Calcium Channel Blockade Enhances Nitric Oxide Synthase Expression by Cultured Endothelial Cells

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Abstract—In a recent study, we found marked increases in nitric oxide (NO) production and endothelial and inducible NO synthase (eNOS and iNOS) expressions with calcium channel blockade in rats with chronic renal failure. This study was undertaken to determine whether enhanced NO production with calcium channel blockade is a direct effect of this therapy or a consequence of the associated hemodynamic and humoral changes. We tested the effects of a calcium channel blocker, felodipine (10\(^{-5}\), 10\(^{-6}\), and 10\(^{-7}\) mol/L), on nitrate and nitrite (NOx) generation, Ca\(^{2+}\)-dependent and -independent NOS activity, and eNOS and iNOS protein masses in proliferating and quiescent rat aortic endothelial cells in culture. Compared with vehicle alone, felodipine significantly increased NOx generation, Ca\(^{2+}\)-dependent NOS activity, and eNOS protein mass in proliferating and quiescent endothelial cells. Felodipine did not modify the stimulatory action of 10% fetal calf serum on DNA synthesis (thymidine incorporation) and cell proliferation. Ca\(^{2+}\)-independent NOS activity and iNOS protein expression were negligible and unaffected by calcium channel blockade. NOx production and NOS expression were greater in proliferating cells than in quiescent cells. Thus, calcium channel blockade upregulates endothelial NO production in vitro, confirming our previous in vivo study. This observation indicates that the reductions in cytosolic [Ca\(^{2+}\)] and vasodilation with calcium channel blockade are not only due to inhibition of Ca\(^{2+}\) entry but also to an NO-cGMP–mediated mechanism. (Hypertension. 1998;32:718-723.)

Key Words: calcium channel blockers • endothelium-derived relaxing factor • nitric oxide • nitric oxide synthase • rats, inbred SHR

Nitric oxide (NO), otherwise known as endothelium-derived relaxing factor (EDRF), plays an important role in blood pressure homeostasis. In addition, NO inhibits platelet adhesion and cell proliferation. Thus, normal production of NO can protect renal and systemic vasculature against pressure-dependent and pressure-independent mechanisms that lead to glomerular and arteriolar sclerosis. In fact, inhibition of NO production by NO synthase (NOS) inhibitor causes sustained hypertension, vascular injury, and glomerulosclerosis. Interestingly, calcium channel blockade has been shown to mitigate hypertension, improve NO production, and prevent vascular and glomerular injury in rats subjected to chronic NOS blockade. In addition, administration of the calcium channel blocker nifedipine has been shown to reverse the age-dependent decline in brain NOS activity and NO production in the senescent mouse. Likewise, chronic administration of the calcium channel blocker RO-5967 mitigated hypertension, reduced intimal inflammatory infiltrate, and improved acetylcholine-induced vasorelaxation of aortic rings in spontaneously hypertensive rats.

In a recent study we demonstrated a marked reduction of NO production together with depressed vascular and remnant kidney NOS activity, as well as endothelial and inducible NOS (eNOS and iNOS) protein abundance, in rats with chronic renal failure. This was associated with a significant elevation of basal cytosolic [Ca\(^{2+}\)] and marked attenuation of stimulated surge in [Ca\(^{2+}\)]. Chronic administration of the calcium channel blocker felodipine ameliorated the associated hypertension, increased NO production, and restored vascular and renal NOS activity and protein abundance. The in vivo nature of the latter study did not allow distinction between a possible direct and an indirect (eg, hemodynamic) effect of calcium channel blockade on NOS expression. The present study was designed to determine the effect of calcium channel blockade on NOS activity and protein expression by cultured endothelial cells in vitro, where hemodynamic and other in vivo factors are necessarily absent. The results showed a significant increase in NO production, eNOS activity, and eNOS protein expression by cultured endothelial cells treated with felodipine when compared with the vehicle-treated cells. These observations are suggestive of a direct stimulatory action of calcium channel blockade on endothelial NO production.

Methods

The following products were purchased from Sigma Chemical Co: minimum essential medium (Eagle, D-Valine modification), collagenases II and IV, rabbit anti-human von Willebrand factor antiserum, rhodamine-conjugated goat anti-rabbit IgG, penicillin, strep-
tonycin, endothelial cell growth supplement, heparin, leupeptin, aprotinin, soybean trypsin inhibitor, PMSF, calmodulin, tetrahydrobiopterin, NADPH, L-arginine, and L-citrulline. Dowex AG 50-WX8 resin was obtained from Bio-Rad Laboratories. L-[3 H]-arginine and Hybond-ECL membranes were obtained from Amersham Life Sciences Inc, and [3 H]-thymidine was purchased from Du Pont–NEN.

Hybond-ECL membranes were obtained from Amersham Life Sciences Inc, and [3 H]-thymidine was purchased from Du Pont–NEN. Anti-eNOS monoclonal antibody, peroxidase-conjugated goat anti-mouse IgG antibody, anti-Mac NOS-I, human endothelial positive control, and mouse macrophage positive control were supplied by Transduction Laboratories. Tris-glycine gel was bought from Novex Inc.

**Methods**

**Cell Culture**

Adult Sprague-Dawley rats were anesthetized with an intraperitoneal injection of sodium pentobarbital, and a thoracotomy was performed. The full length of the thoracic aorta was removed under sterile conditions, rinsed 3 times with PBS, and placed into a 100-mm culture dish filled with serum-free culture medium on ice. The vessel was gently cleaned of periadventitial fat and connective tissue, cut to expose the luminal surface, and rinsed with the culture medium. The surface was then covered with a collagenase solution (II and IV, 1 mg/mL), and incubated at 37°C for 1 hour. After incubation, the solution containing detached endothelial cells was aspirated and placed in a tube with 5 mL medium plus serum to arrest the digestion process. The vessel surface was then subjected to a forceful stream of the culture medium using a 10-mL syringe to collect the remaining cells loosely attached to the surface. Finally, the vessel was rinsed with the medium, and the medium containing additional cells was collected and combined with the initial aspirate. The cell suspension was centrifuged at 2000 rpm for 10 minutes. The cell pellet was washed twice, suspended in medium to a total volume of 2 mL, and placed in a 60-mm culture dish. The dish was then placed in a humidified incubator at 37°C with 5% CO₂. After 2 days, 2 mL of fresh medium was added to the dish, and the incubation was continued for an additional 2 days. Thereafter, the medium was changed every other day. Once the cells had formed a monolayer, the cells were subcultured. The cells were identified by staining with a specific antibody to von Willebrand factor and fluorescent-labeled LDL as described previously.¹

**Study Design**

The cells obtained on passages 4 and 7 were used for the experiments. The cells reaching 70% to 80% confluence were treated with the medium, and the medium containing additional cells was collected and combined with the initial aspirate. The cell suspension was centrifuged at 2000 rpm for 10 minutes. The cell pellet was washed twice, suspended in medium to a total volume of 2 mL, and placed in a 60-mm culture dish. The dish was then placed in a humidified incubator at 37°C with 5% CO₂. After 2 days, 2 mL of fresh medium was added to the dish, and the incubation was continued for an additional 2 days. Thereafter, the medium was changed every other day. Once the cells had formed a monolayer, the cells were subcultured. The cells were identified by staining with a specific antibody to von Willebrand factor and fluorescent-labeled LDL as described previously.¹

**NOS Activity Assay**

NOS activity was measured as previously described.² In brief, the cells were washed 3 times with PBS, scraped with a plastic scraper, collected in centrifuge tubes, and spun at 500 g for 6 minutes. The cells were then resuspended in 200 μL of homogenization buffer (Tris-HCl [50 mmol/L], EDTA [0.1 mmol/L], and EGTA [0.1 mmol/L]) containing the following protease inhibitors (leupeptin [10 μg/mL], aprotinin [2 μg/mL], soybean trypsin inhibitor [10 μg/mL], and PMSF [50 μmol/L], pH 7.4). The homogenates were then assayed for NOS activity. Each sample was incubated in a buffer (Tris-HCl [50 mmol/L], EDTA [0.1 mmol/L], and EGTA [0.1 mmol/L]) containing cofactors (calmodulin [10 μmol/L], tetrahydrobiopterin [3 μmol/L], CaCl₂ [2.5 mmol/L], and NADPH [1 mmol/L]) and the substrate L-arginine 100 μmol/L, combined with L-[3 H]-arginine (0.2 μCi/pmL) at 55 Ci/mmol) for 45 minutes at 37°C. The mixture also contained 1 mmol/L L-citrulline to minimize conversion of newly formed L-[3 H]-citrulline back to L-[3 H]-arginine. After the incubation period, the reaction was quenched by the addition of 1 mL of stop buffer (HEPES [20 mmol/L], pH 5.5, EDTA [2 mmol/L], and EGTA [2 mmol/L]). The reaction mix was applied to a 1-mL column containing Dowex AG 50-WX8 (Na⁺ form) resin that had been preequilibrated with the stop buffer. L-[3 H]-citrulline was eluted twice with 0.5 mL of stop buffer, and radioactivity was determined by liquid scintillation counting. Calcium-independent NOS (iNOS) activity was measured in replicate samples by replacing CaCl₂ with EDTA (1.7 mmol/L). Protein concentration of the cell lysates was determined by using a Bio-Rad kit with bovine albumin used as the standard.

**Western Blot Analysis**

These measurements were carried out to determine the eNOS and iNOS protein masses as previously described.³,⁴ Briefly, the treated cells were washed with PBS, then extracted directly into the sample buffer (2% SDS and 10% glycerol, 0.0025% bromophenol, and 63 mmol/L Tris-HCl, pH 6.8), and the total protein was determined by using a Bio-Rad kit. Cell lysate protein (50 μg) was size-fractionated on 4% to 12% Tris-glycine gel at 130 V for 3 hours. In preliminary experiments, we found that the given protein concentrations were within the linear range of detection for our Western blot technique. After electrophoresis, proteins were transferred onto Hybond-ECL membrane at 400 mA for 120 minutes using the Novex transfer system. The membrane was prehybridized in 10 mL buffer A (10 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 0.1% Tween-20, and 10% nonfat milk powder) for 1 hour and then hybridized for an additional 1 hour in the same buffer containing 10 μL of the given anti-NOS monoclonal antibody (1:1000). Thereafter, the membrane was washed for 30 minutes in a shaking bath; the wash buffer (buffer A without nonfat milk) was changed every 5 minutes before 1 hour of incubation in buffer A plus goat anti-mouse IgG–horseradish peroxidase at the final titer of 1:1000. Experiments were carried out at room temperature. The washes were repeated before the membrane was developed with a light-emitting nonradioactive method using ECL reagent (Amersham Inc). The membrane was then subjected to autoradiography for 1 to 5 minutes. The autoradmo-

![Figure 1. NO production assessed as total NOx, detected in the culture medium, 24 hours after addition of vehicle (control) and felodipine at concentrations of 10⁻⁸ mol/L (felo-5), 10⁻⁶ mol/L (felo-6), and 10⁻⁷ mol/L (felo-7). A, Results of experiments carried out in proliferating endothelial cells cultured in the presence of 10% FCS. B, Results in quiescent cells cultured in a medium containing 0.5% FCS. Data are mean ± SEM of a minimum of 4 to 6 experiments. *P<0.05 vs control, **P<0.01 vs control.](image-url)
Calcium Channel Blockade Increases NOS Expression

Results

NO Production

Data are shown in Figure 1. NO production assessed as total nitrate and nitrite (NOx) recovered in the culture medium was significantly greater in endothelial cells treated with 10^{-7} mol/L felodipine than in the vehicle-treated cells (P<0.01). This was true for both proliferating and quiescent cells. No further increase was observed in NO production at the higher felodipine concentrations (10^{-6} and 10^{-5} mol/L). Under all conditions, NO production by proliferating cells was significantly greater than the corresponding measurement obtained from quiescent cells. On each occasion, similar responses were observed in cells used at passages 4 and 7.

NOS Activity

Both proliferating and quiescent endothelial cells treated with 10^{-6} mol/L felodipine showed a significant increase in calcium-dependent NOS activity when compared with the vehicle-treated cells (P<0.05). The magnitude of the felodipine-induced rise in calcium-dependent NOS activity at 10^{-6} mol/L was similar to that found at the 10^{-5} mol/L concentration. Under each condition, NOS activity was significantly higher in proliferating cells than in the quiescent cells.

The magnitude of iNOS activity detected in our endothelial cell preparation was minimal and was virtually unaffected by calcium channel blockade. Data are illustrated in Figure 2.

NOS Protein Mass

Data are depicted in Figures 3, 4, 5, 6, 7, and 8. Both proliferating and quiescent endothelial cells treated with 10^{-7} mol/L felodipine showed a significant rise in eNOS protein mass when compared with the vehicle-treated cells. Similar effects were observed at 10^{-6} and 10^{-5} mol/L felodipine concentrations. No detectable quantities of iNOS were found by Western blot analysis of protein extracts obtained from either felodipine- or vehicle-treated endothelial cells corresponding with their negligible iNOS activity. A significant direct correlation was found between calcium-dependent NOS activity and eNOS protein mass. Likewise, eNOS protein mass was directly related to NO production. On each occasion, cells studied at passage 7 behaved similarly to those studied at passage 4.

Cell Proliferation

Data are shown in Figures 9 and 10. The addition of felodipine to the culture medium did not significantly alter...
endothelial cell proliferation in response to stimulation with 10% FCS. Similarly, increased DNA synthesis in response to stimulation with 10% FCS as determined by thymidine incorporation was not affected by felodipine at either concentration used. Similar responses were observed with cells used at passages 4 and 7. In contrast, calcium channel blockade significantly depressed 10% FCS–stimulated DNA synthesis in rat mesangial cells as previously reported. This observation points to the disparate actions of calcium channel blockade on proliferative response in the 2 cell systems.

Discussion

NO, once considered a toxic industrial and environmental pollutant, is now regarded as a major endogenous biological modulator with numerous diverse functions. Constitutive generation of NO by the endothelial cells contributes to the vasodilatory tone and as such plays an important role in blood pressure homeostasis, renal and systemic vascular resistance, and tissue perfusion. In addition, NO interferes with platelet adhesion and retards cellular proliferation and serves as a neurotransmitter, an immune effector, an antioxidant, and a free radical.

Impaired NO production can cause hypertension by raising renal and systemic vascular resistances and by promoting sodium and water retention. In addition, NO deficiency can promote glomerulosclerosis and arteriosclerosis by facilitating cell migration and proliferation within the glomeruli and blood vessel walls. Moreover, loss of the inhibitory effect of NO on platelet adhesion can facilitate the formation of microthrombi and the release of thrombogenic, proinflammatory, and mitogenic platelet factors. Thus, NO deficiency can impair renal and vascular function and structure.

As noted above, several earlier observations have suggested that various calcium channel blockers improve endothelial function and enhance EDRF/NO production. However, it is not clear whether the given calcium channel blockers enhance the activity or production of NO. In

Figure 4. Group data illustrating eNOS protein mass in quiescent (A) and proliferating (B) endothelial cells incubated for 24 hours in the presence of vehicle (control), 10^{-5} mol/L felodipine (felo-5), or 10^{-6} mol/L felodipine (felo-6). Data represent mean±SEM of 4 to 6 experiments. **P<0.01 vs control.

Figure 5. Representative Western blot of eNOS protein obtained in quiescent and proliferating rat aorta endothelial cells incubated in the presence of either vehicle (lanes 1 and 2, quiescent cells; lanes 3 and 4, proliferating cells) or 10^{-7} mol/L felodipine (lanes 3 and 4, quiescent cells; lanes 7 and 8, proliferating cells). Incubation was continued for 24 hours.

Figure 6. Group data illustrating eNOS protein mass in quiescent and proliferating endothelial cells incubated for 24 hours in the presence of vehicle (control) and 10^{-7} mol/L felodipine (felo-7). Data are mean±SEM of 4 experiments. **P<0.01 vs control.

Figure 7. Correlation between Ca^{2+}-dependent NOS activity and eNOS protein mass in proliferating (A) and quiescent (B) cells incubated in the presence of vehicle (●), 10^{-5} mol/L (■), and 10^{-6} mol/L (▲) felodipine.
addition, it is not clear whether the effect is caused by a direct action of the drugs on the endothelial cells or whether it is secondary to alterations in blood flow, shear stress, and blood pressure, which are well-known modulators of eNOS expression.1,18–21 In the present study, we tested the effect of the calcium channel blocker felodipine on NO production and NOS protein expression in cultured rat aorta endothelial cells. Consequently, the effect of drug-induced hemodynamic alterations on L-arginine–NO pathway was obviated by the in vitro nature of the experiments. Moreover, possible modulatory influence of drug-induced neurohormonal changes and cell-type interactions were avoided in this system. Thus, the observed increases in NO production, eNOS activity, and eNOS protein mass represent a direct effect of the drug on endothelial cells. This observation can account for the reported improvement in endothelial function with chronic administration of several different calcium channel blockers in animals with hypertension and chronic renal failure.2–5

It is important to note that the addition of felodipine in the given concentrations did not affect 10% FCS–stimulated endothelial cell growth as determined by DNA synthesis and cell count in vitro. This finding contrasted the growth inhibitory effect of calcium channel blockade on the rat mesangial cells shown in this study and by other investigators.12 This is particularly interesting because increased NO production per se may inhibit cell growth.1 The reason for the observed disparity in the effect of calcium channel blockade on endothelial versus mesangial cell growth is uncertain and requires further investigation. Under all conditions, proliferating endothelial cells exhibited greater release of NO and higher eNOS activity than their corresponding quiescent cells. This was not because of differences in cell numbers between the parallel experiment because all data were normalized and expressed per milligram of cellular protein. The effects of cell growth on NO production and NOS protein expression shown in the rat aorta endothelial cells in this study are in complete conformity with the results of an earlier study of bovine aorta endothelial cells.8

In summary, calcium channel blockade with felodipine increased NO production, eNOS activity, and eNOS protein expression by cultured rat aorta endothelial cells. This phenomenon can account for the reported improvement in endothelial function with calcium channel blockade in renal and hypertensive disorders. The data further point to enhanced NO generation as an additional mechanism for the vasodilatory effect of calcium channel blockade.

Figure 8. Correlation between NO production (NOx) and eNOS protein mass in proliferating (A) and quiescent (B) cells incubated in presence of vehicle ( ), 10⁻⁵ mol/L felodipine ( ), and 10⁻⁶ mol/L ( ) felodipine.

Figure 9. Cell count of rat aorta endothelial cells cultured for 24 hours in a medium containing 10% FCS and either vehicle (control) or felodipine at concentrations of 10⁻⁵ mol/L (felo-5) or 10⁻⁶ mol/L (felo-6). No significant difference was found.

Figure 10. [³H]-Thymidine incorporation in rat aorta endothelial cells (A) and rat mesangial cells (B) cultured for 24 hours in medium containing 10% FCS and either vehicle (control) or felodipine at concentrations of 10⁻⁸ mol/L (felo-8), 10⁻⁷ mol/L (felo-7), 10⁻⁶ mol/L (felo-6), or 10⁻⁵ mol/L (felo-5). Data are mean±SEM of 6 experiments. *P<0.05 vs control.

References


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