Amlodipine Modulates THP-1 Cell Adhesion to Vascular Endothelium via Inhibition of Protein Kinase C Signal Transduction

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Abstract—Inflammatory responses play an important role in atherosclerosis. To critically assess the effect of dihydropyridines in inflammatory reactions, we conducted a monocyte-endothelial adhesion assay with monocyctic THP-1 cells treated with amlodipine under flow conditions in vitro. THP-1 cells were incubated in the presence of amlodipine (10 μmol/L) for 48 hours and then perfused over activated (interleukin-1β, 10 U/mL, 4 hours) human umbilical vein endothelial cells. The adhesion of THP-1 cells was significantly reduced after amlodipine treatment (P<0.001); however, flow cytometric analysis revealed that the expression levels of integrins in THP-1 cells were not significantly altered. Furthermore, Western blotting analysis of THP-1 cell lysates revealed that translocation of RhoA from the cytosol to the membrane was significantly diminished after amlodipine treatment. In addition, activation of protein kinase C-α and -β, as well as intracellular calcium influx, induced by phorbol 12-myristate 13-acetate, was diminished after amlodipine treatment. Pretreatment of THP-1 cells with calphostin C, a potent inhibitor of protein kinase C, significantly reduced THP-1 adhesion to vascular endothelium, whereas activation of β1-integrin was reduced after amlodipine treatment in THP-1 cells, based on the immunoreactivity of an activation-specific antibody for β1-integrin. Similar inhibitory effects were observed when we used freshly isolated peripheral blood mononuclear cells. These findings suggest a potential role for amlodipine in monocyte-endothelial interactions by modulation of protein kinase C- and RhoA-dependent mechanisms, which might account for its vascular protective effects. (Hypertension. 2003;42: 329-334.)

Key Words: calcium channel blockers ■ cell adhesion molecules ■ monocytes ■ protein kinases ■ signal transduction

L-type calcium channel antagonists are widely used in the management of hypertension as well as coronary heart diseases, and an increasing number of reports support the therapeutic benefits of these compounds for patients with cardiovascular diseases. Recently, amlodipine, a Ca2+ channel blocker, was shown to reduce the progression of atherosclerotic plaque formation in rabbit models,1,2 suggesting its role in atherosclerosis. In one of those studies, amlodipine caused a significant and dose-dependent reduction in lesion formation in the thoracic aorta,1 whereas in another, it exhibited an atheroprotective effect by acting as an antioxidant and reducing LDL uptake by the vessel wall, which consequently limited the size and extent of lesion areas.3 These two findings have been proposed to show potential mechanisms for the antiatherosclerotic effect of amlodipine. In addition to those findings, the results of several in vitro studies also indicate that treatment with amlodipine enhances nitric oxide production in endothelial cells,4 suggesting an anti-inflammatory role for the compound. In the present study, we attempted to elucidate the molecular mechanism responsible for the anti-inflammatory role of amlodipine by using an in vitro flow-chamber apparatus to examine amlodipine’s effect on monocyte-endothelial interaction. We found that amlodipine reduced the adhesion of THP-1 and human umbilical vein endothelial cells (HUVECs) and also inhibited protein kinase C (PKC) activation and RhoA translocation. Thus, our results provide concrete biologic evidence for the antiatherosclerotic potential of amlodipine.

Methods

Reagents and Cells

THP-1, a human leukemia cell line of monocyte/macrophage lineage, was obtained from American Type Culture Collection (Manassas, Va) and grown in RPMI-1640 medium with 10% fetal bovine serum. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood drawn from healthy volunteers, as previously described.5 HUVECs were isolated from normal-term umbilical cords as previously described.6 All procedures involving human samples were conducted according to the Guidelines for Animal and Human Experimentation of Tokyo Medical and Dental University.
For use in the flow-chamber apparatus, HUVECs (passages 2 and 3) were placed onto 22-mm, fibronectin-coated glass coverslips. The antibodies directed to the following molecules were used in the present study: CD11a (clone 38, Ancell Corp); CD11b (clone 44, YLEM); CD18 (clone MEM48, Southern Biotechnology Associates); CD49d (clone A4-PU1, Upstate Biotechnology); L-selectin (clone FMC46, Serotec); RhoA (Santa Cruz Biotechnology); β1-integrin (clone HUTS21, PharMingen, and clone 4B7R, Santa Cruz Biotechnology); and PKCα, -β, -δ, and -ε (New England Biolabs). 4′,6-diamidino-2-phenylindole (DAPI) and ionophore K23E1 were obtained from Dojindo Japan. Phorbol 12-myristate 13-acetate (PMA) was purchased from Wako Chemicals USA, Inc. Calphostin C was obtained from Calbiochem. Interleukin-1β (IL-1β) was obtained from Genzyme. Dulbecco’s phosphate-buffered saline (DPBS) was obtained from Sigma (D8662).

**Adhesion Assay Under Flow Conditions**

We conducted an in vitro adhesion assay with monocytic THP-1 cells or PBMCNs treated with amlodipine under simulated flow conditions (estimated shear stress = 1.0 dyn/cm²) by using a protocol that has been previously described in detail.5 THP-1 cells or PBMCNs were stained with a 0.25% trypan blue solution or a solution of DAPI (10 mmol/L Tris-HCl, pH 7.4, 10 mmol/L EDTA, 100 mmol/L NaCl, and 500 ng/mL DAPI) for 10 minutes at room temperature after incubation with amlodipine. THP-1 cells and PBMCNs (10⁶ cells/mL) were diluted in the perfused medium (DPBS containing 0.2% human serum albumin) and then perfused over activated (IL-1β, 10 U/mL, 4 hours) HUVEC monolayers. The interactions of THP-1 cells or PBMCNs with HUVECs were observed under an inverted microscope (Olympus, IX70) and then analyzed by image analysis software. In some experiments, a static adhesion assay was performed as previously described.5

**Flow Cytometry**

THP-1 cells were first incubated with the indicated primary antibodies on ice for 45 minutes, washed twice with RPMI-1640 medium containing 5% fetal calf serum, and then incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (1:50 dilution). Fluorescence intensity was analyzed with a fluorescence-activated cell sorting system (FACSCaliber, Becton-Dickinson).

**Translocation of RhoA and PKC in THP-1 Cells**

The expression of RhoA and PKC was detected in the membrane and cytosolic fractions of the THP-1 cell lysate by Western blotting as described previously.5 An equal amount of protein (10 µg) from each fraction was subjected to 12.5% (RhoA) or 8% (PKC) sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and Western blotting analysis was carried out with mouse monoclonal antibodies to RhoA and the indicated PKC isoforms.

**Quantification of Filamentous Actin in THP-1 Cells**

Filamentous actin (F-actin) in THP-1 cells was quantitated as described previously.6 In brief, THP-1 cells (10⁶/mL) were fixed with 1% paraformaldehyde for 5 minutes, permeabilized with 0.1% Triton X-100 for 60 seconds, and incubated with FITC-conjugated phalloidin for 60 minutes. The fluorescence intensity of the THP-1 cells was quantified by using a fluorescence plate reader and was also observed under a fluorescence microscope.

**Calcium Concentration in THP-1 Cells**

Next, we attempted to determine the effect of amlodipine on cytosolic calcium concentrations in THP-1 cells. THP-1 cells (2 × 10⁶ cells/mL) were preincubated in the presence or absence of amlodipine for 48 hours, washed with DPBS (1.2 mmol/L Ca²⁺), and incubated in the dark at 37°C for 20 minutes in the presence of fura 2-AM (5 µg/mL). The cells were then washed and resuspended in DPBS at a density of 10⁶ cells/mL. To measure intracellular calcium ([Ca²⁺]i), 1 mL of the cell suspension was placed in the cuvette of a CAF-110 fluorescence spectrophotometer (Jasco Japan). PMA (10 ng/mL) was directly injected into the cuvette, and [Ca²⁺], was measured by excitation at 340 and 380 nm and fluorescence emission at 500 nm.

**Activity of β1-Integrin in THP-1 Cells**

The activity of β1-integrin in THP-1 cells was examined by Western blotting analysis with the use of two independent monoclonal antibodies against human β1-integrin (CD29), which were HUTS21 (recognizes an activation-dependent epitope) and 4B7R (recognizes activated and resting β1-integrin), as described earlier.

**Statistical Analysis**

Results are presented as mean ± SD. Data were analyzed by ANOVA, with P < 0.05 considered significant.

**Results**

**Amlodipine Inhibits Adhesion of THP-1 Cells or PBMCNs to Activated HUVECs Under Flow Conditions**

We examined the effect of amlodipine on monocyte-endothelial interactions under flow conditions (shear stress of 1.0 dyn/cm²). When THP-1 cells or PBMCNs were incubated in the presence of amlodipine, the amount of adhesion to HUVECs (IL-1β, 10 U/mL, 4 hours) was decreased (Figure 1A). The inhibitory effect of amlodipine on THP-1 adhesion was statistically significant at a concentration of 10 µmol/L, compared with the control (amlodipine, 6.25 ± 1.75/high-power field [HPF] vs control, 9.63 ± 1.30/HPF; n = 8, P < 0.001), and similar effects were observed with PBMCNs (amlodipine, 7.11 ± 1.76/HPF vs control, 12.78 ± 1.86/HPF; n = 8, P < 0.001). These inhibitory effects were observed in a dose-dependent manner; however, they were not statistically significant with doses lower than 10 µmol/L (data not shown), and amlodipine treatment for <48 hours failed to exhibit a significant reduction of adhesion by THP-1 cells (data not shown). Furthermore, preliminary experiments with trypan blue and DAPI staining revealed that THP-1 cells were not dramatically damaged by amlodipine treatment up to a concentration of 10 µmol/L (data not shown). As a result, we chose to treat THP-1 cells with a concentration of 10 µmol/L for 48 hours, unless otherwise noted. On the other hand, when HUVECs were treated with amlodipine, no significant inhibitory effect was found in the adhesion assays (data not shown).

**Integrin Expression in THP-1 Cells**

To elucidate the molecular mechanism of the observed inhibitory effect of amlodipine toward THP-1 cell adhesion, integrin expression levels were examined by flow cytometric analysis. THP-1 cells were incubated in the presence or absence of amlodipine (10 µmol/L, 48 hours); however, the expression levels of CD11a, CD11b, CD11c, CD18, and CD49d were not significantly different between the two conditions (Figure 1B).

**Amlodipine Reduces RhoA GTPase Activation in THP-1 Cells**

We next examined the effects of amlodipine on the intracellular cytoskeleton networks in THP-1 cells. F-actin content was estimated by using fluorescently labeled phalloidin after treatment with amlodipine. As shown in Figure 2A, amlodip-
Amlodipine treatment significantly reduced F-actin (amlodipine, 417.3±23.0 vs control, 564.8±4.8; P<0.001, n=6). Next, the activation of RhoA GTPase was also examined, because RhoA GTPase is regarded as crucial for cell motility and thus, for adhesive interactions. Western blotting analysis revealed that the translocation of RhoA from the cytosol to the membrane was significantly decreased after incubation with amlodipine (10 μmol/L, 48 hours; Figure 2B).

**Amlodipine Reduces PKC Activation in THP-1 Cells**

The involvement of PKC in amlodipine-dependent RhoA GTPase modulation was further investigated. To monitor PKC activation, the translocation of PKC from the cytosol to the membrane was examined. Figure 3A revealed that the activated forms of other PKC isoforms, such as PKC-βII and PKC-δ, were not significantly reduced after pretreatment (data not shown).

To assess critically the involvement of the PKC-dependent mechanism in the adhesion of THP-1 to vascular endothelium, THP-1 cells were pretreated with 500 nmol/L calphostin C, a specific inhibitor of PKC, as judged from their translocation into the membrane fraction, was observed in THP-1 cells after PMA stimulation; however, pretreatment with amlodipine significantly reduced this PMA-induced PKC activation (Figure 3A). Additional experiments revealed that the activated forms of other PKC isoforms, such as PKC-βII and PKC-δ, were not significantly reduced after pretreatment (data not shown).

PKC activation, the translocation of PKC from the cytosol to the membrane was examined. Activation of PKC-α and PKC-β, as judged from their translocation into the membrane fraction, was observed in THP-1 cells after PMA stimulation; however, pretreatment with amlodipine significantly reduced this PMA-induced PKC activation (Figure 3A). Additional experiments revealed that the activated forms of other PKC isoforms, such as PKC-βII and PKC-δ, were not significantly reduced after pretreatment (data not shown).

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Intracellular Concentration of Calcium Is Modulated by Amlodipine in THP-1 Cells

To investigate the effect of amlodipine on [Ca\(^{2+}\)]\(_i\) in THP-1 cells, [Ca\(^{2+}\)]\(_i\) was measured in THP-1 cells after stimulation with PMA. When THP-1 cells were preincubated with amlodipine, the increase in [Ca\(^{2+}\)]\(_i\) in response to PMA was dramatically diminished (Figure 4A). Furthermore, when THP-1 cells were pretreated with the calcium ionophore K23E1 to increase the level of [Ca\(^{2+}\)]\(_i\), the adhesion of THP-1 cells to activated HUVECs was increased (Figure 4B).

Amlodipine Reduces Activated \(\beta_1\)-Integrin in THP-1 Cells

To investigate the involvement of integrin activation, Western blotting analysis was performed with the monoclonal antibody HUTS21 to detect an activation-dependent epitope of \(\beta_1\)-integrin in THP-1 cells. HUTS21-positive \(\beta_1\)-integrin was significantly increased after incubation with amlodipine in THP-1 cells, whereas immunoreactivity against 4B7R, a monoclonal antibody that detects constitutively expressed \(\beta_1\)-integrin, was not changed (Figure 5).

Discussion

We investigated the effects of amlodipine on the adhesion of monocytes to vascular endothelium under flow conditions. Incubation of monocyctic THP-1 cells with amlodipine significantly inhibited their adhesion to HUVECs in the presence of flow. Recent study results have indicated that certain calcium channel blockers might possess an ability to prevent atherosclerosis in vivo, by several in vitro findings have shown an inhibition of smooth muscle cell proliferation and cytokine production by amlodipine. Moreover, a...
recent prospective study of amlodipine that used a randomized evaluation method clearly suggested its dramatic efficacy in reducing cardiac events.17 However, the mechanism by which this calcium channel blocker modulates atherosclerosis is not yet fully understood, although recent results have indicated a reduction of plasma soluble vascular cell adhesion molecule-1 after treatment with amlodipine for 3 weeks,18 suggesting an anti-inflammatory role for this compound. Thus far, nonexcitable cells, including monocytes and lymphocytes, have been shown to possess a store-operated calcium channel (SOC), however, not a voltage-operated calcium channel (VOC) or an L-type Ca$^{2+}$ channel. As a result, dihydropyridines, which are potent antagonists of the L-type Ca$^{2+}$ channel, are not considered to be effective with these cell types. On the other hand, a recently identified SOC has been proposed as a mammalian homologue of the transient receptor potential gene product of Drosophila photoreceptors, which shares a strong homology with VOC.19,20 These structural similarities indicate that specific compounds that target the VOC might also function against the SOC. In fact, in the present study, we demonstrated that amlodipine was able to reduce the PMA-induced calcium influx in THP-1 cells, which might potentially explain the antiatherosclerotic effect of this compound, although the precise mechanism of this effect remains to be elucidated.

Our present observations of the inhibitory effects of amlodipine toward THP-1 adhesion to vascular endothelium might be important evidence in support of the antiatherosclerotic potential of this compound, because, as has already been shown, the adhesion of monocytes to vascular endothelium is believed to be one of the crucial steps of atherogenesis. However, it is important to note that cell surface expression of adhesion receptors was not changed by amlodipine treatment. Although the dynamic interaction between leukocytes and endothelial cells is mainly regulated by physical binding of adhesion molecules on both sides,21 the intracellular environment, such as the cytoskeleton and related signal-transduction cascades, has also been shown to play an equally important role in this mechanism.7-22 Therefore, we investigated the effects of amlodipine on the relevant intracellular mechanism(s) of THP-1 cells that might modulate monocyte-endothelial interactions.

We also examined the potential participation of RhoA GTPase in amlodipine-induced antiadhesive effects in THP-1 cells, because RhoA GTPase has been shown to be one of the critical regulators of cell motility and cytoskeleton functions.6,23 We previously documented the importance of RhoA GTPase in the regulation of monocyte adhesion to vascular endothelium by using monocytes pretreated with a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, or statin.6 In the present study, we demonstrated for the first time that amlodipine, a dihydropyridine, was able to modulate the activation of RhoA GTPase in monocytes. Although a similar inhibitory action toward RhoA has been shown with statin, the responsible mechanism underlying the observed effects of amlodipine are quite distinct, as dihydropyridines are not likely to inhibit intracellular cholesterol synthesis or the several important intermediates required for activation of small GTP proteins, including RhoA GTPase.24,25 Recent studies with intestinal epithelial cells have also suggested that the [Ca$^{2+}$], has an effect on the regulation of RhoA activation.26 Therefore, as we showed, manipulation of the [Ca$^{2+}$], was able to modulate cell migration via RhoA activation.

Knowledge of the participation of PKC-α and -β upstream of RhoA GTPase has further advanced our understanding of these effects, and recent observations suggest that PKC is the molecular target of ischemia-induced endothelial cell permeability, which is protected by dihydropyridines.27 We observed an effect of amlodipine on PKC isoforms α and β, but not δ or ζ. The importance of PKC-α and -β during cell adhesion has been previously reported,27,28 as Sun et al28 showed that overexpression of PKC-α enhanced the motility and adhesion of breast cancer cells and Nonaka et al29 found that inhibition of PKC-β resulted in reduced entrapment of leukocytes in rat diabetic retina models. Using a specific inhibitor of PKC, we were able to document a critical role for PKC in monocyte-endothelial interactions in the present study.

It is of great interest to elucidate how amlodipine modulates the activation of PKC in THP-1 cells. One possible explanation is that amlodipine interferes with the release of phospholipid components, such as diacylglycerol, from the plasma membrane to activate PKC.27 As previously reported, disturbance of this phospholipid would dramatically affect PKC signaling.30 Furthermore, the unique characteristics of amlodipine that cause it to exhibit a strong and sustained affinity to the lipid bilayer might play a role in the amlodipine-dependent reduction of THP-1 cell adhesion.

**Perspectives**

We found that treatment with amlodipine, a calcium channel antagonist, significantly inhibited monocytes THP-1 cell adhesion to cytokine-activated vascular endothelium under flow conditions. The potential mechanisms seemed to involve inhibition of PKC (α and β), RhoA GTPase, and the actin cytoskeleton by reducing [Ca$^{2+}$]. Although we did not examine other compounds of this class, the lipophilic property of amlodipine might be important to exert this effect. Our results
indicate a novel antiatherosclerotic role for this compound, though at relatively high concentrations, which might be independent of its effect on L-type calcium channels.

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