Synergistic Antihypertensive Effects of Nifedipine on Endothelium
Concurrent Release of NO and Scavenging of Superoxide

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Abstract—Recent studies have suggested that part of the vasorelaxation caused by nifedipine, a 1,4-dihydropyridine Ca2+ antagonist, depends on the endothelium. To study the effect of endothelium-dependent vasorelaxation, the release of NO and superoxide (O2-) in the presence of nifedipine in isolated cultured rabbit endothelial cells was measured. Highly sensitive electrochemical microsensors were placed onto the cell membrane, and the kinetics of NO and O2- were measured simultaneously with time resolutions of 0.1 and 0.05 ms, respectively. Nifedipine at its therapeutical concentrations stimulated NO release and scavenged O2- in endothelial cells. The linear relationship between NO concentration and nifedipine concentration was observed in the range between 0.01 and 1 nmol/L. NO concentration reached a maximum of 200±10 nmol/L at 1.2 nmol/L of nifedipine. The NO concentration was ≈50% and 30% of the concentration measured in the presence of receptor-dependent (acetylcholine) and the receptor-independent (Ca2+ ionophore A23187) NO synthase (eNOS) agonists, respectively. NO release stimulated by eNOS agonists was followed by the generation of the NO scavenger superoxide. The concentration of O2- was significantly lower after stimulation with nifedipine (peak 5±0.5 nmol/L) than after stimulation with acetylcholine (15±1 nmol/L) and Ca2+ ionophore (25±1 nmol/L). The average rate of NO release by nifedipine is relatively slow (17 nmol/L per second). This is in sharp contrast to the fast rate of NO release by acetylcholine and Ca2+ ionophore (40 and 300 nmol/L per second, respectively). These experiments show that nifedipine, apart from its well-known Ca2+ antagonistic properties in vascular smooth muscle cells, stimulates the release of significant concentration of NO in endothelium and also preserves NO concentration. Both these effects may be beneficial in the treatment of hypertension. (Hypertension. 2001; 37:34-39.)

Key Words: nifedipine • endothelium • nitric oxide • superoxide

The 1,4-dihydropyridine known as nifedipine is a commonly used cardiovascular drug that is applied for the control of angina, hypertension, and other vascular diseases. Nifedipine can be viewed as a prototypical drug for several generations of 1,4-dihydropyridine antagonists and activators, which potently activate L-type Ca2+ channels, thus modulating the vascular tone via the Ca2+ influx into smooth muscle cells.

It has been also shown that NO is the endothelium-derived relaxing factor. NO is normally generated in the circulation via stimulation of vascular endothelium NO synthase (eNOS) by endothelial mechanochemical receptors. A variety of stimuli, including increased vascular flow, and pharmacological agents, such as acetylcholine (ACh), bradykinin, substance P, ATP, and histamine, produce vascular relaxation by the release of NO. Many vasodilator drugs are thought to act predominantly through either endothelium-dependent or -independent pathways. There is conflicting evidence as to whether 1,4-dihydropyridines may alter the synthesis or release of NO. Pretreatment with nifedipine has inhibited the maximal endothelium-dependent response to methacholine in isolated rabbit aorta. Furthermore, the release of NO from bovine aortic endothelial cells was reported to be inhibited by nifedipine. In more recent studies, nifedipine, unlike amloidipine, did not cause the release of NO from coronary arteries and aortas of normal dogs. In contrast, the dilating and flow-increasing effects of 1,4-dihydropyridines in isolated mesenteric small resistance vessels of guinea pigs were significantly antagonized by treatment with N'-nitro-L-arginine (L-NNA), a competitive NO synthase inhibitor. It has also been reported that L-NNA inhibited nifedipine-induced NO release from porcine coronary arteries with intact endothelium, and this could be partly restored by the addition of L-arginine, the substrate of NO synthase; moreover, after
mechanical denudation of endothelium from the vessels, no nifedipine-induced NO release was detected. This finding was supported by studies with the other form of 1,4-dihydropyridine, nitrendipine, which showed NO release from pig coronary arteries. The limitation of these studies is that the release of NO was suggested on the basis of the comparison of vascular smooth muscle relaxation. This approach is problematic because the 1,4-dihydropyridine Ca2+ antagonists likely have unavoidable direct effects on the vascular smooth muscle of either intact rings or the bioassay tissue. This is important because the 1,4-dihydropyridine Ca2+ antagonists may decrease the bioactivity of released NO compounds from the endothelium by reducing the activity of the enzyme responsible for NO production or by either direct or indirect inactivation of NO.

In the present study, the release of NO and its scavenger superoxide (O2−) were measured in the presence of nifedipine. Electrochemical microsensors were used for direct simultaneous measurement of O2− and NO release on the surface of a single endothelial cell. These microsensors, designed for cell cultures, allow the direct quantification of NO and O2− with high sensitivity. Superoxide is a major scavenger of NO in endothelium. O2− can be generated simultaneously with NO, and its excessive production may be a major determinant of NO availability. It was also worthwhile to evaluate a generation of O2− in the presence of nifedipine because the chemical structure of 1,4-dihydropyridine possesses several properties that are characteristic of antioxidants.

**Methods**

**Cell Culture**

Cultured endothelial cells were derived from rabbit aorta. Cells were grown in T-75 tissue culture flasks (Corning) in MEM (Cellgro) containing 10% FBS (Biocell Laboratories, Inc) and 0.004% gentamicin. The culture was incubated in 5% CO2 at 37°C and passed twice a week by enzymatic (trypsin) procedure. The confluent cells were dissociated by treatment of xanthine with xanthine oxidase was used). Solutions of 3 agonists of endothelial NO synthase (eNOS) were tested: ACh (1 μmol/L), CaI (1 μmol/L ACh), and L-nitro-arginine methyl ester (L-NAME) or 200 μmol/L Nω-nitro-arginine (L-NMA). For experiments in Ca2+-free solution, Ca2+ was omitted from HBSS solution and 2 mmol/L EGTA was added. NO and O2− releases were measured electrochemically from single endothelial cells. All chemicals were purchased from Sigma, unless otherwise noted.

**NO and Superoxide Measurement**

NO and superoxide microsensors were prepared according to the procedures published previously. The sensors operated in a 3-electrode system, consisting of the sensor working electrode, a platinum wire (0.1-mm) counter electrode, and a standard calomel reference electrode. The current proportional to NO concentration was measured by a porphyrinic sensor, which operated in amperometric mode (EG&G PAR model 283 Potentiostat/Galvanostat was used) at constant potential of 0.75 V versus the standard calomel electrode. The response time used in these measurements was 0.1 ms, and the detection limit was 10−9 mol/L. The superoxide sensor also operated in amperometric mode at constant potential of −0.23 V versus the standard calomel electrode. The response time of the superoxide sensor was 0.05 ms, and the detection limit was 10−9 mol/L. The NO and O2− concentrations were determined from the measured current by means of a calibration curve (NO standard—saturated aqueous solution or O2− stoichiometrically generated by treatment of xanthine with xanthine oxidase was used).

Working electrodes (NO or O2− sensor) were placed close to the surface (20±5 μm) of the cell membrane with the help of a computer-controlled micromanipulator. Solutions of 3 agonists of endothelial NO synthase (eNOS) were tested: ACh (1 μmol/L) was dissolved in HBSS, and Ca2+ ionophore (Cal) A23187 (1 μmol/L) and nifedipine (0.01 mmol/L to 1.6 μmol/L) were dissolved in absolute ethanol. The concentration of the eNOS agonists, Cal and ACh, used in all experiments was selected on the basis of the dose-response curve (maximal response). NO and O2− were measured as an increase of the current from its background level. Whether nifedipine has O2− scavenger properties in a xanthine-xanthine oxidase superoxide anion–generating system has been assessed. Briefly, 20 μL of a given concentration of nifedipine was mixed with 5 μL of 0.5 mmol/L xanthine in 2 mL HBSS. The reaction was initiated by adding 10 μL of 0.02 U xanthine oxidase.

**Statistical Analysis**

Statistical evaluation was performed by ANOVA followed by the Student-Newman-Keuls test. Values are expressed as mean±SEM, with a value of P<0.05 considered statistically significant.

**Results**

A typical high-resolution amperogram obtained for NO release from endothelial cells after injection of Cal is shown in Figure 1a. The peak concentration of NO is 600±30 mmol/L (n=6). The peak concentration stimulated by a receptor-independent NO agonist (Cal) represents the maximum of
NO concentration that can be released by this particular endothelial cell (rabbit aortic endothelial cell). A rapidly increasing NO concentration was observed 750 ms after injection of Cal (1 μmol/L). The rate of NO production was 300 nmol/L per second, and the maximum NO concentration was reached after 2.75 seconds. After ~2.9 seconds, a rapid decrease (152 nmol/L per second) of NO concentration was observed.

An amperogram recorded after stimulation of NO release by ACh, a receptor-dependent NO agonist, shows a peak concentration of 410±20 nmol/L (n=6, Figure 1b). This represents ~70% of maximum concentration recorded in the presence of Cal. Moreover, the kinetics of NO release stimulated by ACh is distinctively different from that observed after stimulation of NO release with Cal. An increase of NO concentration was observed 5 seconds after injection of ACh (1 μmol/L). The rate of increase of NO concentration was 40 nmol/L per second (~8 times slower than that observed for Cal). A semiplateau was established after 16 seconds, and after ~25 seconds, a slow decrease of NO concentration (rate 27 nmol/L per second) was observed.

Figure 1c shows a high-resolution amperogram recorded during nifedipine-stimulated NO release with a peak concentration of 200±10 nmol/L (n=6), which is ~70% and 50% lower than the peak concentration observed in the presence of Cal and ACh, respectively. The kinetics of NO release by nifedipine is somewhat between that observed for Cal and ACh. The NO release was observed 1 second after injection of nifedipine. An initial rate of NO release was high (25 nmol/L per second). After ~2.5 seconds, the rate decreased significantly (10 nmol/L per second), and after 8 seconds, a semiplateau was established. After ~13 seconds, a slow decay of NO concentration was observed. The decrease of NO concentration occurred initially at a rate of 5.6 nmol/L per second, which is ~30 times lower than the rate of NO decay observed after stimulation with Cal and 5 times lower than that observed after stimulation with ACh. After 30 seconds, a further decrease of the rate of NO decay was observed (1.6 nmol/L per second). Forty seconds after injection of nifedipine, a recorded NO concentration was two thirds of the maximum (peak) concentration. This is in contrast to Cal- and ACh-stimulated NO release, in which NO concentration decreased to zero level after 13 and 40 seconds, respectively.

To assess the efficiency of the NO synthase agonists, the rate of superoxide (O$_2^-$) release during production of NO was also evaluated. Amperograms of O$_2^-$ release were recorded simultaneously with amperograms of NO release after the addition of the NO synthase agonists. After injection of Cal, a peak of O$_2^-$ concentration (25±1.25 nmol/L, n=6) was produced 1 second later than the maximum of NO concentration (Figure 1a). The rate of O$_2^-$ release was 12.5 nmol/L per second. ACh-stimulated O$_2^-$ production reached a maximum of 15±1 nmol/L (n=6) after 20 seconds (Figure 1b). The rate of O$_2^-$ release was 2 nmol/L per second (~6 times lower than the rate of Cal-stimulated O$_2^-$ release). After the injection of nifedipine, only a trace concentration of O$_2^-$ was recorded (peak 5±1 nmol/L O$_2^-$ observed 10 seconds after injection of nifedipine, n=6; Figure 1c). The peak O$_2^-$ production was 5 times lower after stimulation with nifedipine than after stimulation with Cal. The peak concentration of 5 nmol/L of O$_2^-$ was the lowest O$_2^-$ concentration ever recorded in our laboratory after stimulation of NO release with eNOS agonist.

A linear increase of peak NO concentration with an increasing concentration of nifedipine from 0.01 to 1 nmol/L was observed (Figure 2a). A maximum NO concentration of 200±10 nmol/L (n=6) was recorded at 3 nmol/L nifedipine. After that, the concentration of NO decreased and reached a plateau (130 nmol/L) at nifedipine concentrations >100 nmol/L. The shape of the dose-response curve for O$_2^-$ was similar to that observed for NO (n=6, Figure 2b). The generation of O$_2^-$ from endothelial cells increased with nifedipine concentrations up to 10 nmol/L. At concentrations of nifedipine >10 nmol/L, a steady decrease of O$_2^-$ concentration was observed. Moreover, as can be clearly seen from Figure 3 (n=6), nifedipine scavenges the O$_2^-$ generated either by cells or by the xanthine/xanthine oxidase system. In the experiments with endothelial cells, there were significant differences in the ratio of NO/O$_2^-$ concentrations between the tested eNOS agonists: 40±3 nmol/L for nifedipine versus 24±1 and 27±2 nmol/L for Cal and ACh, respectively (Figure 3a). Also, nifedipine scavenged the O$_2^-$ generated by the xanthine/xanthine oxidase system (Figure 3b).

A typical decrease of NO production was observed (~65±5% and 75±5% inhibition in the presence of
L-NAME and L-NMA, respectively; \( n = 6 \) for Cal and nifedipine-stimulated NO release in the presence of NO inhibitors (Figure 4). Superoxide production after injection of either Cal or nifedipine was inhibited by 35\% to 6\% in the presence of L-NAME and by 75\% to 6\% in the presence of L-NMA. In the free Ca\(^{2+}\) buffer with a Ca\(^{2+}\)-chelating agent (2 mmol/L EGTA), the peak concentration of NO stimulated by Cal and nifedipine decreased by \( \approx 85\% \) and 45\% to 8\%, respectively \( (n = 6) \). In the absence of extracellular Ca\(^{2+}\), the inhibition of \( \mathrm{O}_2^- \) production was observed after the addition of Cal as well as nifedipine. The percentage of \( \mathrm{O}_2^- \) decrease was similar to that observed for NO.

**Discussion**

The 1,4-dihydropyridine Ca\(^{2+}\) channel antagonist nifedipine stimulated NO release and scavenged \( \mathrm{O}_2^- \) in endothelial cells. The nifedipine-dependent NO release in endothelial cells was due to eNOS activation, which is supported by the fact that the release of NO was inhibited by an analogue of L-arginine: L-NAME or L-NMA. Biochemical characterization of eNOS revealed a strong requirement for calmodulin and Ca\(^{2+}\).\(^{14}\) The Ca\(^{2+}\) ions that activate the eNOS may originate from intracellular stores or from the extracellular space. Several groups have shown that removal of extracellular Ca\(^{2+}\) can markedly inhibit the release of NO.\(^{2,14}\) This concept can be confirmed by our findings that removal of extracellular Ca\(^{2+}\) prevented Cal-stimulated or ACh-stimulated NO release from cultured rabbit endothelial cells. In the experiments with nifedipine, removal of extracellular Ca\(^{2+}\) also significantly affected the nifedipine-stimulated NO release.

Ca\(^{2+}\) influx and a resultant activation of eNOS in endothelial cells may be associated with dihydropyridine-like receptor(s), but it probably depends on an additional mechanism other than that related to L-type Ca\(^{2+}\) channels. Only a nonselective cation channel in endothelial cells was characterized in previous studies.\(^{15}\) Any other 1,4-dihydropyridine–nonspecific membrane effect resulting in the changes of the NO concentrations in endothelium, ie, direct or indirect stimulation of the eNOS by other agonists, should also be taken into account.

The capability to scavenge \( \mathrm{O}_2^- \) at the time of NO release is an important feature of the action of nifedipine in endothelial cells. Data related to kinetics of NO and \( \mathrm{O}_2^- \) release have suggested that nifedipine is very efficient in scavenging \( \mathrm{O}_2^- \) at the time superoxide is released during NO production; the NO/\( \mathrm{O}_2^- \) ratio, when calculated with peak concentrations, was almost twice higher for nifedipine versus Cal or ACh. However, the concentration of \( \mathrm{O}_2^- \) measured by the sensor depends on the kinetics of its generation and the kinetics of the scavenging process and the kinetics of the electrode reaction. These processes are concentration dependent. Therefore, it is very difficult to assess the scavenging process.
on the basis of the data obtained from experiments on endothelial cells. To limit the number of variables, the measurements of the scavenging process were performed by using the xanthine/xanthine oxidase system. The data obtained from these measurements clearly indicate that nifedipine is a scavenger of $O_2^-$. The generation of $O_2^-$ is $Ca^{2+}$ dependent, as is the production of NO by eNOS. Even though NO production can be high in endothelium, especially shortly after the injection of an NO agonist, the concomitant progressively produced $O_2^-$ rapidly reacts (rate constant $k=9.6\times10^9$ L/mol per second) with it to produce the stable product peroxynitrite (OONO$^-$. The near diffusion limited reaction of $O_2^-$ with NO to form OONO$^-$ is even faster than the reaction of $O_2^-$ with superoxide dismutase ($k=2\times10^9$ L/mol per second). When OONO$^-$ becomes protonated ($pK_a=6.8$), the formed HOONO usually undergoes isomerization (half-time <1 second) to form hydrogen cation and nitrate anion. However, as the HOONO concentration increases as maximal $O_2^-$ accumulations react with freshly synthesized NO, local HOONO concentration may become sufficient to ensure its efficient transport to reactive sites as far as several cell diameters away. In the vicinity of certain reactive centers, HOONO may undergo homolytic cleavage to a hydroxyl free radical (OH$^-$) and nitrogen dioxide free radical (NO$_2^-$) or heterolytic cleavage to a nitronium cation (NO$_2^+$) and hydroxide anion (OH$^-$). Three of these cleavage products (OH$^-$, NO$_2^-$ radicals, and NO$_2^+$) are among the most reactive and damaging species in biological systems. It has been suggested that $O_2^-$ can be produced by eNOS.$^{16}$ The process of $O_2^-$ production by eNOS can be triggered by an insufficient concentration of L-arginine in the proximity of the enzyme. Therefore, it is conceivable that the extent of $O_2^-$ production during the production of NO is a crucial factor in the assessment of the efficiency of NO synthase agonists. Confirmation that eNOS rather than other sources produced most of the $O_2^-$ in endothelial cells has come from experiments showing $O_2^-$ release after treatment with known NO agonists as well as its inhibition after incubation with known eNOS inhibitors. Of course, there are many other sources of $O_2^-$ besides disarranged $Ca^{2+}$-dependent eNOS. Normally these ($Ca^{2+}$-independent) other sources contribute to the basal concentration of $O_2^-$ and are efficiently scavenged by superoxide dismutase or basal NO.$^{18}$ Moreover, it is worthwhile to note that enhanced NO concentration may actually inhibit other enzymatic sources of $O_2^-$, such as NADPH oxidase.$^{17}$

The present in vitro studies show some very unusual and highly favorable kinetics of NO release by nifedipine. This unusual kinetics is due to the modest stimulation of NO release by nifedipine with concomitant scavenging of $O_2^-$. The rapid kinetics of NO release by most eNOS agonists is always followed by extensive release of $O_2^-$ and the NO release stimulated by CaI is a good example of such kinetics. In contrast, the rate of NO production stimulated by nifedipine was 10 times lower than the NO production stimulated by CaI. The slow kinetics of NO release is associated with the decrease of $O_2^-$ generation. Nifedipine not only stimulates NO release with favorable rate and prevents the high production of $O_2^-$ but also scavenges $O_2^-$. Therefore, the action of NO is prolonged in the presence of nifedipine, and the eNOS system operates with high efficiency. As mentioned above, the $O_2^-$ consumes NO in a rapid chemical reaction to form OONO$^-$; thus, nifedipine also reduces the level of OONO$^-$, which may initiate a cascade of cytotoxic reactions. The nifedipine concentrations that stimulate NO release and scavenge $O_2^-$ in endothelium may be considered clinically relevant, inasmuch as the nifedipine therapeutic plasma concentration in humans is 0.07 to 0.2 $\mu$mol/L.$^{18}$ In an early report, Singer and Peach$^3$ observed the decrease of smooth muscle relaxation in the presence of nifedipine in the rabbit aorta. The difference between the effect of nifedipine described in the present study and that reported previously is probably due to sequential addition of 2 eNOS agonists, nifedipine and either CaI or methacholine in the study of Singer and Peach. In the procedure involving the sequential addition of 2 eNOS agonists, the second agonist will always produce much less NO (depletion of the NO pool). At the time the study of Singer and Peach was published, the effect of NO action on endothelium was not known; therefore, the data were interpreted as just a simple inhibition of endothelium-dependent vasodilation by nifedipine.

The evidence that nifedipine stimulates NO release and is also a potent $O_2^-$ scavenger in endothelium has potential therapeutic implications in the treatment of cardiovascular endothelium-impaired function disorders. Endothelial dysfunction is associated with reduced NO production and/or enhanced inactivation of NO after its release from endothelial cells by $O_2^-$ or oxidized LDL.$^{19,20}$ Also, nifedipine can be a significant therapeutic agent that prevents the development of atherosclerosis; it has been shown that in early hypercholesterolemia, the production of NO is still unaltered but that NO is destroyed by $O_2^- \text{ before having an opportunity to leave the endothelium (a major source of } O_2^- \text{ is the endothelium itself).}^{18}$ Nifedipine can be potentially used in combination with chronic nitrate treatment because endothelium-derived $O_2^-$ is responsible, at least in part, for mediating nitrate tolerance. The elevated NO level generated by nifedipine during chronic treatment would, in addition to the improvement of smooth muscle relaxation, also inhibit smooth muscle proliferation. The lack of sufficient basal systemic NO activity in the endothelium-impaired function disorders, eg, essential hypertension, leads to smooth muscle proliferation and thickening of the arterial wall.$^{19,20}$ In addition, luminal release of NO inhibits platelet adhesion and aggregation, thus contributing to the beneficial effects of nifedipine.

In conclusion, we present evidence for a dual mode of action of nifedipine, a 1,4-dihydropyridine derivative $Ca^{2+}$ antagonist, ie, the vasorelaxing effect produced by the release of NO and the scavenging of $O_2^-$ in endothelium, apart from its well-known vasorelaxing effect produced by inhibition of the smooth muscle L-type $Ca^{2+}$ channel influx. This dual action of nifedipine is important in the preservation of eNOS and consequently in the prevention of the dysfunction of the endothelium.

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References

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