Calcium Antagonists Ameliorate Ischemia-Induced Endothelial Cell Permeability by Inhibiting Protein Kinase C

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Background—Dihydropyridines block calcium channels; however, they also influence endothelial cells, which do not express calcium channels. We tested the hypothesis that nifedipine can prevent ischemia-induced endothelial permeability increases by inhibiting protein kinase C (PKC) in cultured porcine endothelial cells.

Methods and Results—Ischemia was induced by potassium cyanide/deoxyglucose, and permeability was measured by albumin flux. Ion channels were characterized by patch clamp. $[Ca^{2+}]$ i, was measured by fura 2. PKC activity was measured by substrate phosphorylation after cell fractionation. PKC isoforms were assessed by Western blot and confocal microscopy. Nifedipine prevented the ischemia-induced increase in permeability in a dose-dependent manner. Ischemia increased $[Ca^{2+}]$ i, which was not affected by nifedipine. Instead, ischemia-induced PKC translocation was prevented by nifedipine. Phorbol ester also increased endothelial cell permeability, which was dose dependently inhibited by nifedipine. The effects of non–calcium-channel–binding dihydropyridine derivatives were similar. Analysis of the PKC isoforms showed that nifedipine prevented ischemia-induced translocation of PKC-α and PKC-ζ. Specific inhibition of PKC isoforms with antisense oligodeoxynucleotides demonstrated a major role for PKC-α.

Conclusions—Nifedipine exerts a direct effect on endothelial cell permeability that is independent of calcium channels. The inhibition of ischemia-induced permeability by nifedipine seems to be mediated primarily by PKC-α inhibition.

Anti-ischemic effects of dihydropyridine calcium antagonists could be due in part to their effects on endothelial cell permeability. (Circulation. 1999;99;2523-2529.)

Key Words: cells □ ischemia □ proteins □ calcium

Calcium antagonists ameliorate ischemia in patients with chronic stable angina, unstable angina, variant angina, and silent ischemia and are used effectively in the treatment of hypertension. Calcium antagonists also prevent myocardial injury after ischemia and reperfusion. Calcium antagonists enhance vasodilatation, improve regional blood flow, and reduce ischemia-induced injury. Their actions have generally been attributed to specific L-type calcium channel blocking effects on vascular smooth muscle cells. However, calcium antagonists may also influence endothelial cells, which have not been shown to possess L-type calcium channels. Direct effects on endothelial cells could be important, because the endothelium plays a major role in ischemia-induced tissue injury. Adhesion molecule expression, NO release, and the maintenance of endothelial cell–layer permeability are disturbed with ischemia. Orth et al recently demonstrated that calcium antagonists inhibit the proliferation of mesangial cells, which also have no L-type calcium channels. Possible L-type calcium channel–independent mechanisms include inhibition of ATP utilization, blockade of K+ channels, inhibition of agonist–receptor interactions, inhibition of Na+/H+ exchange, and inhibition of hydrophobic compound traffic via multidrug resistance proteins.

Block et al previously reported a calcium antagonist–mediated inhibition of protein kinase C (PKC) in vascular smooth muscle cells. Because PKC plays a central role in several endothelial cell functions, such as adhesion and permeability, we tested the hypothesis that the calcium antagonist nifedipine influences ischemia-induced permeability in endothelial cells via PKC inhibition.

Methods

Phorbol ester TPA (phorbol 12-myristate 13-acetate), histone type III-S, DEAE cellulose, and all other materials, if not stated otherwise, were purchased from Sigma Chemical Co. γ-[32P]ATP was obtained from Amersham. 1,2-Diolein and phosphatidylinerse were purchased from Avanti Polar Lipids. CY3-conjugated anti-mouse IgG antibodies were obtained from Dianova. The PKC inhibitor Goe 7624 was obtained from Calbiochem. The fluorescent probe fura 2-AM was purchased from Serva. All dihydropyridines were a gift from Bayer, Leverkusen, Germany.

Porcine aortic endothelial cells were isolated and cultured as previously described in detail.11 Experiments were performed with confluent monolayers, 4 days after they were seeded onto filters. The purity of these cultures was >98% endothelial cells. Permeability across the endothelial cell monolayer was studied in a...
2-compartment system separated by a filter membrane. Both compartments contained modified Tyrode solution supplemented with 10% (vol/vol) FCS. The luminal compartment containing the monolayer had a volume of 2.5 mL, whereas the abluminal compartment had a volume of 10.5 mL. The fluid in the abluminal compartment was constantly stirred. Trypan blue–labeled albumin (60 μmol/L) was added to the luminal compartment. We continuously monitored the appearance of Trypan blue–labeled albumin in the abluminal compartment by pumping the liquid through a 2-wavelength photometer (model Specord S-10; Carl Zeiss; wavelength of 580 nmol/L for Trypan blue and 720 nm for the reference wavelength). The concentration of Trypan blue–labeled albumin in the luminal compartment was determined every 10 minutes of incubation. The albumin flux (F) across the monolayer was determined from the increase in albumin concentration ([A2]) in the abluminal compartment (volume V): F = d[A2]/dt × V, where t is time. Data were expressed as percentage of a defined control situation.

The term ischemia (from the Greek ische, to keep back) refers to local anemia due to mechanical vascular obstruction. In our cell system, we actually performed energy depletion to mimic some of the features of ischemia in a cell culture system. Effects of energy depletion were studied in the presence of potassium cyanide (KCN; 1 mmol/L), an inhibitor of oxidative phosphorylation, and 2-deoxy-D-glucose (DG; 1 mmol/L), an inhibitor of glycolysis, as described previously. The effect of KCN/DG on endothelial cell permeability was fully reversible.

PKC activity was measured in cultured confluent cells after isolation of a particulate fraction by previously described techniques. Data presented in Figure 6 were calculated by subtraction of nonspecific activity. Western blot analysis was performed as described elsewhere. The antisense methods were described in previous publications. Phosphorothioate oligodeoxynucleotides (ODNs) were purchased from TIB Moleb. For transfection, cells were incubated with lipofectin (10 μg/mL) and ODN (1 μmol/L) in the absence of FCS at 37°C for 4 hours, washed 2 times with medium, and then incubated with medium and ODN (1 μmol/L) for another 4 hours. Afterward, the medium was changed to 10% FCS for 24 hours before the start of the experiments.

For [Ca2+]i measurements, cells were sowed on coverslips and incubated at 37°C for 4 days. The calcium measurements were performed with a Spex fluorolog 2 spectrofluorometer, which was connected to a Nikon Diaphot 300 microscope and a variable-aperture photometer to isolate individual cells on the microscope stage (Spex Industries Inc). Cultured endothelial cells were loaded with fura 2-AM with 15 minutes’ incubation in PBS containing 5 μmol/L fura 2-AM (added from a 5 mmol/L stock solution in DMSO). 

Statistical Analysis
Statistical analysis was performed with the commercially available statistics SPSS (SPSS Inc). Because the data comprise time series, general linear modeling with repeated measures and between-subject effects were calculated. In case of significant group effects, data obtained at 30 minutes were compared between groups by ANOVA with post hoc Scheffé tests to allow for multiple testing.

Results
Administration of KCN (1 mmol/L) and DG (1 mmol/L) resulted in a rapid decrease in ATP from 15±2 to 8±2.4 nmol/mg protein within 10 minutes (n = 5; P ≤ 0.05; data not shown). Ischemia increased permeability within minutes, reaching a maximum at 30 minutes (148±9% of control values; n = 7; P ≤ 0.01) and remaining stable thereafter (Figure 1). Afterward, endothelial cell permeability declined to a plateau phase. The ischemia-induced increase in permeability was totally reversible after the KCN/DG-containing medium was removed (data not shown). Figure 2 shows the effect of nifedipine on the time course of ischemia-induced increases in permeability. Nifedipine decreased both the initial peak and the plateau phase of ischemia-induced permeability. The ameliorating effect of nifedipine was observed at a concentration of 10×10-6 mol/L (n = 7; P ≤ 0.01 compared with KCN/DG). We observed a nonspecific cation current in the endothelial cells. Characteristics of t-type calcium channels were not detected (n = 7; data not shown). We then analyzed whether calcium influx could occur by other mechanisms. Cadmium (5×10-5 mol/L) was used as a nonspecific inhibitor of calcium flux (Figure 3). Cadmium had no effect on basal permeability and did not influence the ischemia-
Induced alteration (n=5). In addition, cadmium did not interfere with the effects of nifedipine (Nif) on endothelial cells. Possible effects of nifedipine on intracellular free calcium concentrations in endothelial cells were investigated in the next set of experiments. A representative experiment is shown in Figure 4. Incubation of endothelial cells with KCN/DG led to a rapid and sustained increase in intracellular free calcium concentration from 143±29 to 472±35 nmol/L (n=8; P<0.01). Concomitant incubation with nifedipine did not influence the rapid increase, nor did incubation with nifedipine alter the sustained elevation in intracellular free calcium concentration during ischemia (472±35 versus 458±39 nmol/L; n=8; P=NS). After removal of KCN/DG from the medium, intracellular free calcium concentrations returned to baseline (data not shown).

Figure 3. Effect of nonspecific calcium channel blocker cadmium (Cadm.) on ischemia-induced permeability in endothelial cells. Cadmium had no effect on basal permeability or ischemia-induced increase in permeability and did not interfere with the effects of nifedipine (Nif) on endothelial cells. Data are mean±SD after 30 minutes of incubation (n=5 separate experiments; *P<0.05 vs KCN/DG). C indicates control.

Figure 4. Nifedipine (Nif) had no effect on [Ca\(^{2+}\)] in endothelial cells after KCN/DG-induced ischemia.

Figure 5. Effect of PKC inhibitors on ischemia-induced endothelial cell permeability (n=6). Staurosporine (Stauro; 10\(^{-8}\) mol/L) and Goe 6976 (G6; 10\(^{-7}\) mol/L) reduced ischemia-induced permeability almost completely. Preincubation with TPA (100 nmol/L) for 24 hours also abolished the effect, as did nifedipine (Nif; 10\(^{-8}\) mol/L). Data are mean±SD after 30 or 60 minutes of incubation (n=6 separate experiments; *P<0.05 vs KCN/DG). C indicates control.
PKC activity at 10 minutes (n=6; P≤0.05). Preincubation with nifedipine (10−3 mol/L) reduced PKC activity in the membrane fraction significantly (n=6; P≤0.05). We next investigated whether nifedipine specifically inhibits the translocation of specific PKC isoforms in endothelial cells. In Figure 8, the results of translocation experiments for PKC-α, -δ, -ε, and -ζ are shown. PKC-α (Figure 8A) showed a single band at 82 kDa and was mostly located in the cytosolic fraction under resting conditions. Endothelial cell exposure to KCN/DG resulted in a translocation of PKC-α from the cytosolic fraction to the membrane or particulate fraction. Preincubation with nifedipine prevented the ischemia-induced translocation. Densitometric analysis of the translocation experiments (n=3; P≤0.05 compared with control and nifedipine) is also shown in Figure 8. In Figure 8B, the effects of KCN/DG on PKC-δ are shown. Ischemia had no significant effect on the translocation of PKC-ε. In Figure 8C, the effects of ischemia on PKC-ε are shown. Ischemia had no effect on the translocation of PKC-ε. PKC-ζ (Figure 8D) showed a double band in both the cytosol and the particulate fractions. KCN/DG induced an increase in the upper band in the particulate fraction and a concomitant decrease in the cytosolic fraction. This effect was completely prevented by nifedipine (n=3; P≤0.05 compared with control and nifedipine).

To analyze which PKC isoform is responsible for the increase in ischemia-induced permeability, we used antisense ODN to specifically suppress expression of the respective PKC isoform (Figure 9; n=4). Endothelial cells were incubated with lipofectin (10 µg/mL) and antisense ODN, sense ODN, or reversed ODN against PKC-α, -ε, or -ζ before exposure to KCN/DG. Antisense ODN for PKC-α almost completely inhibited the increase in ischemia-induced endothelial cell permeability. In contrast to the effects of antisense against PKC-α, antisense ODN against PKC-ε and -ζ did not reduce ischemia-induced permeability significantly.

Finally, we examined whether the effects of nifedipine are stereoselective with a dihydropyridine nifedipine enantiomer (BAY K005552). In addition, a dihydropyridine compound that does not bind to L-type calcium channels (BAY R001223) was investigated. These compounds are similar in structure but have no effect on the L-type calcium channel (personal communication from Bayer Inc, 1998). The drugs (Figure 10) were similar in efficacy to nifedipine in terms of blocking ischemia-induced endothelial cell–layer permeability increases (n=4; P≤0.05 compared with KCN/DG alone).

**Discussion**

Ischemia increased endothelial cell permeability, which was associated with an increase in [Ca²⁺], and PKC activation. Nifedipine inhibited this effect dose dependently without changes in [Ca²⁺], homeostasis but with inhibition of PKC. PKC-α and -ζ inhibition may be responsible for the ameliorating effect of nifedipine on ischemia-induced endothelial cell permeability. Our findings suggest that PKC is the molecular target that influences ischemia-induced increases in endothelial cell permeability. Lynch et al. were the first to observe that PKC activation is an important signal transduction pathway to increase macromolecular transport across endothelial monolayers. Several reports subsequently supported this finding in vitro. PKC activation plays a role in the increased endothelial cell permeability induced by bradykinin, hydrogen peroxides, thrombin, and endothelins. Thus, in addition to an increase in [Ca²⁺], the activation of PKC appears to be a major determinant of an increase in endothelial cell permeability. Increased PKC activity may be subsequent to or actually induced by the increase in [Ca²⁺]. On the other hand, increased endothelial cell permeability after direct activation of PKC via phorbol ester suggests a mechanism independent of changes in [Ca²⁺]. However, the exact mechanism of PKC action within endothelial cells is not clear. Exposure to phorbol ester leads to phosphorylation and redistribution of the cytoskeletal proteins caldesmon and vimentin, in concert with agonist-
mediated endothelial cell contraction and resultant barrier dysfunction. This observation indicates that PKC activation increases endothelial cell permeability by an interaction with cytoskeletal proteins. This hypothesis is supported by recent observations in epithelial cells, in which PKC-dependent actin reorganization led to modulation of intercellular permeability.

We observed an effect of KCN/DG on the PKC isoforms α and ζ but not δ and ε. Translocation of PKC-α after ischemia has also been observed by others. Wang and coworkers demonstrated that PKC-α is responsible for the opening of ion channels with ischemia. Others have found a PKC-α translocation in ischemic myocardium and myocardial cells. We showed previously that PKC-α plays an important role in the regulation of endothelial cell permeability. Our results with KCN/DG support a role for this PKC isoform in mediating endothelial cell permeability during ischemia. Other investigators have also observed an effect of ischemia on the PKC isoform ε. However, we did not observe an effect on the ischemia-induced increase in endothelial cell permeability by downregulation of PKC-ε. Because we used a different model and induced metabolic rather than perfusion-related ischemia, it is possible that model-dependent characteristics play a role in these discrepant findings. Our finding with respect to the PKC isoform ζ during ischemia is in accordance with the recent observation of Mizukami et al.

Block and coworkers showed that 3 calcium antagonists (nifedipine, verapamil, and diltiazem) all inhibited the action of recombinant platelet-derived growth factor in vascular smooth muscle cells and reduced PKC activation. They also showed that calcium antagonists modulate expression of HMG-CoA reductase and LDL-receptor genes stimulated by platelet-derived growth factor. These calcium antagonist-mediated effects on gene expression were observed at pharmacological concentrations that were 1 to 2 orders of magnitude lower than those required for inhibition of depolarization-induced opening of voltage-sensitive L-type calcium channels. Similar observations have been made by other groups. However, in the above-mentioned studies, the effects of calcium antagonists were investigated in cell types that possess L-type calcium channels, such as vascular smooth muscle cells. Recently, Orth et al investigated the effects of calcium antagonists in mesangial cells and could not associate the observed inhibitory effects on cell proliferation with changes in intracellular calcium. Our data demonstrate that the inhibitory effect of calcium channel blockers on PKC is not dependent on L-type calcium channels or on changes in intracellular calcium regulation. PKC inhibition by calcium antagonists is conceivably responsible for the effects of these compounds in other cell types that do not express L-type calcium channels.

A direct interaction between nifedipine and PKC isoforms has not been observed in in vitro investigations. However, the
inhibitory effect of nifedipine on TPA-induced permeability would favor such an explanation. Our findings suggest that nifedipine blocks the proximal part of the PKC activation pathway. Because \([Ca^{2+}]_i\) was not influenced by nifedipine in the present study, we would rather speculate that nifedipine interferes with the release of phospholipids from the plasma membrane or other intracellular lipid pools. Possible candidates are diacylglycerol, ceramide, or other products of the phosphoinositide cycle. An increased production of these compounds in ischemia has been shown. Decreased generation of these compounds would then lead to a decrease in PKC activation. Another possible mechanism is the inhibition of PKC binding to intracellular sites and cell membranes. Our results may explain previous experimental studies in which calcium antagonists were useful in protecting from ischemic injury in various tissues. Translocation of PKC isoforms in ischemic cardiomyocytes has been shown by Yoshida et al. Translocation of protein kinase C isoforms has been observed in both ischemic neuronal tissue and blood vessels. A role for PKC activation is also suggested by the observation that this isoform opens ion channels in ischemic tissue. The finding that calcium antagonists influence PKC activity during ischemia could be important, because PKC inhibition has been found to be beneficial in protecting the heart, lung, and brain from ischemia. The link between ischemia and increased endothelial cell–layer permeability is also relevant. For instance, the prevention of ischemia-induced permeability increases could be useful therapeutically.

In summary, we observed that the dihydropyridine calcium antagonist nifedipine prevented the ischemia-induced in-
crease in endothelial cell permeability. We ruled out the possibility that this was dependent on L-type calcium channels or was mediated by changes in \([Ca^{2+}]_i\) regulation. Instead, we observed PKC inhibition in endothelial cells by nifedipine, specifically, the inhibition of PKC-\(\alpha\). Although the exact molecular mechanism of this effect is unclear, our findings provide a rationale for the observed vascular effects of calcium antagonists after ischemia and may also explain the effects of calcium antagonists in other cell types that do not express L-type calcium channels.

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