Antioxidant Effects of Photodegradation Product of Nifedipine

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Recently, increasing evidence suggests that the antihypertensive drug nifedipine acts as a protective agent for endothelial cells, and that the activity is unrelated to its calcium channel blocking. Nifedipine is unstable under light and reportedly decomposes to a stable nitrosonifedipine (NO-NIF). NO-NIF has no antihypertensive effect, and it has been recognized as a contaminant of nifedipine. The present study for the first time demonstrated that NO-NIF changed to a NO-NIF radical in a time-dependent manner when it interacted with human umbilical vein endothelial cells (HUVECs). The electron paramagnetic resonance (EPR) signal of NO-NIF radicals in HUVECs showed an asymmetric pattern suggesting that the radicals were located in the membrane. The NO-NIF radicals had radical scavenging activity for 1,1-diphenyl-2-picrylhydrazyl, whereas neither NO-NIF nor nifedipine did. In addition, the NO-NIF radical more effectively quenched lipid peroxides than NO-NIF or nifedipine. Furthermore, NO-NIF attenuated the superoxide-derived free radicals in HUVECs stimulated with LY83583, and suppressed iron-nitrilotriacetic acid (Fe-NTA)-induced cytotoxicity in rat pheochromocytoma (PC12) cells. Our findings suggest that NO-NIF is a candidate for a new class of antioxidative drugs that protect cells against oxidative stress.

Key words nifedipine; nitrosonifedipine; antioxidant; electron paramagnetic resonance; reactive oxygen species; endothelial cell

Calcium channel blockers have been used for more than 30 years to treat a variety of cardiovascular diseases including angina, arrhythmias, and hypertension. Nifedipine [1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester] is a 1,4-dihydropyridine-derivative calcium channel blocker widely used for treatment of hypertension and angina. Over the past decade, many clinical studies have suggested that nifedipine has not only hypotensive effects but also cardiovascular organ-protective effects in patients with heart and circulatory diseases.[1—4)] In addition, it has been reported that nifedipine ameliorates endothelial dysfunctions[5—9)] through an anti-apoptotic effect[10,11)] in human endothelial cells. However, it is unlikely that nifedipine exerts these effects through its calcium-blocking property because endothelial cells do not have voltage-dependent L-type calcium channels, which are thought to be the target of 1,4-dihydropyridine derivatives.[12)] The antioxidant activity may be one possible mechanism responsible for the organ-protective effects of 1,4-dihydropyridine calcium channel blockers.[13—15)] However, the antioxidant activity of nifedipine is reported to be less prominent than that of other 1,4-dihydropyridine calcium channel blockers.[13,14)]

Nifedipine is extremely sensitive to light and can be converted to its nitroso analog, nitrosonifedipine [2,6-dimethyl-4-(2-nitrosophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester] (NO-NIF), under normal room illumination.[16—22)] On the other hand, NO-NIF is also enzymatically produced from nifedipine without exposure to light.[23)] The ability of NO-NIF to block calcium channels is quite weak or non-existent compared with that of nifedipine.[24—26)] Meanwhile, Yanez *et al.* reported that NO-NIF scavenged 2,2′-azo-bis(2-amidinopropane)-derived alkylperoxyl (ABAP) radicals and that the activity was 2.3 times greater than that of trolox, a water-soluble vitamin E analogue.[27)] In addition, Mišík *et al.* postulated that NO-NIF interacts with unsaturated lipids to form NO-NIF radicals that are responsible for the antioxidant properties of NO-NIF.[28)] We therefore hypothesized that the antioxidant activity of NO-NIF is a critical element in the organ-protective effects of nifedipine. Therefore, in this study, we investigated the ability of NO-NIF to protect cells from oxidative stress using cultured human umbilical vein endothelial cells (HUVECs) and rat pheochromocytoma (PC12) cells.

Experimental

Materials Nifedipine, methanol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), palmitic acid, linoleic acid, iron(III) nitrate nonahydrate($Fe(NO_3)_3 \cdot 9H_2O$), 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (Tempol), superoxide dismutase (SOD; from bovine erythrocytes), and nitrilotriacetic sodium (NTA-Na) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Linolenic acid was obtained from Tokyo Chemical Industries (Tokyo, Japan). Arachidonic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) were from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Stearic acid was from MP Biomedicals, Inc. (OH, U.S.A.). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Labotec (Tokyo, Japan). LY83583 was obtained from Calbiochem (Darmstadt, Germany). All other reagents were of analytical grade. Water was demineralized and further purified using a Milli-Q system from Millipore (Bedford, MA, U.S.A.). The stock solution of 5 mM iron-nitrilotriacetic acid (Fe-NTA) was prepared by mixing 10 ml of 10 mM $Fe(NO_3)_3 \cdot 9H_2O$ with 10 ml of 20 mM NTA-Na immediately before use.[29)]

Preparation of NO-NIF NO-NIF was prepared according to a previous report on nifedipine.[19)] Briefly, 500 ml of nifedipine solution (10 mM) in

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methanol was placed in glass beaker and then exposed to a halogen light (500 W, Kodak Ektagraphic III Projector, Kodak, Rochester, NY, U.S.A.) with constant stirring. The UVA intensity of the source was 1.0 mW cm^{-2} when measured with a UV radiometer UVR-3036/S (Topcom, Tokyo, Japan) at the position of the sample. Every 2 h a sample was removed and subjected to HPLC with UV detection. HPLC was performed using a JASCO 880-PU pump (Tokyo, Japan) and a manual injector with a 20 μl loop. The separation was carried out on a Cosmosil $_5C_{18}$-AR-II (4.6×100 mm) column. The mobile phase consisted of a mixture of methanol (60%, v/v) and water filtered through a 0.45 μm filter (Millipore). HPLC analysis was performed at room temperature under isocratic conditions with a flow rate of 0.7 ml/min, then monitored using a UV spectrum detector (Hitachi, L-7400, Tokyo, Japan) at 292 nm. The eluent corresponding to newly observed peaks was collected, evaporated for determination of its structure by ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) in $CDCl_3$ solution, and referenced to tetramethylsilane (TMS) (0.00 ppm) using a JEOL GSX400 spectrometer (Tokyo, Japan). IR spectra were determined by a JASCO FT/IR-420 spectrometer (Tokyo, Japan), melting point by a Yanaco MP-500D apparatus (Kyoto, Japan), and a mass spectrometry by a Waters Micro Mass LCT-Premier spectrometer (Japan Waters, Tokyo, Japan). The data were as follows; mp 94.2—94.6 °C; IR (KBr) 1730, 1558, 1493 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.67 (s, 6H), 3.39 (s, 6H), 6.55 (dd, J=8.0, 1.2 Hz, 1H), 7.44 (dt, J=8.0, 1.2 Hz, 1H), 7.52 (dd, J=8.0, 1.2 Hz, 1H), 7.72 (dd, J=8.0, 1.2 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 23.4×2 (CH_3), 51.9×2 (CH_3), 107.7 (CH), 127.7 (Cq), 128.8 (CH), 130.6 (CH), 135.0 (CH), 139.9 (Cq), 144.3 (Cq), 156.4 (Cq), 161.6 (Cq), 167.6 (Cq); high resolution-mass spectrometry (HR-MS) (electrospray ionization (ESI)) m/z Calcd for $C_{17}H_{17}N_2O_5$ $[M^+ + H^+]$ 329.1137.

After 18 h irradiation under our experimental conditions, nifedipine had been completely converted to NO-NIF with a purity of more than 99%. Then, the reaction mixture was evaporated nearly to dryness and recrystallized several times from methanol.

Cell Culture HUVECs (Takara Bio, Shiga, Japan) were cultured at a density of 1×10^5 cells/ml in 60 mm dishes (Iwaki Glass Co., Ltd.) in Endothelial Basal Medium 2 (EBM-2, Takara Bio) supplemented with 10% fetal bovine serum (FBS), gentamicin sulfate (50 μg/ml), and amphotericin-B (50 μg/ml) in addition to human fibroblast growth factor B (10 ng/ml), recombinant human epidermal growth factor (20 ng/ml), recombinant human vascular endothelial growth factor (rhVEGF; 1 ng/ml), insulin-like growth factor 1 (IGF-1; 1 ng/ml), ascorbic acid (1 μg/ml), heparin (3 ng/ml), and hydrocortisone (0.4 μg/ml) at 37 °C in an incubator containing 5% CO_2. After 2 d in culture, HUVECs were washed with prewarmed (37 °C) phosphate-buffered saline (PBS) without Ca/Mg. The cells were harvested by trypsinization and washed first in cold complete medium and subsequently in PBS with Ca/Mg (PBS(+)), then suspended at cell density of 1×10^5 cells/ml in PBS(+).

Rat pheochromocytoma PC12 cells were inoculated at a density of 1×10^5 cells/ml in 24-well plastic plates (Iwaki). Each well contained 600 μl of RPMI 1640 (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% FBS, and cells were cultivated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h, then starved in RPMI 1640 without FBS for 16 h prior to experiments.

Lactate Dehydrogenase Assay The cell damage induced by oxidative stress and its attenuation by NO-NIF was evaluated by a lactate dehydrogenase (LDH) assay (Cytotoxicity Detection kit, Roche, Grenzach-Wyhlen, Germany). Briefly, the RPMI 1640 medium was aspirated from PC12 cells, and the plates were washed twice with 2—3 ml of Krebs–Ringer Hepes (KRH) buffer (NaCl 125 mM, KCl 4.7 mM, $CaCl_2$ 2.2 mM, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) 10 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, and glucose 6 mM, pH 7.4). The PC12 cells were then incubated in KRH buffer containing either 10 μM NO-NIF or 10 μM nifedipine for 30 min at 37 °C in a dark environment. The KRH buffer was then removed, and the cells were oxidized by the addition of Fe-NTA complex dissolved in KRH buffer (10 μM) and then incubated for 4 h at 37 °C. The plates were centrifuged at 400 ***g*** and 4 °C for 5 min, and 50 μl aliquots were taken to quantify the LDH. The assay was performed according to the manufacturer's instructions. The release of intracellular LDH to the extracellular medium was measured by determining the enzyme activity and expressed as a percentage of total cellular activity. The absorbance was measured at 490 nm using a plate reader. We chose Fe-NTA complex as the oxidant because Fe-NTA is a good reagent for generation of lipid peroxidation and reactive oxygen species (ROS).[29]

Free Radical Analysis by EPR Spectroscopy The free radical metabolites of NO-NIF were examined using a Bruker EPR spectrometer (EMX Plus, Bruker Bio Spin GmbH, Rheinstetten, Germany) with an X-band cavity (ER4119HS, Bruker) to collect all EPR spectra. All EPR signals were obtained at 9.5 GHz with 100 kHz modulation. Samples were transferred to glass capillary tubes (10 μl, Drummond Co., Broomall, PA, U.S.A.) and set into the EPR cavity for the measurements. The spin concentration of NO-NIF-derived radicals was determined by the double integration using Bruker WinEPR software, and radical concentrations were calculated by reference to the double integral of signals from a known concentration of the freshly-prepared stable radical Tempol (0—0.1 mM) run under identical conditions, as described previously.[30] HUVEC-derived ROS formation was studied by DMPO spin trapping using the methods of Souchard *et al.* with slight modifications.[31] Instrument conditions are described in each figure legend.

Leucomethylene Blue Assay The leucomethylene blue (LMB) assay[32,33] is based on the hemoglobin-catalyzed oxidation of colorless benzoyl leucomethylene blue to detect the existence of lipid hydroperoxide. Reduction of the lipid hydroperoxide by an antioxidant to the corresponding alcohol results in a reduction of LMB oxidation, which is an indication of anti-peroxide activity. The LMB assay was performed in 96-well microtiter plates. Sample solutions were mixed with LMB solution (5 mg *N*-benzoyl leucomethylene blue in 8 ml dimethylformamide), then 100 μl of the solution was mixed with 50 μl of *S*-13-hydroperoxyoctadecadienoic acid (13-HPODE; 240 μM in PBS containing 5% EtOH) and 100 μl of the catalytic reagent (50 mM potassium phosphate buffer pH 5.0 containing 1.4% Triton X-100 and 5.5 mg hemoglobin) in the microtiter plate. After 10 min incubation at room temperature, absorbance at 660 nm was monitored using a microtiter plate reader.

DPPH Radical Assay The antioxidant activities of NO-NIF in the presence or absence of fatty acids were assayed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical dismutation technique with modification.[34] Briefly, 100 μl of DPPH methanol solution (0.5 mM) was diluted with 300 μl of methanol, then mixed with 80 μl of buffer solution (50 mM acetate buffer, pH 5.5) and 20 μl of sample solution diluted by methanol. After 30 min incubation, the reaction mixture was transferred to the EPR quartz flat cell, and the EPR spectrum of DPPH radicals was measured. The relative concentration of DPPH radicals was obtained by double integration of each spectrum. EPR spectrometer settings are given in the figure captions.

Statistical Analysis Values are expressed as means±S.D. for 3—6 separate experiments. One-way analysis of variance was used to determine significance among groups, after which a modified *t*-test with the Bonferroni correction were used for comparison between groups. Values of $p<0.05$ were accepted as statistically significant.

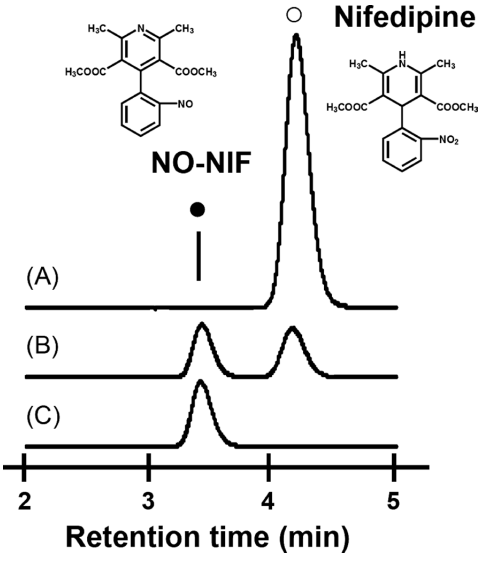

Fig. 1. Effect of Light on Nifepedine

Nifedpine (10 mM) methanol solution was exposed to a halogen light (1.0 mW cm^{-2}), and aliquots were sampled at 0 (A), 7 h (B), and 18 h (C). Open and closed circles represent nifedipine and NO-NIF, respectively. The injection volume was 20 μl, and chromatographic peaks were monitored at 292 nm. Eluent at t=3.6 was collected and then evaporated for NMR, IR, and MS.

Results

Synthesis of NO-NIF When nifedipine was exposed to light, it (retention time=4.3 min) was gradually changed to another compound (retention time=3.4 min) over time (Fig. 1). In order to determine the structure of the compound produced, we conducted ^{1}H-NMR, ^{13}C-NMR, IR, and MS measurements. According to the data, (Found 329.1148), it was concluded that the new compound was NO-NIF as reported[19] (Fig. 1).

Lipid Peroxidation In order to measure the scavenging activity of nifedipine and NO-NIF toward lipid peroxides, we conducted LMB assays. This method uses the hemoglobin-catalyzed oxidation of colorless *N*-benzoyl leucomethylene blue to detect the presence of 13-HPODE. The mixture of NO-NIF with linoleic acid was prepared by mixing 5 mM NO-NIF with 5 mM linoleic acid and then incubated for one hour at 37 °C. As shown in Fig. 2, NO-NIF with linoleic acid showed more potent activity than NO-NIF alone, and NO-NIF had higher potency, depending on anti-lipid peroxidation, than nifedipine did ($p<0.01$).

Free Radical Production by NO-NIF It was reported that NO-NIF interacts with oleic acid[23] or dioleoyl phosphatidylcholine[28] to form NO-NIF radical adducts. Therefore, we investigated whether other fatty acids that comprise the cell membrane and related fatty acids generate NO-NIF radicals or not. As shown in Figs. 3B—F, co-incubation of NO-NIF with unsaturated fatty acids (linoleic, linolenic, arachidonic, eicosapentaenoic, or docosahexaenoic acid) generated apparent three-line EPR signals. Neither NO-NIF (Fig. 3A) nor NO-NIF with saturated fatty acids (Figs. 3G, H) gave EPR signals under the same conditions.

Subsequently, we investigated the time course of NO-NIF radical generation in the presence of various concentrations of linoleic acid. As shown in Fig. 4, the NO-NIF-derived EPR signal stimulated by linoleic acid was augmented in a time-and concentration-dependent manner.

Reduction of DPPH Radical by NO-NIF DPPH is considered to be a model compound of a lipophilic radical, and it has been used to verify the radical scavenging activity of various sorts of antioxidants. The DPPH radical is scavenged by antioxidants through donation of a hydrogen to form the stable non-radical DPPHH molecule on the basis of following equation[35]: DPPH$^{\cdot}$+HX→DPPHH+$^{\cdot}$X (where HX represents an antioxidant). The more the DPPH radical concentration decreased, the more potent the antioxidant activity of the compound through its hydrogen-donating property. Neither NO-NIF nor NO-NIF with saturated fatty acids (Fig. 5F: palmitic acid and Fig. 5G: stearic acid) affected the DPPH radical intensity. However, in the presence of NO-NIF with unsaturated fatty acids (Fig. 5C: linoleic acid, Fig. 5D: linolenic acid, and Fig. 5E: arachidonic acid), the DPPH signal intensity significantly decreased. Nifedipine did not affect the DPPH radical signal intensity (data not shown).

Formation of NO-NIF Radical in HUVECs Next, we

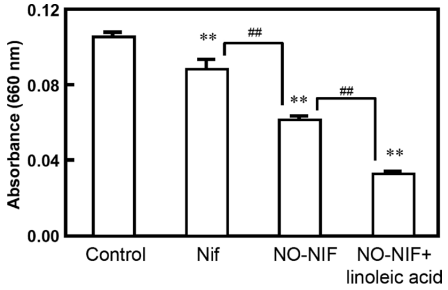

Fig. 2. Effect of Various Agents on Reduction of 13-HPODE

13-HPODE (48 μM) was mixed with 250 μM of nifedipine (Nif), NO-NIF, or a mixture of NO-NIF plus linoleic acid (1 : 1), then incubated for 10 min at room temperature. The remaining 13-HPODE was measured at 660 nm by LMB assay. Values are means±S.D. of four independent experiments. ** $p<0.001$ *vs.* control, and ## $p<0.01$ between samples.

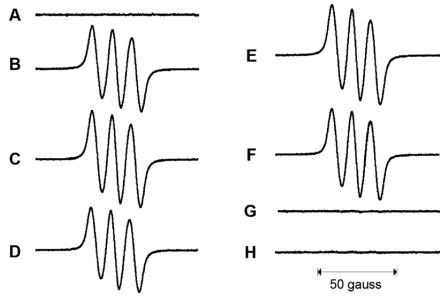

Fig. 3. EPR Spectra of NO-NIF with Fatty Acids

NO-NIF (A; final conc. 5 mM) and 5 mM (final conc.) fatty acid (B, linoleic acid; C, linolenic acid; D, arachidonic acid; E, eicosapentanoic acid; F, docosahexaenoic acid; G, palmitic acid; H, stearic acid) were mixed with methanol and incubated for 4 h at 37 °C. The EPR spectrometer instrument settings were power 20 mW, center field 3521 gauss, sweep width 100 gauss, sweep time 4 min, modulation frequency 100 kHz, modulation width 1 gauss, and time constant 0.163 s.

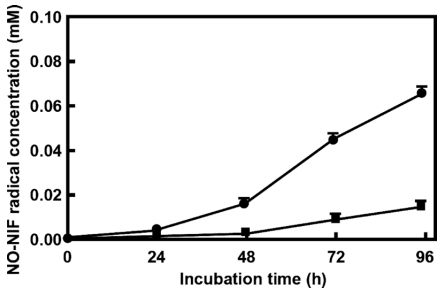

Fig. 4. Time Course of Linoleic Acid-Induced NO-NIF Radical Formation

NO-NIF (1 mM) was mixed with linoleic acid (closed square, 0.1 mM; closed circle 1 mM) in methanol and kept in airtight brown glass vials for the indicated periods at 37 °C. Each EPR signal was double-integrated to obtain arbitrary units of spin concentration, then compared with that of a known concentration of Tempol as described in Experimental. Values are means±S.D. of four independent experiments. EPR spectrometer instrument settings were power 20 mW, center field 3362 gauss, sweep width 50 gauss, sweep time 4 min, modulation frequency 100 kHz, modulation width 1 gauss, and time constant 0.1 s.

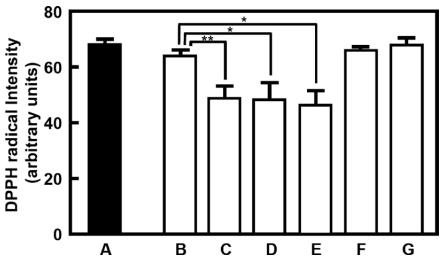

Fig. 5. Effect of NO-NIF with or without Fatty Acids on DPPH Radical Intensity

NO-NIF (0.1 mM) was mixed with 0.1 mM fatty acids for 144 h at 37 °C, then with 0.1 mM DPPH. A, 0.1 mM DPPH; B, 0.1 mM DPPH with 0.1 mM NO-NIF; C, B with 0.1 mM linoleic acid; D, B with 0.1 mM linolenic acid; E, B with 0.1 mM arachidonic acid; F, B with 0.1 mM palmitic acid; and G, B with 0.1 mM stearic acid. * $p<0.05$ and ** $p<0.01$ *vs.* DPPH with NO-NIF(B). Data are expressed as the means±S.D. of three separate experiments. EPR spectrometer instrument settings were the same as in Fig. 3.

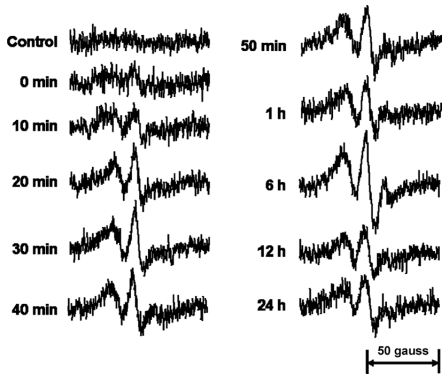

Fig. 6. EPR Spectra of NO-NIF-Derived Radicals in the Presence of HUVECs

HUVECs (1×10^5 cells/ml) were incubated in PBS(+) with 1 mM NO-NIF at 37 °C in a dark environment. Aliquots (30 μl) of cell suspension were subjected to EPR measurement at the indicated time points. EPR spectrometer instrument settings were same as in Fig. 3 except that four scans were conducted to improve the signal/noise ratio.

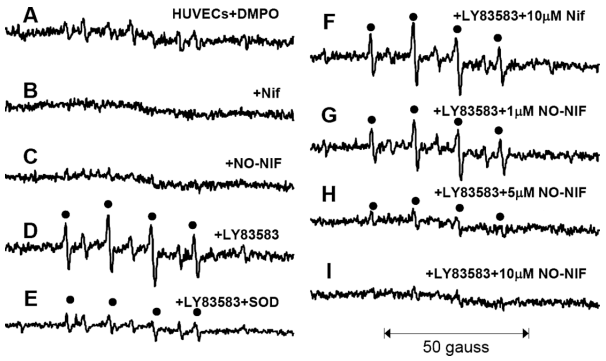

Fig. 7. Effects of Nifedipine and NO-NIF on DMPO Spin Adducts Obtained from LY83583-Stimulated HUVECs

HUVECs (1×10^5 cells/ml) were incubated in the presence of the spin-trap agent DMPO (100 mM) and stimulated with LY83583 for ROS generation. Spectrum A: HUVECs with DMPO; spectrum B: same as A but+10 μM nifedipine; spectrum C: same as A but+10 μM NO-NIF; spectrum D: same as A but+100 μM LY83583; spectrum E: same as D but+100 U/ml SOD; spectrum F: same as D but+10 μM nifedipine; spectrum G: same as D, but+1 μM NO-NIF; spectrum H: same as D but+5 μM NO-NIF; spectrum I: same as D but+10 μM NO-NIF. Closed circles represent DMPO/˙OH signals. EPR spectrometer instrument settings were the same as in Fig. 3.

investigated whether the NO-NIF radical was produced during incubation with HUVECs. One milliliter of the cell suspension was mixed with 1 mM NO-NIF, then incubated for up to 24 h at 37 °C in a dark environment for NO-NIF radical measurement. Aliquots of the suspension were transferred to 60 μl glass capillary tubes at the times indicated in Fig. 6, and each EPR spectrum was measured. When HUVECs were incubated with NO-NIF, non-symmetrical EPR signals of NO-NIF radicals were observed (Fig. 6), and the shapes of the EPR signal were identical to those from NO-NIF with liver[23] and heart.[28] The EPR signal reached the maximum at 6 h, although EPR signals were still observed till the end of observation period (24 h).

Effect of Nifedipine or NO-NIF on Production of Reactive Oxygen Species We previously reported that LY83583 generated superoxide anion radicals ($O_2^{\cdot-}$) from cultured cells using the EPR-spin trapping technique.[36] Here, we used the technique to investigate whether NO-NIF could scavenge reactive oxygen species (ROS) in cultured HUVECs. Nifedipine or NO-NIF was added to the cell suspension

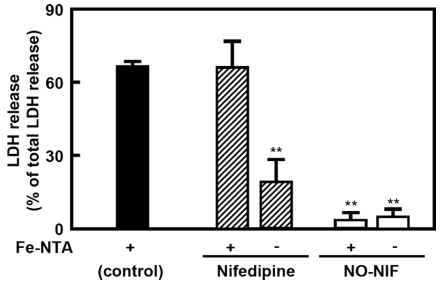

Fig. 8. Protective Effect of NO-NIF on Cultured PC12 Cells against Fe-NTA-Induced Cytotoxicity

PC12 cells were pretreated with 10 μM nifedipine or 10 μM NO-NIF for 30 min before being exposed to 10 μM Fe-NTA for 4 h or were treated with Fe-NTA without any pretreatment for 4 h as a control. Then, the release of LDH into the medium was measured as described in "Experimental." Data are expressed as the means±S.D. of three experiments. ** $p<0.001$ *vs.* control.

5 min prior to the addition of DMPO and LY83583. When HUVECs were incubated with 100 mM DMPO alone, no EPR signal was observed (Fig. 7A) with either nifedipine (10 μM) or NO-NIF (10 μM) (Figs. 7B, C). However, after 5 min incubation of HUVECs with 100 mM DMPO, followed by addition of 100 μM LY83583, the EPR signal typical of DMPO/˙OH ($a^N=a^H=14.7$ gauss) was observed (Fig. 7D) as reported previously.[36] When SOD (100 U/ml) was co-incubated with HUVECs and stimulated by LY83583, the EPR signals of DMPO/˙OH were suppressed (Fig. 7E). NO-NIF decreased the DMPO/˙OH adducts induced by LY83583 in a concentration-dependent manner (Figs. 7G—I), whereas nifedipine did not (Fig. 7F).

Effects of NO-NIF on Fe-NTA Cytotoxicity When PC12 cells were treated with 10 μM Fe-NTA, 66.2±2.1% of LDH was released from cells. NO-NIF treatment significantly attenuated the cytotoxicity induced by Fe-NTA (2.5±3.6%), whereas nifedipine did not (66.2±10.8%). The intrinsic cytotoxicity of NO-NIF (5.1±3.0%) was negligible compared with that of nifedipine (19.5±8.7%) under this experimental condition (Fig. 8).

Discussion

Nifedipine is one of the most widely used dihydropyridine (DHP)-based calcium antagonists, and it has been used for the treatment of hypertension and angina pectoris since the 1960s. In addition to its hypotensive and anti-anginal effects, pleiotropic effects of nifedipine were recently reported: anti-apoptotic and anti-inflammatory effects on endothelial cells that control the development and progression of atherosclerosis.[1,6—11,37—41] However, it is not clear how nifedipine exerts these pleiotropic effects because its antioxidative activity, which is responsible for the cardiovascular protective effect,[13,14,42] is weak in comparison to other DHPs.[43—46] Incidentally, nifedipine is light-sensitive, and is converted completely to its nitroso analog, 2,6-di-methyl-4-(2-nitrosophenyl)-3,5-pyridine-carboxylic acid dimethyl ester (NO-NIF, Fig. 1) without further photochemical degradation under ordinary light in 24 h.[23] In addition, it has been reported that NO-NIF was enzymatically generated from nifedipine.[23] To date, it has been reported that NO-NIF reduced the alkylperoxyl radicals on its nitroso aromatic group, the kinetic rate constant was ten and two times higher than that of nifedipine and Trolox,[27] respectively, and NO-NIF reacted with unsatu-

rated fatty acids to form nitroxide radicals *via* a pseudo-Diels–Alder mechanism.[18,28] These observations led us to hypothesize that NO-NIF was responsible for the additional beneficial effect of nifedipine through its antioxidant activity. However, it was unclear which species, NO-NIF or NO-NIF radicals, contributed to its antioxidative activity or how NO-NIF exerts its effects. Therefore, in the present study, we investigated the antioxidant activities of nifedipine, NO-NIF, and mixtures of NO-NIF with various unsaturated fatty acids.

As reported previously, NO-NIF gave three-line EPR signals when mixed with unsaturated fatty acids,[28] whereas it did not when mixed with a saturated fatty acid (Fig. 3). These phenomena indicated that the NO-NIF radical was responsible for the interaction of its nitroso group with unsaturated double bounds of lipids through pseudo-Diels–Alder reactions.[23,28,47—50] In addition, the signal intensities of NO-NIF radicals with unsaturated fatty acids were all the same, suggesting that the formation of NO-NIF radicals was unrelated to the number of unsaturated double bonds. In addition, the NO-NIF radical was stable[18,21] enough to measure by EPR spectrometry, and gradually increased with time as 6% of NO-NIF was converted to NO-NIF radicals over a 4-d incubation when 1 mM NO-NIF was mixed with 1 mM linoleic acid (Fig. 4). In the previous study, NO-NIF showed a potent scavenger effect towards ABAP-derived alkylperoxyl radicals by electron transfer reaction.[27] However, whether NO-NIF mixed with a fatty acid acts as a free radical scavenger was unknown. Therefore, we studied the radical scavenging activity of reaction mixtures containing NO-NIF with or without fatty acids by DPPH assay[35] and LMB assay. When NO-NIF was mixed with DPPH, little scavenging activity was observed (Fig. 5). Nifedipine did not interact with DPPH radicals as reported previously[43] (data not shown). However, mixtures containing NO-NIF and unsaturated fatty acids significantly scavenged DPPH radicals, suggesting that radical–radical interactions might be involved. In the other hand, the mixtures containing NO-NIF and unsaturated lipids have electrons to offer electrophiles.[51]

The scavenging activity against 13-HPODE increased in the order of nifedipine, NO-NIF, and NO-NIF plus linolenic acid (Fig. 2). 13-HPODE is a significant component of oxidatively-modified low-density lipoprotein (LDL) and has been shown to be present in atherosclerosis lesions.[52] Natarajan *et al.* reported that 13-HPODE not only activates the mitogen activated protein kinases (MAPKs) (extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK)), but nuclear factor-kappa B (NF-κB) DNA binding activity and Ras as well as vascular cell adhesion molecule-1 (VCAM-1) promoter activation, and these phenomena were blocked by antioxidants.[53] This led us to predict that NO-NIF plus unsaturated fatty acids would be able to suppress the inflammation-related cell signaling that promotes atherosclerosis, which NO-NIF and nifedipine do not.

Next, we investigated whether the NO-NIF interacts with living cell systems to produce NO-NIF radicals. In these experiments, we chose HUVECs because of endothelial cell dysfunction leads to the development of atherosclerosis, and it is known that nifedipine has pleiotropic effects on these cells.[37] Apparent EPR signals of NO-NIF radicals appeared after 10 min incubation, then gradually increased for 6 h, and were maintained for 24 h. Because the shape of the EPR signal of NO-NIF radicals was not symmetrical, as shown in Fig. 3, but asymmetrical, it was evident that the NO-NIF radical species was located in the membrane environment.[28,54]

Nifedipine selectively accumulates in the membrane, and it was reported that the membrane-based partition coefficient was 1000.[55] Although the retention time of NO-NIF (3.4 min) was shorter than that of nifedipine (4.3 min) in the octadecylsilyl (ODS) column system, it is conceivable that NO-NIF is still lipophilic and locates in the membrane,[19] where it is changed to NO-NIF radicals by reaction with unsaturated fatty acids in the lipid bilayer, because cell membranes contain 40 mol% unsaturated fatty acids.[56]

When nifedipine was mixed with HUVECs instead of NO-NIF, no EPR signal was observed (data not shown), although NO-NIF radical production from nifedipine in liver tissue was previously reported.[23] The discrepancy between our data and previous works suggest that hepatic enzymes contribute to the generation of NO-NIF from nifedipine.[23]

Incidentally, it was reported that nifedipine did not inhibit ROS production from cells stimulated by 7-ketocholesterol or TNF-α in endothelial cells.[43] When HUVECs were incubated with 10 μM LY83583 for 5 min, formation of DMPO/$^{\cdot}$OH adducts was observed (Fig. 7D). This EPR signal was decreased by the addition of SOD (Fig. 7E), suggesting that $O_2^{\cdot -}$ was produced from HUVECs in the culture media after LY83583 stimulation. We did not detect the DMPO/$O_2^{\cdot -}$ adduct from HUVECs because it decomposed rapidly to the DMPO/$^{\cdot}$OH adduct in the cell system.[57] In this study, NO-NIF but not nifedipine decreased the formation of DMPO/$^{\cdot}$OH adducts in a concentration-dependent manner (Figs. 7F—I), suggesting that NO-NIF and/or NO-NIF radicals may contribute to the suppression of $O_2^{\cdot -}$ generation from HUVECs stimulated by LY83583. We could not distinguish which species contributes to the suppression of $O_2^{\cdot -}$ production by HUVECs stimulated with LY83583 because NO-NIF gradually changed to NO-NIF radicals during incubation (Fig. 6), although our present data suggest that NO-NIF has the ability to reduce $O_2^{\cdot -}$-mediated oxidative stress in endothelial cells independent of calcium channel blockage activity.

Finally, we investigated whether NO-NIF protected cultured cells from the oxidative stress induced by Fe-NTA complex. We chose the Fe-NTA complex because we previously demonstrated that a solution of the Fe^{3+}-NTA complex changed to the Fe^{2+}-NTA complex during storage under light and that the Fe^{2+}-NTA complex produced ROS including $O_2^{\cdot -}$ and hydroxyl radical ($^{\cdot}$OH).[58] Likewise, Fe^{2+}-NTA induced oxidation of linoleic acid in the presence of a linoleic acid hydroperoxide (LOOH) such as 13-HPODE as follows.[59]

$$Fe^{3+}\text{-NTA} \rightarrow Fe^{2+}\text{-NTA (photo irradiation)}$$

$$Fe^{2+}\text{-NTA} + O_2 \rightarrow Fe^{3+}\text{-NTA} + O_2^{\cdot -}$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{2+}\text{-NTA} + H_2O_2 \rightarrow Fe^{3+}\text{-NTA} + OH^- + {}^{\cdot}OH$$

$$Fe^{2+}\text{-NTA} + LOOH \rightarrow LO^{\cdot} + Fe^{3+}\text{-NTA} + OH^-$$

$$LO^{\cdot} + LH \rightarrow LOH + L^{\cdot} \text{ (initiation reaction)}$$

Therefore, it was conceivable that NO-NIF diminished the cytotoxicity induced by Fe-NTA because, as we demonstrated, NO-NIF possessed scavenging activity due not only to lipid hydroperoxide (Fig. 2) but also ROS (Fig. 7).

As expected, pretreatment with NO-NIF completely prevented the Fe-NTA-induced LDH release from PC12 cells, but nifedipine did not attenuate the cell injury, suggesting that the antioxidative activity of NO-NIF is superior to that of its mother compound, nifedipine. In 1992, Mak *et al.*, reported that lipophilic DHPs exhibited antioxidant activity and that the mechanism might be mediated by membrane lipid antiperoxidation.[14,60] However, our results indicate that nifedipine seems to have less potent antioxidative activity than other DHPs.[14,61] Furthermore, recent studies demonstrated that azelnidipine, a kind of DHP, inhibited or scavenged ROS in both *in vivo* and *in vitro* systems.[43,62,63]

In conclusion, we found that NO-NIF accumulated in the cell membrane and was partially but gradually converted to stable NO-NIF radicals. These compounds may participate in inhibition of lipid peroxidation of the lipid membrane by direct interaction with lipid hydroperoxide and dismutation of lipid radicals, and may suppress ROS production. Because NO-NIF is one of the metabolites of nifedipine *in vivo*[23] and its intrinsic toxicity was less than or equal to that of nifedipine in PC12 cells (Fig. 8), we expect that NO-NIF may be a candidate for an antioxidant drug to protect the cell membrane.

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