Nifedipine Increases Endothelial Nitric Oxide Bioavailability by Antioxidative Mechanisms

Reinhard Berkels, Guido Egink, Tobias A. Marsen, Henning Bartels, Renate Roesen, Wolfgang Klaus

Abstract—Short-term treatment of the endothelium with dihydropyridine calcium antagonists resulted in an increased release in NO that is not due to a modulation of L-type calcium channels, because macrovascular endothelial cells do not express this channel. We investigated whether long-term (48 hours) treatment of porcine endothelial cell cultures with the dihydropyridine calcium antagonist nifedipine resulted in a similar enhanced NO liberation. Regarding to the underlying mechanism, we examined whether (1) nifedipine changed the mRNA and protein levels of the constitutive endothelial NO synthase (NOS) in endothelial cell cultures or (2) nifedipine exerts an NO protective effect via its antioxidative properties, as revealed in a cell culture model and with native endothelium from porcine coronary arteries. Nifedipine induced a significant time- and concentration-dependent increase (132 ± 47%, 1 µmol/L, 40 minutes’ incubation) in the basal NO liberation (oxyhemoglobin assay). This increased NO release was not due to elevated NOS (type III) mRNA (Northern blot analysis) and protein (Western blot analysis) levels. However, nifedipine (both short- and long-term treatment) significantly reduced the basal and glucose (20 and 30 mmol/L)-stimulated formation of reactive oxygen species (lucigenin assay) of endothelial cell cultures and native cells. We conclude that the calcium antagonist nifedipine enhances the bioavailability of endothelial NO without significantly altering the NOS (type III) mRNA and protein expression, possibly via an antioxidative protection. This increased NO availability may cause part of the vasodilation and might contribute to the antithrombotic, antiproliferative, and antiatherosclerotic effects of dihydropyridine calcium antagonists. (Hypertension. 2001;37:240-245.)

Key Words: antioxidants ■ calcium antagonists ■ endothelium ■ nitric oxide ■ nitric oxide synthase ■ protein ■ nifedipine

Dihydropyridine (DHP)-type calcium antagonists are important drugs in the treatment of hypertension and coronary heart disease. They induce their specific pharmacological effects by binding to L-type calcium channels,1,2 which results in a reduced calcium influx with impaired electromechanical coupling both in vascular smooth muscle cells and in the heart.3 A few years ago, however, it was observed that removal of the endothelium or blockade of the guanylate cyclase of the vessel wall reduced the efficacy of the DHP-induced vasorelaxation,4 which indicated an endothelium-responsive cGMP-mediated process as part of the DHP action. Because macrovascular endothelial cells lack voltage-operated L-type calcium channels,5,6 the DHPs must exert these effects via other mechanisms. In this context, it is worth mentioning that DHPs may also exert antithrombotic7,8 and antiatherosclerotic9,10 effects in different experimental and clinical settings, of which the underlying signal transduction remains obscure.

With this background, it is tempting to speculate that NO, one of the most prominent endothelium-derived factors,11,12 which relaxes smooth muscle cells via the cGMP signal cascade,13 might be involved in these DHP actions. In fact, in various models, evidence has accumulated that DHPs stimulate the endothelial NO release,14–19 which may mediate or at least contribute to the above-mentioned calcium channel-independent effects.

Up to now, however, all of these findings were obtained only after acute exposure to DHPs (lasting minutes to hours). Because patients usually take calcium antagonists for a longer period of time, we investigated whether long-term treatment of endothelial cell cultures with nifedipine may alter the basal endothelial NO release as well as the expression of the constitutive endothelial NO synthase (ecNOS) mRNA and protein. Furthermore, because NO is rapidly deactivated by reactive oxygen species (ROS)20 and the DHPs may act as scavengers as known from different in vitro models,21–23 we also determined the antioxidative potency of nifedipine in endothelial cell cultures to reveal a potential NO-protection effect as an underlying mechanism of the increased NO bioavailability.

Methods

Cell Culture of Porcine Aortic Endothelial Cells

Porcine aortas were obtained fresh from a local abattoir. Immediately after removal, the vessels were stored in sterile PBS (4°C) with...
penicillin (200 mg/L) and streptomycin (200 mg/L) (Sigma Chemical Co). The endothelial cells were isolated enzymatically (1 mg/mL dispase; Boehringer Mannheim) and cultured in Medium 199 (GIBCO) with 10% FCS (GIBCO), 100 mg/L penicillin, 100 mg/L streptomycin, and HEPES. Cells were grown in an incubator (Heraeus) at 37°C in a 5% CO₂ atmosphere and saturated humidity. The culture medium was replaced every day to compensate for decomposition of the substances during treatment. On confluence (2-4 days), the cells were detached through treatment with trypsin (0.05% and 0.02% EDTA; Sigma Chemical Co), washed once, and seeded into 25-cm² flasks and again to confluence. Only passage 2 was used for the experiments. The purity of the porcine aortic endothelial cell (PAEC) culture was tested by uptake of Dil-labeled acetylated LDL, factor VIII immunostaining, and anti–smooth muscle actin immunostaining (Sigma Chemical Co). To avoid decomposition of the light-sensitive drug, nifedipine was added under yellow light, and the cultures were kept in the dark. Nifedipine was dissolved in DMSO (stock solution 10 mmol/L) and subsequently diluted in medium, resulting in final concentrations of 0.1 and 1.0 µmol/L and a maximum DMSO concentration of 0.01%, which did not cause any solvent effects on the parameters tested.

Measurement of Antioxidative Effects

The release of ROS from endothelial cells was measured in cultured endothelial cell suspensions and in native endothelial cells with a lucigenin chemiluminescence assay. Briefly, coronary arteries of female pigs were prepared immediately after excision of the hearts and stored in Tyrode's solution saturated with oxygen. During the removal of connective tissue, special care was taken to preserve the endothelium. The arteries were cut into rings of 0.3 cm (wt weight 2 to 3 g), and the rings opened with a longitudinal cut. The pieces were incubated in 450 µL HEPES-buffered saline solution (in mmol/L) (HEPES 5, NaCl 140, KCl 5, CaCl₂ 2, MgCl₂ 1, and glucose 5, adjusted to pH 7.4) with or without nifedipine or superoxide dismutase (SOD; 70 U/mL; Sigma Chemical Co) and with different glucose concentrations (5, 10, or 30 mmol/L) for 20 minutes at 37°C. Basal ROS formation of native cells and cell cultures was measured at 5 mmol/L glucose. Hyperglycemia (30 mmol/L) was used to induce an increase in the ROS release from native endothelial cells. To determine the NO release of cultured cells, we used the microtechnology method of measurement of NO. Measurement of NO

**Nonradioactive Northern Blot Analysis**

Before isolation of the mRNA from confluent PAEC monolayers, the cells were treated with nifedipine (untreated cells as control) for the entire passage (48 hours). Total cellular mRNA was extracted from endothelial cells according to a modification of a mRNA isolation kit (Boehringer Mannheim). Briefly, endothelial cells were solubilized in lysis buffer consisting of 5 mol/L guanidium thiocyanate, 0.7 mol/L Tris-HCl, pH 8.0, 1.3 mol/L LiCl, 65 mmol/L EDTA, 0.25% dithiothreitol, and 0.13% laurylsarcosine. Poly(A) mRNA was subsequently incubated with biotin-labeled oligo(dt)₂₀ probe and magnetic streptavidin particles at 37°C for 5 minutes. Streptavidin-mRNA complex was resuspended in buffer (10 mmol/L Tris buffer, 0.2 mol/L LiCl, 1 mmol/L EDTA, pH 7.5), repetitively washed, and heat dissociated (65°C, solvent H₂O) before being magnetically separated.

RNA was then size-fractionated in a 1.2% agarose gel electrophoresis before being transferred and immobilized on a nylon membrane (Boehringer Mannheim). Hybridization was performed as RNA-RNA hybrids because of superior strength and higher affinity. Membranes were hybridized simultaneously with a linearized 1.5-kb fragment of digoxigenin-UTP-labeled MOS RNA and with digoxigenin-UTP–labeled β-actin RNA (Boehringer Mannheim) for verification of equal amounts of RNA. Digoxigenin-UTP labeling had been performed through in vitro transcription of the 1.5-kb human MOS cdNA insert with T7 RNA polymerase. The ecNOS-specific probe was kindly donated by Kenneth Bloch (Department of Anesthesiology, Massachusetts General Hospital, Boston, Mass). Sequencing of 200 bp of the probe confirmed the identity of the ecNOS-RNA-RNA hybrids were visualized with chemiluminescence after binding of anti-digoxigenin alkaline phosphatase conjugate with CDP-Star and were exposed to Lumi-Film x-ray film (Boehringer Mannheim). The amount of mRNA expression was quantified through densitometry with National Institutes of Health Image software on an Apple Macintosh computer equipped with a Microtech scanner and corrected for β-actin expression.

**Cell Lysis and Immunoblot Analysis**

Treated and untreated confluent PAECs were washed with ice-cold PBS and immediately solubilized in 500 µL lysis buffer (150 mmol/L NaCl, 50 mmol/L HEPES, 1% SDS, and 1 tablet/50 mL complete protease inhibitor cocktail; Boehringer Mannheim). The amount of protein was determined with a Bio-Rad protein kit before equal amounts of protein were used in 1 blot. Cell lysate protein was size-fractionated on an 8% SDS gel and blotted onto a PVDF (Bio-Rad) membrane with wet blotting. These immunoblots were then blocked with nonfat milk powder (2%, 30 minutes), incubated with the primary monoclonal mouse anti–endothelial cell NOS antibody (1: 3000, 1 hour), and washed in buffer (TBS-T) before being incubated with the secondary horseradish peroxidase–conjugated anti-mouse IgG antibody (1: 3000, 1 hour) (Affinity Research). Protein-antibody conjugates were visualized with chemiluminescence reagent (Boehringer Mannheim), which uses horseradish peroxidase to oxidize luminol and exposed to Kodak medical x-ray film for 0.5, 1, 1.5, and 2 minutes. The protein amount was quantified through densitometry with National Institutes of Health Image software on an Apple Macintosh computer equipped with a Microtech scanner.

**Measurement of NO**

To determine the NO release of cultured cells, we used the oxyhemoglobin assay. This technique measures the conversion of oxyhemoglobin to met-hemoglobin by NO. Briefly, PAECs were cultured in 60-cm² Petri dishes and treated with nifedipine during the entire passage (untreated cells as a control). The cells were washed three times with HEPES-buffered saline solution, and the cells were prepared oxygen-free (0.25 mol/L LiCl in HEPES buffer) and exposed to 2 mL of the dishes and directly measured. The cells were kept at 37°C during the measurement. The spectra were measured with a single-wavelength spectrophotometer (DU 7500; Beckman Instruments). To access the antioxidation of the oxyhemoglobin, a dish without cells was identically prepared for comparison. The oxyhemoglobin was measured every 10 minutes for 40 minutes. The antioxidation spectra were subtracted to obtain difference spectra, and the increasing met-hemoglobin formation was measured at 402 nm compared with the isosbestic point at 411 nm.

**Preparation of Oxyhemoglobin**

Oxyhemoglobin was prepared by dissolving rabbit hemoglobin (containing <50% met-hemoglobin) in phosphate buffer. During
stirring, the mixture was exposed to oxygen (95% plus 5% CO₂) for 20 minutes. Then, a molar excess of sodium dithionite was added and gassed for 15 minutes. The resulting oxyhemoglobin was purified through gel chromatography to remove the sodium dithionite. The purity was controlled with spectroscopy, and the concentration was determined with the Lambert-Beer law.

Statistics
All data are expressed as mean±SEM for n experiments. The results were analyzed by the nonparametric Wilcoxon test for matched pairs and the Mann-Whitney test for nonmatched pairs. A value of P,<0.05 was considered statistically significant.

Results

NO Measurement
The treatment of PAECs with nifedipine for 48 hours (throughout the entire passage) resulted in an increased basal NO release of these monolayers (cells from the same animal). During the measurement, nifedipine was not present in the supernatant. At 20 minutes after integration of the NO liberation through the formation of met-hemoglobin, a significant concentration-dependent increase in the NO release by nifedipine (0.1 and 1 μmol/L) could be demonstrated, in contrast to untreated cells (n=6) (Figure 1), which persisted for the entire course of the experiment. Preincubation with NNA (100 μmol/L) resulted in a complete inhibition of the NO formation of the cells (n=3) (Figure 1).

Northern Blot Analysis
The treatment of endothelial cells with nifedipine did not result in a significant alteration in the NOS mRNA expression (Figure 2), although at a concentration of 1 μmol/L, a slight increase could be shown (n=4) (Figure 2).

Western Blot Analysis
The Western blot analysis revealed the same behavior as the Northern blot analysis. Nifedipine at a concentration of 0.1 μmol/L did not alter the NOS protein expression (n=4), whereas at the higher concentration (1 μmol/L), a slight but nonsignificant increase was seen (n=4) (Figure 2).

Antioxidative Properties of Nifedipine
Nifedipine (1 μmol/L) induced a significant decrease in the basal ROS release (lucigenin assay) of endothelial cell cultures (n=6) (Figure 3) and of native cells of porcine coronary arteries (−85±21% at 1 μmol/L, n=4). This effect could be achieved by both short-term (20 minutes) and long-term (48 hours) incubation of the cells with nifedipine (1 μmol/L) (n=6) (Figure 3).

Because in different pathological situations, such as diabetes and atherosclerosis, a marked increase in the ROS concentration has been found,24,31,32 we induced an increased ROS release in native vessels and in cell suspensions by increasing the glucose concentrations (30 or 20 mmol/L).

Figure 1. Nifedipine (NIF) induced a significant concentration- and time-dependent increase in the basal NO release (oxyhemoglobin assay) from cultured PAECs (n=6). This effect could be blocked with N-nitro-L-arginine (NNA), a specific NOS inhibitor (n=3). * Indicates P<0.05 from control group.

Figure 2. Influence of nifedipine (0.1 and 1 μmol/L; 48 hours) (NIF) on the ecNOS mRNA and protein expression of PAEC cultures (passage 2) compared with untreated control cells (n=4). The relative optical density of the bands was determined and related to the untreated control cells (100%). Nifedipine induced a slight but not significant increase in the mRNA and protein levels. Representative images of a Northern and Western blot are depicted (top of the columns).

An antibody against the inducible NOS (type II NOS) (Western blot) showed no results (data not shown).

Figure 3. Short-term (20 minutes) and long-term (48 hours) treatment with nifedipine (1 μmol/L) (NIF) caused a significantly reduced basal ROS formation (lucigenin chemiluminescence assay) of endothelial cell culture suspensions (n=6 to 8). * Indicates P<0.05 from control group.
Inhibition of the glucose (20 mmol/L)-induced increase in ROS (lucigenin chemiluminescence assay) in native endothelial cells of porcine coronary arteries was concentration dependently diminished by treatment (20 minutes) with nifedipine (n=6 to 8). A similar inhibition could be achieved by incubation (20 minutes) with SOD (70 U/mL) (n=8). * Indicates P<0.05 from control group.

The glucose-induced ROS release of native endothelium from porcine coronary arteries as well as from suspended endothelial cultures was significantly reduced by nifedipine (short-term treatment, 20 minutes) in a concentration-dependent manner (Figures 4 and 5) (n=6 to 8). This short-term effect could be validated with suspensions of nifedipine-treated (48 hours, no nifedipine present during measurement) endothelial cells (Figure 5). In cells that had been pretreated with nifedipine (0.1 and 1 μmol/L), the glucose-induced ROS release was significantly reduced (n=6) (Figure 5). Furthermore, ≈50% of the ROS release could be abolished by incubation with SOD (70 U/mL) (Figures 4 and 5), indicating that superoxide anions or the product of superoxide anions and NO forms a substantial part of the ROS. Denudation of the endothelium from the artery segments completely abolished (−87±10%; n=4) the ROS release, indicating that the endothelium is the source of the ROS.

**Discussion**

It is known that various DHP calcium antagonists enhance the NO release from endothelial cells. Using an NO-sensitive electrode, we previously demonstrated that nifedipine in the micromolar and submicromolar concentration ranges stimulates the NO release from the native endothelium. These findings were obtained after acute exposure to DHP calcium antagonists. Therefore, we investigated whether (1) a long-term treatment (48 hours, entire passage 2) of porcine endothelial cell cultures with nifedipine resulted in a similarly altered NO release and (2) an increased formation and/or an impaired inactivation of NO might be involved by measuring the ecNOS (NOS isoform III) mRNA and protein expression and the antioxidative potency of nifedipine, respectively.

In contrast to the short-term measurements during long-term treatment, nifedipine was not present in the buffer during the antioxidative and NO experiments.

We could demonstrate that treatment with nifedipine (concentration and time dependently) significantly increased the NO liberation of endothelial cells. The NO release could be completely blocked by N-nitro-L-arginine, an NOS inhibitor. This is consistent with findings of Ding and Vaziri, who showed by measuring nitrate and nitrite (the degradation products of NO) that the treatment of endothelial cells with the DHP calcium antagonist felodipine (0.1 to 10 μmol/L, 24 hours) increased the endothelial NO release from rat aortic endothelial cells. To investigate whether this increased NO release is due to a modulation of the expression of the ecNOS gene, we performed Northern and Western blotting. We found slightly but not significantly increased mRNA and protein levels after treatment with nifedipine. This stands in contrast to the study of Ding and Vaziri, who found a significant rise in the ecNOS protein mass after felodipine treatment. This may be due to differences in the cell culture model or to the use of a different calcium antagonist. They used rat endothelial cells in passage 7, whereas we studied porcine endothelial cells in passage 2, which may be closer to the physiological situation because the cell physiology may change during advanced passages. Moreover, others could show that the activity of the ecNOS enzyme is not altered by nifedipine, which could be confirmed with the use of another DHP calcium antagonist (pranidipine).

The maximum concentration (1 μmol/L) used in our study is higher than the plasma concentrations of nifedipine in vivo (∼0.2 μmol/L). It is not an unusual phenomenon that ex vivo higher concentrations of calcium antagonists are required to achieve pharmacological effects. Furthermore, in vivo the lipophilic nifedipine accumulates in membranous structures, resulting in markedly higher local concentrations. Nifedipine at the concentrations used in our study did not significantly alter the cell proliferation of PAECs, so there were no differences in cell numbers that could explain the different NO release (data not shown).
From our data, we cannot conclude that nifedipine directly interferes with the expression of ecNOS mRNA and protein. On the other hand, it might be possible that nifedipine alters the signal transduction responsible for NOS activation. For example, Zhang and coworkers showed that amloidipine induced an increased nitrite (degradation product of NO) production in microvessels and macrovessels of dog hearts (acute addition of the DHP). This increase could be abolished with Hoe 140, a kinin receptor blocker, indicating that a kinin-mediated mechanism plays an important role. In contrast to our results, these authors did not find an enhanced nitrite production by nifedipine. This may be explained by species differences or the different preparation, especially of the microvessels (sequential-dissection and homogenization). Moreover, the measurement of nitrite by the Griess assay may be less sensitive than measurement by the oxyhemoglobin assay. Very recently, Kitakaze et al described increased cardiac NO levels by nifedipine in an open chest ischemia-reperfusion canine model.

In addition, there are reports (acute treatment of endothelial cells with DHP) that kinases, as well as an increased Ca\(^{2+}\) influx, may play a role in the DHP-induced NO release. It is known that NO is rapidly deactivated by ROS, and because there are different findings that DHPs exert antioxidative properties, we re-investigated this aspect in both native endothelial cells from porcine coronary arteries and suspended endothelial cells by using a lucigenin assay. The previous studies usually used in vitro assays with enzymes generating superoxide anions; investigated the oxidation of LDL in vitro or ex vivo, or investigated the lipid peroxidation of sarcolemmal membrane preparations. These studies, high micromolar (≈ 10 \(\mu\)mol/L) concentrations were always needed to induce an antioxidative effect. In contrast, we used native cells or cell cultures, which are more physiological and offer the possibility that nifedipine accumulates in membranes, thus reaching higher concentrations. In addition, we stimulated the ROS release of the endothelial cells because this is a pathological situation that often occurs in persons with diabetes and atherosclerosis. We used acute hyperglycemia to induce an ROS increase. The basal and glucose-induced ROS releases of endothelial cell suspensions and native cells were significantly reduced by short-term treatment with nifedipine, and long-term treatment (48 hours) of endothelial cell cultures showed an even more pronounced effect of nifedipine. Using the lucigenin assay, we could not determine which ROS radicals are produced by nifedipine, but a large part of the ROS seems to be either superoxide anions (inhibition by the SOD) or products that result from a reaction of superoxide anions with NO, such as peroxynitrite. It seems that at least part of the increased NO release by nifedipine is due to a protection from ROS, which deactivates NO. One could argue that the ROS level may also be reduced due to a reaction with NO (increased by nifedipine), but the studies conducted with nifedipine in cell-free systems confirm the antioxidative properties of the nifedipine molecule per se.

In summary, we showed that the treatment of endothelial cells with nifedipine increased NO release that is not due to an altered expression of ecNOS mRNA and protein. Instead, it seems that there is an enhanced availability of NO by an antioxidative protection. This will not rule out that other mechanisms, such as kinin-mediated actions, play an important role, too, or act synergistically. The increased NO availability may contribute to beneficial effects of DHP calcium antagonists that were found by others, such as antiaggregatory, antiproliferative, and antiatherosclerotic effects.

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