Discussion

Calcium antagonists are widely used in the treatment of hypertension and angina pectoris. Recent evidence suggests...
that these drugs improve the clinical outcome in patients with certain cardiovascular diseases.\textsuperscript{20} Generally, nifedipine and other dihydropyridine derivatives are considered to retard VSMC proliferation by reducing the cellular availability of calcium and interfering with the calcium-calmodulin complex to inhibit VSMC proliferation and migration. In addition, nifedipine has been shown to modulate low-density lipoprotein metabolism by macrophages. However, it has not been clear whether the Akt signaling is involved in the beneficial effect of calcium antagonists on the development of cardiovascular diseases.

In the present study, we showed that nifedipine inhibits VSMC dedifferentiation and suppresses neointimal thickening after balloon injury. The nifedipine concentration used in this study does not reduce the blood pressure; this suggests that it has an antiatherogenic effect that is independent of its influence on blood pressure. This finding is consistent with that of studies that have reported the inhibition of atherosclerosis through a direct antioxidant effect of calcium antagonists on endothelial cells\textsuperscript{10,11}; these studies have also reported that calcium antagonists exhibit an antiatherogenic action without causing any reduction in the blood pressure or changes in the plasma lipid profile.\textsuperscript{21}

PDGF-BB was overexpressed at sites of VSMC proliferation after balloon injury of the vessels and at sites of atherosclerosis, suggesting its role in the development of intimal thickening. In fact, blocking of PDGF-BB or its receptor has been reported to inhibit neointimal thickening.\textsuperscript{1} Therefore, PDGF-BB seems to be responsible for the phenotypic changes and VSMC dedifferentiation in vivo. Recently, costimulation with PDGF-BB and interleukin 1\beta has been proved to induce sustained activation of Akt and p70S6K.\textsuperscript{22} In the present study, we showed that nifedipine suppresses PDGF-induced increases in phospho-Akt expression. Our preliminary experiments also showed that nifedipine inhibits increases in both phospho-Akt1 and phospho-Akt2 (data not shown). These findings suggest that nifedipine inhibits PDGF-induced neointimal thickening by suppressing Akt phosphorylation and VSMC dedifferentiation.

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**Figure 3.** Downregulation of Akt inhibits VSMC dedifferentiation. Differentiated VSMCs were infected with LacZ or DN-Akt for 48 hours. VSMC dedifferentiation was induced in these cells, and VSMCs were then harvested at 0, 30, and 180 minutes after induction of dedifferentiation. SMemb expression (A) and SM2 expression (C) by were determined by Western blot analysis. Then, the blot was reprobed for α-tubulin to confirm equal loading of protein in each well. SMemb expression (B) and SM2 expression (D) were quantified by densitometric analysis from 5 independent experiments. Data are normalized for α-tubulin and expressed as the mean±SEM. *P<0.05 vs VSMCs treated with LacZ.

**Figure 4.** Nifedipine inhibits upregulation of the Akt signaling in intimal VSMCs but not in the endothelium after the rat balloon injury model. Nifedipine was administered for 3 weeks at 0.3 mg/kg per day. The balloon injury was performed 1 week after the administration of nifedipine. Expression for α-actin, phosphor-Akt, and von Willebrand factor was immunostained in injured arteries at 2 weeks after the balloon injury.
Nifedipine suppressed the increased phospho-Akt expression in vitro. Moreover, treatment of injured arteries with nifedipine reduced Akt phosphorylation in the neointimal VSMCs in vivo. These results indicate that nifedipine suppresses the activation of Akt signaling and thereby inhibits VSMC dedifferentiation. In contrast, nifedipine did not affect the Akt signaling in the endothelial cells of injured arteries. This finding is reasonable because endothelial cells are not known to have any receptors for calcium antagonists. This result is also supported by our previous finding that nifedipine indirectly enhances NO production by endothelial cells by stimulating vascular endothelial growth factor release from the VSMCs.23 Our present findings have the important implication that regeneration of endothelial cells and upregulation of endothelial NO synthase expression via Akt signaling activated by vascular endothelial growth factors may not be suppressed by calcium antagonists.24,25

The number of functional L-type calcium channels significantly decreased in dedifferentiated VSMCs and increased on differentiation.26 This is consistent with our finding that nifedipine inhibits the dedifferentiation of differentiated VSMCs, thereby suppressing the development and progression of atherosclerosis.

**Perspectives**
We demonstrated that nifedipine, an L-type calcium channel antagonist, inhibits upregulation of the Akt signaling in VSMCs but not in the regenerating endothelium. Our results also suggest that modulation of the Akt signaling by nifedipine leads to an inhibition of VSMC dedifferentiation in injured arteries. These findings may provide new insights into the mechanisms underlying the beneficial effects of calcium antagonists in the treatment of cardiovascular diseases.

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**Disclosures**
None.
References


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Supplemental Methods

Materials
Recombinant human PDGF-BB was obtained from Oncogene Research Products (San Diego, CA). Nifedipine and anti-α smooth muscle actin (Clone1A4) mouse IgG2a monoclonal antibody was purchased from Sigma-Aldrich (Saint Louis, MO). Anti-SMemb and SM2 mouse IgG monoclonal antibodies were obtained from Yamasa Corporation (Chiba, Japan), anti phospho-Akt (Ser473) rabbit IgG polyclonal antibody and total-Akt1 rabbit IgG polyclonal antibody were from Cell Signaling Technology, Inc. (Danvers, MA), and anti-PDGF-B (H-55) rabbit IgG polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-von Willebrand factor rabbit IgG polyclonal antibody was purchased from Dako Cytomation (Kyoto, Japan). N-acetyl-L-cysteine (NAC) was purchased from Sigma (St. Louis, MO).

Cell Culture
For VSMC differentiation, VSMCs were cultured in HuMedia-SD2 medium (Kurabo) supplemented with 1% FBS, 50 µg/ml gentamycin, and 50 ng/ml anphotericin B, and 30 µg/ml heparin on 100-mm diameter laminin-coated dishes (BD Biosciences). Cells were maintained at 37°C in an atmosphere of 95% air and 5% CO2. For induction of VSMC dedifferentiation, differentiated VSMCs were transferred to laminin-uncoated dishes (2 chamber culture dishes or 100 mm diameter dishes) and were cultured in the presence or absence of nifedipine (50 µmol/L). For PDGF or IGF-1 treatment, VSMCs were incubated overnight in serum-free Dulbecco’s modified Eagle’s medium, and then were stimulated with each drug with or without calcium antagonists. Cells were used between passages 5 and 10. Because nifedipine is extremely susceptible to degradation by light, all nifedipine solutions were prepared and added to the cultures under yellow light and the cells were incubated in the dark.

Animal models
Male Sprague-Dawley rats aged 12 weeks (n=12) were anaesthetized with pentobarbital (40 mg/kg, i.p.) and incisions were made in the neck and back. Then a cannula and an osmotic pressure pump (model 2ML4; Alzet, Palo Alto, Calif., USA) were implanted. The pump was set to a rate of 2.5 µL/h and contained sufficient solution for 3 weeks of treatment. It was filled with nifedipine dissolved in ethanol or with the vehicle. The cannula (PE50, Clay Adams) was connected to the pump and then inserted into the left jugular vein, where it was fixed with a suture to allow continuous infusion mediated by
osmotic pressure. Then the wounds were disinfected and sutured. After seven days, incisions were made in the neck and lower limb to expose the left carotid artery and femoral artery. A 2F Fogarty balloon catheter (Baxter Health Care, Irvine, Calif., USA) was introduced into the femoral artery and was advanced to the bifurcation of the left internal and external carotid artery. The balloon was inflated with 0.3 mL of air after confirming that it was inside the carotid artery by visual inspection. Then the balloon catheter was pulled back to the aortic arch with the balloon inflated and this procedure was done three times to cause intimal injury. Next, the balloon catheter was removed and the femoral artery was sutured. Finally, the incisions were disinfected and sutured. In each animal, the right carotid artery was used as an intact control sample. The animals were randomly allocated to one of the following two groups: (1) the untreated group or (2) the nifedipine-treated group (0.3 mg/kg/day for 3 weeks). At 2 weeks after balloon angioplasty, the rats were anaesthetized and the right and left carotid arteries were harvested. These samples were fixed overnight in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4), and stored in 20% (w/v) sucrose in 0.1 M phosphate buffer for 2 days at 4°C. Then the samples were embedded in Tissue Tek O.C.T. compound (Sankura Finetek, Torrance, CA), and 10 µm thick sections were cut on a cryostat at –20°C. Serial sections were mounted on three separate glass slides coated with silan, and were stained with haematoxylin and eosin or subjected to immunohistochemistry. After being photographed through a microscope, the neointimal area was measured.

Adenoviral vector-mediated gene delivery
In cell culture study, the adenoviral construct expressing DN-Akt was infected for 2 hours into differentiated VSMCs cultured on laminin-coated dishes with serum-free media and 16 hours in serum-containing media. Then, these cells were transferred to laminin-uncoated dished to induce the dedifferentiation. In another in vivo study, rats underwent balloon injury of the carotid artery were divided into 5 groups (n=5–6/group), which were the LacZ, nifedipine + LacZ, DN-Akt, CA-Akt, and nifedipine + CA-Akt groups. After balloon injury, 1×10¹⁰ pfu of adenovirus diluted in 50 µL of PBS (final volume) was delivered into the injured segment and incubated for 10 minutes with vascular clamping. The influence of gene transfection was assessed at 7 days after injury, when rats were killed by an overdose of pentobarbital and their carotid arteries were removed.
Figure S1. Nifedipine suppresses changes of SMemb expressions after induction of dedifferentiation. (A) Western blot analysis of SMemb was performed after induction of dedifferentiation in the presence or absence of nifedipine (50 µmol/L). The blot was reprobed for α-tubulin to confirm equal loading of protein in each well. (B) SMemb expression was quantified by densitometric analysis from 5 independent experiments. Data are normalized for α-tubulin and expressed as the mean ± SEM (n=5). *P<0.05 vs. VSMCs without nifedipine.
Figure S2. Nifedipine suppresses changes of SM2 expressions after induction of dedifferentiation.
(A) Western blot analysis of SM2 was performed after induction of dedifferentiation in the presence or absence of nifedipine (50 μmol/L). The blot was reprobed for α-tubulin to confirm equal loading of protein in each well.
(B) SM2 expression was quantified by densitometric analysis from 5 independent experiments. Data are normalized for α-tubulin and expressed as the mean ± SEM (n=5). *P<0.05 vs. VSMCs without nifedipine.
Figure S3. Nifedipine inhibits the Akt signaling in VSMCs up-regulated by incubation with PDGF. (A) Phosho-Akt expression after treatment with PDGF-BB (10 ng/ml) with or without nifedipine (50 µmol/L) was determined by western blot analysis. (B) Phospho-Akt expression was quantified by densitometric analysis from 4 independent experiments. Data are normalized for α-tubulin and expressed as the mean ± SEM (n=5). *P<0.05 vs. VSMCs without nifedipine.
Figure S4. Both Nifedipine and amlodipine inhibits the Akt signaling up-regulated by incubation with IGF-1. (A) Phosho-Akt expression after treatment with IGF-1 (50 ng/ml) with or without nifedipine (50 µmol/L) or amlodipine (10 µmol/L) was determined by western blot analysis. (B) Phospho-Akt expression was quantified by densitometric analysis from 4 independent experiments. Data are normalized for α-tubulin and expressed as the mean ± SEM (n=5). *P<0.05 vs. VSMCs with neither nifedipine nor amlodipine.