Original Article

Nifedipine-Induced Vascular Endothelial Growth Factor Secretion from Coronary Smooth Muscle Cells Promotes Endothelial Tube Formation *via* the Kinase Insert Domain-Containing Receptor/Fetal Liver Kinase-1/NO Pathway

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Endothelial cells (ECs) are the critical cellular element responsible for postnatal angiogenesis. Since the calcium channel blocker (CCB) nifedipine indirectly upregulates endothelial superoxide dismutase expression by stimulating the production of vascular endothelial growth factor (VEGF) from smooth muscle cells (SMCs), we examined whether nifedipine would induce human coronary artery endothelial cell (HCEC) tube formation *via* an increase in VEGF production from human coronary artery SMCs (HCSMCs) in an *in vitro* model. Nifedipine stimulated VEGF production from HCSMCs, and this stimulation was abolished by protein kinase C (PKC) inhibitors and a bradykinin B2 receptor antagonist. In addition, supernatant derived from nifedipine-treated HCSMCs induced HCEC tube formation. This tube formation was inhibited by pretreatment with a specific inhibitor of kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/Flk-1) tyrosine kinase and an inhibitor of nitric oxide (NO) synthase. In conclusion, nifedipine increases VEGF secretion through PKC activation *via* the B2 receptor. The VEGF secretion directly induces HCEC tube formation *via* the KDR/Flk-1/NO pathway. CCBs may thus have novel beneficial effects in improving coronary microvascular blood flow in addition to their main effect of reducing blood pressure. (*Hypertens Res* 2005; 28: 147–153)

Key Words: endothelial cells, vascular endothelial growth factor, kinase insert domain-containing receptor/fetal liver kinase-1, nifedipine, bradykinin B2 receptor

Introduction

Calcium channel blockers (CCB) are widely used for the treatment of hypertension and coronary artery disease because these drugs induce an effective vasodilation, including in coronary arteries (1–3). CCB are believed to increase coronary blood flow by inhibiting the entry of Ca^{2+} into smooth muscle cells (SMCs) through specific L-type calcium

channel-blocking effects (4, 5). However, CCB may also influence endothelial cells (ECs), which have not been shown to possess L-type calcium channels (6, 7). In addition, Orth *et al.* demonstrated that CCBs inhibited the proliferation of mesangial cells, which also lack L-type calcium channels (8). Thus, CCBs are thought to exert blood pressure-lowering effects independent of L-type calcium channel blockade.

Since angiogenesis, the process of postnatal neovascularization, is a critical component of several human diseases,

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including ischemic heart disease, cancer, diabetic microvascular disease, rheumatoid arthritis and psoriasis, we focused on the effect of the CCB nifedipine on angiogenesis. Angiogenesis is believed to be mediated by the proliferation, migration, and remodeling of fully differentiated endothelial cells (9, 10). Several lines of evidence support the putative role of nifedipine in the modulation of angiogenesis; for example, Fukuo *et al.* reported that nifedipine indirectly upregulates endothelial superoxide dismutase (SOD) expression by stimulating vascular endothelial growth factor (VEGF) production from SMCs (11). In addition, nifedipine upregulates SOD expression in SMC *via* nitric oxide (NO) production from ECs (12). Increased NO production in ECs is important for protecting organs against ischemic or hypertensive stress (13). More recently, we reported that stimulation of the bradykinin (BK) B2 receptor leads to the transactivation of VEGF receptor kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/Flk-1) as well as to endothelial NO synthase (eNOS) activation, which induces angiogenesis in human coronary artery ECs (HCECs) (14). These reports indicate that the precise role of nifedipine in angiogenesis is likely to be complex, and that nifedipine may be critical to the angiogenic process.

In the present study, using an *in vitro* model of HCEC tube formation on a matrix gel, we showed that an increase in VEGF production from nifedipine-treated human coronary artery SMCs (HCSMCs) may be a potent signal for inducing angiogenesis through the KDR/Flk-1/NO pathway in HCECs. In addition, we showed that protein kinase C (PKC) inhibitors and a B2 receptor antagonist could attenuate nifedipine-induced angiogenesis.

Methods

Materials

The following antibodies and reagents were purchased: VEGF and an inhibitor of NO synthase L-NAME (*N*^ω-nitro-L-arginine methyl ester hydrochloride) from Sigma (St. Louis, USA); a specific inhibitor of KDR/Flk-1 tyrosine kinase (4-[(4'-chloro-2'-fluoro)phenylamino]-6,7-dimethoxy-quinazoline; Tki), inhibitors of PKC Go6850 (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide) and Go6980 (3-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide) from Calbiochem (La Jolla, USA); and Hoe140 (D-arginyl-[Hyp³,Thi⁵,D-Tic⁷,Oic⁸]-BK and BK from Peptide Institute Inc. (Osaka, Japan).

Cell Culture

HCSMCs and HCECs were purchased from Clontech (Palo Alto, USA). SMCs and ECs were cultured in media supplemented with 5% fetal bovine serum (FBS), penicillin/streptomycin, SMC growth supplement or endothelial cell growth supplement (Takara Co., Osaka, Japan) at 37°C under 5%

CO₂, and used after 3–5 passages. In the experiments, SMCs and ECs cultured in the same manner but without cell growth supplement were used as controls.

Cell Proliferation Assay

SMCs or ECs (1×10^3 cells) were plated on a 96-well plate and cultured under 5% serum conditions. After 48 h, the cells were cultured for 18 h in the presence or absence of different kinds of reagents in medium supplemented with 0.2% FBS and without cell growth supplement at 37°C under 5% CO₂. The cells were stained with CellTiter 96 One Solution Reagent (a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS assay] (Promega, Madison, USA) for 4 h at 37°C under 5% CO₂, and absorbance at 490 nm was recorded with a 96-well plate reader.

Angiogenesis Assay on Matrigel

An angiogenesis assay on Matrigel was performed as described previously (14, 15). Briefly, the matrix gels (Chemicon International, Inc., Temecula, USA) were allowed to polymerize in the plate. ECs (1×10^3 cells) were seeded and grown on Matrigel for 18 h in a humidified 37°C, 5% CO₂ incubator. In experiments, ECs were grown with the supernatants from SMCs that had been treated with different kinds of reagents in medium supplemented with 0.5% FBS and without growth supplement. After washing, tube formation was observed using a light microscope, and pictures were captured with a computer system. We performed a “pixel analysis” of the tube formation area according to a procedure described previously (14, 15).

Apoptosis Assay

SMCs or ECs (1×10^5 cells) were cultured for 48 h in a 10-cm dish containing 5% FBS. Then, the medium was exchanged for medium containing nifedipine, 0.2% FBS, and no cell growth supplement, and the cells were cultured an additional 18 h at 37°C under 5% CO₂. Staining of cells with fluorescence-labeled annexin V (FITC-Annexin V) (MedSystems, Burlingame, USA) was used as a functional index of early apoptosis. The percentage of stained cells was analyzed quantitatively by flow cytometry.

Enzyme Immunoassay

Concentrations of VEGF in SMCs cultured and treated with different kinds of reagents in medium supplemented with 0.5% FBS and without growth supplement for 18 h in a humidified atmosphere at 37°C were determined as described previously (16) in duplicate by specific enzyme immunoassays (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Fig. 1. Culture medium derived from nifedipine-treated HCSMCs induced HCEC tube formation, and this formation was inhibited by Tki and L-NAME. ECs were seeded and grown on Matrigel for 18 h in supernatants from SMCs that had been treated with different kinds of reagents. Representative pictures of HCECs plated on Matrigel (A–J). Nif, nifedipine. Data show the % change in tube formation compared with that in the control (A) ($n = 3$, mean $\pm$ SEM). * $p < 0.05$ vs. A. † $p < 0.05$ vs. E.

Preparation of Protein Extract and Immunoblotting

Untransfected or transfected SMCs were cultured in medium containing 0.2% FBS in the presence or absence of different kinds of reagents for 18 h at 37°C under 5% CO₂. Cells were then scraped. The procedure for cell lysis and Western blot analysis of signaling proteins on Immobilon-P membranes (Millipore Corp., Bedford, USA) has been described previously (17). Horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence system (Amersham, Buckinghamshire, UK) were used for detection, and band intensity was quantified by digital image analysis.

Statistical Analysis

Data are shown as the mean $\pm$ SD. Differences in individual variables were analyzed by an unpaired *t*-test as appropriate. A value of $p < 0.05$ was considered statistically significant. Data were analyzed using commercially available statistical software (Statview-J 4.11; Abacus Concepts Inc., Berkeley, USA).

Results

Culture Medium Derived from Nifedipine-Treated HCSMCs Induced HCEC Tube Formation, and This Effect Was Inhibited by Tki and L-NAME

We first analyzed the ability of nifedipine to stimulate and stabilize tube formation, with ECs cultured on Matrigel. Since the clinical plasma concentration of nifedipine is about 0.2 μmol/l (18), the concentration of nifedipine used in the experiments was from 0.1 to 5 μmol/l. As shown in Fig. 1, we examined whether a cell culture medium derived from nifedipine-treated or untreated SMCs could stimulate EC tube formation. The medium derived from nifedipine-treated SMCs dose-dependently led to the formation of a capillary-like structure on the Matrigel surface. We optimized the dose for the maximum dose required to induce tube formation in experiments, in which the dose-response showed that the maximum effective dose of nifedipine was 2 μmol/l. When 2 μmol/l of nifedipine was indirectly incubated with ECs on a Matrigel surface for 18 h, tube formation was not observed (data not shown). To analyze the mechanism responsible for this finding, we analyzed whether nifedipine-induced tube

Fig. 2. *a*: While nifedipine at up to 2 $\mu\text{mol/l}$ did not affect cell proliferation, nifedipine at 5 $\mu\text{mol/l}$ had an anti-proliferative effect in HCSMCs (open circle) and HCECs (closed circle) as assessed by MTS assay. The graph shows the % cell proliferation compared with that in the untreated control sample ($n=3$, mean $\pm$ SEM). * $p < 0.05$ vs. the control. Nifedipine time- and dose-dependently induced VEGF secretion from HCSMCs. *b*: The graph shows VEGF secretion from HCSMCs treated with (open circle) or without (closed circle) 2 $\mu\text{mol/l}$ of nifedipine for the indicated time ($n=3$, mean $\pm$ SEM). * $p < 0.05$ vs. 0 h. *c*: The graph shows VEGF secretion from HCSMCs treated with (closed square) or without (open square) the indicated concentration of nifedipine for 18 h ($n=3$, mean $\pm$ SEM). * $p < 0.05$ vs. no treatment.

formation in ECs was blocked by Tki and L-NAME. Nifedipine (2 $\mu\text{mol/l}$)-induced tube formation was blocked by Tki and L-NAME, suggesting that the supernatant derived from nifedipine-treated SMCs stimulated EC tube formation through the KDR/Flk-1/NO pathway.

Nifedipine at up to 2 $\mu\text{mol/l}$ Did Not Affect Cell Proliferation in HCSMCs or HCECs

To analyze another possible mechanism by which nifedipine might stimulate cell proliferation, we analyzed whether nifedipine induced the proliferation of HCSMCs and HCECs (Fig. 2a). Although nifedipine at up to 2 $\mu\text{mol/l}$ did not affect the proliferation of HCSMCs or HCECs, at 5 $\mu\text{mol/l}$ it significantly inhibited the proliferation of both HCSMCs and HCECs.

Nifedipine Time- and Dose-Dependently Induced VEGF Secretion from HCSMCs

Since nifedipine-induced tube formation was blocked by Tki, we examined whether nifedipine increased the release of VEGF from HCSMCs. As shown in Fig. 2b and c, nifedipine induced a significant increase in the release of VEGF from HCSMCs: time- and concentration-dependent increases in VEGF secretion were observed, and these increases were significant from 8 h and 1 $\mu\text{mol/l}$, respectively. However, the mRNA expression of VEGF isoforms as assessed by reverse transcription-polymerase chain reaction (RT-PCR) was not increased by exposure to nifedipine for up to 18 h (data not shown), suggesting that nifedipine regulated VEGF secretion by some post-transcriptional mechanisms. When ECs were incubated with 2 $\mu\text{mol/l}$ of nifedipine for 18 h, VEGF secretion was not observed in this cell system. Although the culture medium derived from nifedipine (5 $\mu\text{mol/l}$)-treated SMCs

induced the greatest VEGF secretion, tube formation was lower than that with medium treated with 2 $\mu\text{mol/l}$ of nifedipine. We also measured the apoptotic effect under the same conditions. Although CCB inhibited glomerular cell apoptosis (19), $10 \pm 3\%$ of cells showed apoptosis in the presence of a high concentration of nifedipine (5 $\mu\text{mol/l}$) in this study. Therefore, 5 $\mu\text{mol/l}$ of nifedipine blocked HCSMC proliferation accompanied by apoptosis. Tube formation caused by the nifedipine (5 $\mu\text{mol/l}$)-induced increase in VEGF secretion may be blocked by the anti-proliferative effect of nifedipine (5 $\mu\text{mol/l}$) on HCSMCs and HCECs.

PKC Inhibitor Decreased Nifedipine-Induced VEGF Secretion from HCSMCs

Since stretching and BK have been shown to increase VEGF secretion through PKC activation (20, 21), we next examined whether nifedipine induces VEGF secretion through a PKC pathway (Fig. 3). We preincubated cells for 1 h with the selective PKC inhibitors Go6983 and Go6850. These inhibitors blocked nifedipine (2 $\mu\text{mol/l}$)-induced VEGF secretion from HCSMCs.

B2 Receptor Antagonist Blocked Nifedipine-Induced VEGF Secretion from HCSMCs

Since nifedipine induced VEGF secretion from SMCs and this stimulation was abolished by Hoe140 (11), we also examined whether nifedipine induces VEGF secretion through a B2 receptor-PKC pathway (Fig. 4). We preincubated cells for 1 h with or without Go6983 and Hoe140, then incubated them with nifedipine or BK for 18 h. BK-induced VEGF secretion was blocked by Go6983 and Hoe140 as a positive control. Nifedipine-induced VEGF secretion was also blocked by Go6983 and Hoe140, suggesting that nifedipine-induced

Fig. 3. The PKC inhibitors, Go6850 and Go6976, decreased nifedipine-induced VEGF secretion from HCSMCs. The graph shows VEGF secretion in HCSMCs ($n=3$, mean $\pm$ SEM). * $p < 0.05$ vs. the untreated control samples.

secretion was mediated by a B2 receptor-PKC pathway.

Discussion

There are several findings in our study. First, the CCB nifedipine was shown to indirectly induce EC tube formation. We also found that PKC and B2 BK receptor played key signaling roles in nifedipine-induced VEGF secretion. Our findings suggest that HCSMC-derived VEGF plays a role in mediating coronary artery angiogenesis, at least in the *in vitro* model used here. Nifedipine may improve coronary microvascular blood flow in addition to its main effect of reducing blood pressure.

Several agents have been shown to increase VEGF production in a diverse range of cell systems. VEGF expression can be upregulated by hypoxia, platelet-derived growth factor (PDGF), transforming growth factor β (22), BK (20), and phorbol esters (23). We found that stimulation of HCSMCs with nifedipine resulted in a time- and concentration-dependent increase in VEGF secretion. We also characterized the BK receptor involved in this effect using a B2 receptor antagonist. Two subtypes of BK receptors, B1 and B2, have been defined based on their pharmacological properties. Most of the effects of BK have been reported to be linked to B2 receptor activation, whereas the functions of the B1 receptor are largely unknown. In our *in vitro* system, B2 receptor, but not B1 receptor, is expressed in HCSMCs, based on RT-PCR (Miura *et al.*, unpublished observation). Although this does not mean that the B1 receptor plays no role in VEGF secretion, nifedipine induced VEGF secretion through the B2 receptor in our system. The finding that VEGF secretion was mediated by the B2 receptor was consistent with a previous study (11) by Fukuo *et al.* in SMCs. They found that nifedipine indirectly upregulated superoxide dismutase expression in ECs by stimulating VEGF secretion from adjacent vascular SMCs. In this study, we focused on whether nifedipine indirectly stimulates HCEC tube formation by stimu-

Fig. 4. Hoe140, a B2 receptor antagonist, blocked nifedipine-induced VEGF secretion from HCSMCs. The graph shows VEGF secretion in HCSMCs ($n=3$, mean $\pm$ SEM). * $p < 0.05$ vs. the untreated control samples.

lating VEGF secretion from HCSMCs. In addition, we found a clear interaction between VEGF and the KDR/Flk-1/NO pathway.

PKC is a family of isoenzymes that transduce a wide range of biological signals in diverse cell systems (24). To probe the role of PKC in nifedipine-mediated BK-induced VEGF secretion, we studied the effect of two PKC inhibitors, Go6983 and bis-indolyl maleimide (24). We found that these inhibitors blocked nifedipine- or BK-induced VEGF secretion, which suggests that PKC plays a role in VEGF secretion. Our results are consistent with those of previous studies which have shown that PKC plays a role in VEGF production in response to stretching and cytokines in other biological systems (21, 22). On the other hand, Block *et al.* showed that nifedipine inhibited the action of recombinant PDGF in vascular SMCs and reduced PKC activation (25, 26). In addition, the inhibition of ischemia-induced permeability by nifedipine may be mediated by PKC- α inhibition independent of calcium channels (27). These effects were observed at pharmacological concentrations that were 1 to 2 orders of magnitude lower than those required for inhibition of the depolarization-induced opening of voltage-sensitive L-type calcium channels (28). Nifedipine may accumulate in membranous structures, resulting in higher localized concentrations (29). Thus, the concentrations of nifedipine used in our experiments are comparable to the clinical plasma concentration (18) used in the treatment of patients with cardiovascular disease. The effects of higher or lower concentrations of nifedipine may be quite different. More detailed investigations will be required to determine the details of nifedipine-induced PKC inhibition, including a consideration of PKC isoforms.

CCBs inhibit the proliferation of mesangial cells, which have no L-type calcium channels (8). Nifedipine has also been shown to inhibit ischemia-induced permeability in ECs, which also have no L-type calcium channels (27). These reports suggest that nifedipine-induced signaling may be independent of L-type calcium channels. In this study, nife-

Fig. 5. Proposed mechanisms of the effects of nifedipine on endothelial tube formation.

dipine did not directly increase VEGF secretion from ECs and did not induce EC tube formation. Therefore, our results suggest that cellular interaction between HCECs and HCSMCs is very important for nifedipine-induced angiogenesis, and nifedipine-induced VEGF secretion might be dependent of L-type calcium channels in HCSMCs.

As shown in Fig. 5, the cascade we propose is that nifedipine activates PKC through the B2 receptor, and the activated PKC increases VEGF secretion. The VEGF secretion then directly induces HCEC tube formation via the KDR/Flk-1/NO pathway. CCBs may thus have novel beneficial effects in improving coronary microvascular blood flow in addition to their main effect of reducing blood pressure.

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