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Spin-Trapping and Antioxidant Properties of Illuminated and Nonilluminated Nifedipine and Nimodipine in Heart Homogenate and Model System

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SUMMARY

Nifedipine [1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester] and nimodipine [1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid 2-methoxyethyl 1-methylethyl ester], incorporated into diheptanoylphosphatidylcholine liposomes, which were used as a drug carrier system, slightly inhibited lipid peroxidation (induced by *tert*-butylhydroperoxide and Fe^{2+}) in rat heart homogenate. Illumination of nimodipine had no effect on its antioxidant potency, whereas illuminated nifedipine was several times more effective than nonilluminated drug. On illumination, nifedipine converts to 2,6-dimethyl-4-(2-nitrosophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (NTP). NTP formed stable radicals when interacting with the rat heart homogenate and dioleoylphosphatidylcholine, as detected by EPR spectroscopy. No radical formation was observed if nonilluminated nifedipine and nimodipine or illuminated nimodipine were used. The spin density of the unpaired electron in the NTP-adduct was centered on the nitrogen derived from its nitroso group. The motion of the NTP-adduct radical was restricted, indicating that the radicals were located in the membrane of the homogenate and not in the buffer system. Only NTP (not nifedipine or nimodipine) was effective in trapping free radicals formed by the thermal or photoinduced decomposition of 2,2′-azobisisobutyronitrile and radicals formed by photolysis of di-*tert*-butylperoxide. The antioxidant and spin-trapping properties of NTP in our systems were attributed to its nitroso group.

The calcium-blocking agents nifedipine and nimodipine were found to afford myocardial protection during and/or after ischemia (1–3). Free radicals and lipid peroxidation have been implicated in inducing membrane damage associated with a possible role in hypoxia and reoxygenation (4, 5). The principal biological defenses against lipid peroxidation and membrane damage are antioxidants, which have the capacity to scavenge free radicals (6). Nifedipine was found to depress lipid peroxidation of methyloleate (7), phosphatidylcholine liposomes (8), heart and liver microsomes, kidney homogenate (9–11), and sarcolemmal membrane (12). The mechanism of the depression of lipid peroxidation by the drugs studied is not yet clear.

Nifedipine is unstable under daylight conditions (12). This property may have an influence on its antioxidant activity. In some experiments, the requirement of darkness may accidentally be not observed. Therefore, in the present work, we studied the antioxidant activities of illuminated and nonilluminated nifedipine and nimodipine and studied the molecular mechanism of their action.

Materials and Methods

Chemicals. Nifedipine was from Sigma, nimodipine from Drug Research Institute (Modra, ČSFR), and DOPC and DHPC from Avanti Polar Lipids. tBuOOH, AIBN, and DTBP were from Fluka (Bucks, Switzerland).

Samples. Illuminated nifedipine and nimodipine were prepared by illumination of their ethanol solution (5 mg/ml) under daylight or under 500-W lamplight for 120 min. The UV spectra of nifedipine changed upon illumination, as described (13). No changes were found in the UV spectra after illumination of nimodipine. A buffer of (in mM) 140 NaCl, 4 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 0.44 KH_2PO_4, 0.34 Na_2HPO_4, and 21 $NaHCO_3$, pH 7.4, was used.

DHPC- or DOPC-drug liposomes were prepared by mixing lipids and the given drug in ethanol, at a lipid/drug molar ratio of 3. The solvent was evaporated, followed by evacuation. Dry samples were hydrated with the buffer, at a buffer/lipid weight ratio of 17, and vortexed for 1 min. The final drug concentration in the sample was 19.3 mM.

Rat heart (1 g) was homogenized in the buffer (10 ml) with a Teflon-glass homogenizer. DHPC-drug liposomes (50 μl) were added to 300 μl of the homogenate and vortexed for 1 min. The samples were stored at

ABBREVIATIONS: nifedipine, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester; nimodipine, 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid 2-methoxyethyl 1-methylethyl ester; NTP, 2,6-dimethyl-4-(2-nitrosophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester; DHPC, diheptanoyl phosphatidylcholine; DOPC, dioleoyl phosphatidylcholine; tBuOOH, *tert*-butylhydroperoxide; AIBN, 2,2′-azobisisobutyronitrile; TBA, 2-thiobarbituric acid; DTBP, di-*tert*-butylperoxide.

−80° for 24 hr. The final drug concentration in the sample was 2.7 mM. Lipid peroxidation of the homogenate was induced by addition of 7.5 μl of $FeSO_4$ (2 mM) and 7.5 μl of tBuOOH (2 mM). The final concentration of each compound was 42 μM. Relative lipid peroxidation of the homogenate was estimated as TBA-reactive products, according to the method in Ref. 8.

Spin-trapping properties of the drugs were studied in samples of 20 mg of drug/1 ml of benzene, to which 50 μl of either DTBP or a saturated solution of AIBN in benzene were added as the initiation radical system. The samples were then illuminated by xenon lamp for 2 min (AIBN and DTBP) or heated directly in an EPR spectrometer to 57° (AIBN). The EPR spectra were measured and simulated on a Bruker 200 D spectrometer equipped with an Aspect 2000 computer.

Results

Lipid peroxidation. Nifedipine and nimodipine had a minor inhibitory effect on lipid peroxidation, measured as TBA-reactive products, in the rat heart homogenate (Fig. 1). NTP, formed by illumination of nifedipine, had several times higher potency in depressing lipid peroxidation than did nonilluminated nifedipine. However, illumination of nimodipine had no effect on its antioxidant potency.

Free radical formation. To evaluate the molecular mechanism of the antioxidant properties of NTP, we measured the EPR spectra of the drugs in the rat heart homogenate. Only the homogenate containing illuminated nifedipine (NTP) had the EPR signal of the stable radical, as shown in Fig. 2B and Fig. 3. When NTP in 50 μl of ethanol was added to 300 μl of the homogenate, the same radical as shown in Fig. 2B, yet of about 4 times lower intensity, was formed. The EPR signal in the homogenate-DHPC samples was not seen in the samples with nonilluminated nifedipine prepared in the dark or with nimodipine or illuminated nimodipine (Fig. 2C). However, when the samples of the homogenate with nonilluminated nifedipine (spectrum shown in Fig. 2A) were illuminated by daylight, in a glass EPR capillary, for 30 min, an EPR spectrum similar to that shown in Fig. 2B was obtained.

The EPR signal intensity of the NTP-adduct radical depended on the peroxidation of the homogenate, measured as TBA-reactive products (Fig. 4). The EPR signal intensity of the NTP-adduct radical in the homogenate was relative high and stable ($t_{1/2} \approx 120$ min). When the homogenate containing

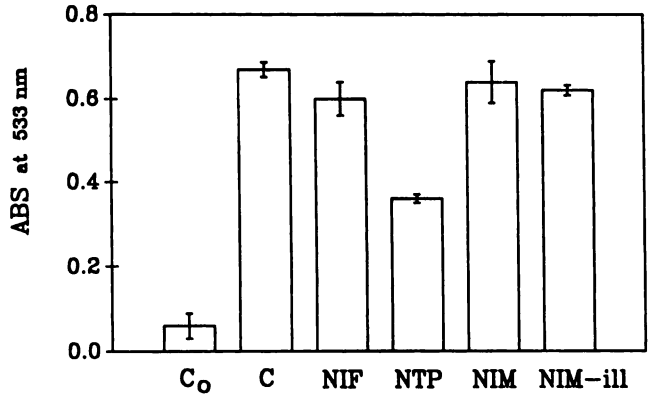

Fig. 1. Effect of the drugs (2.7 mM) on lipid peroxidation induced by $FeSO_4$ and tBuOOH in the heart homogenate at 25°, evaluated as absorbance (*ABS*) at 533 nm of TBA-reactive products. Control before (C_o) and after 18 min of peroxidation without drugs (*C*), and samples in the presence of nifedipine (*NIF*), illuminated nifedipine (*NTP*), nimodipine (*NIM*), and illuminated nimodipine (*NIM-ill*).

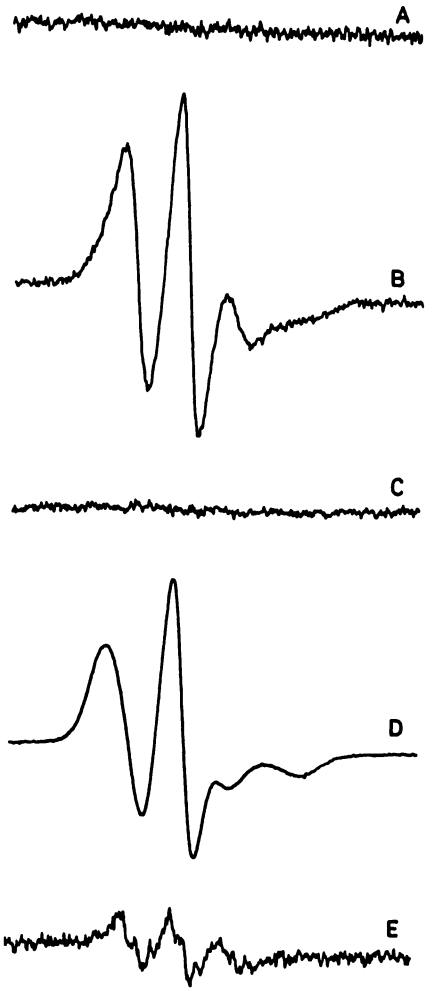

Fig. 2. EPR spectra observed in the presence of the drugs in the homogenate (2.7 mM) and lipid liposomes (19.3 mM). A, Nonilluminated nifedipine in the homogenate. B, NTP in the homogenate. C, Illuminated nimodipine in the homogenate. D, NTP in DOPC, E, NTP in DHPC. The spectrometer gain was 5×10^5 (A–C), 1.25×10^5 (D), or 1×10^6 (E). Spectrum width, 10 mT; temperature, 25°.

Fe^{2+} and tBuOOH (to induce lipid peroxidation) was incubated for 10 or 20 min at 25° and NTP was added after the incubation, the EPR signal intensity was lower (Fig. 4). There was a correlation between the decrease in the EPR signal of the NTP-adduct radicals and the increase of lipid peroxidation measured as TBA-reactive products. The relative EPR signal intensity of the NTP-adduct in the homogenate was 15.5. The intensity of the NTP-adduct in the homogenate with Fe^{2+} and tBuOOH, incubated for 10 or 20 min, was 4.4 and 1.4, respectively. The relative TBA-reactive product concentration was 12 in the homogenate and 69 and 77 in the homogenate with Fe^{2+} and tBuOOH, incubated for 10 or 20 min, respectively.

NTP in DHPC formed a radical in very low concentrations, and its splitting constants (Fig. 2E) differed from those of the NTP-adduct in the homogenate (Fig. 2B).

To evaluate the nature of NTP-adducts in the homogenate, we measured also the EPR spectra of the drugs in DOPC liposomes. Only NTP was found to form a radical in DOPC liposomes. Its EPR spectrum is shown in Figs. 2D and 5. The EPR spectrum of the NTP-DOPC radical was similar to that of the NTP-homogenate radical (Figs. 2 and 3). Because the

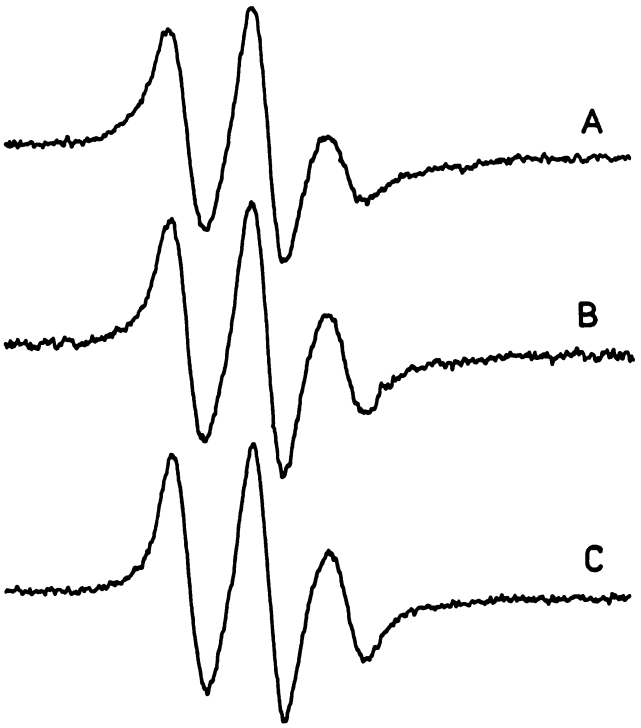

Fig. 3. EPR spectra observed in the homogenate in the presence of NTP (2.7 mM) at the temperatures 37° (A), 50° (B), and 60° (C). Spectrum width, 10 mT; gain, 2×10^5.

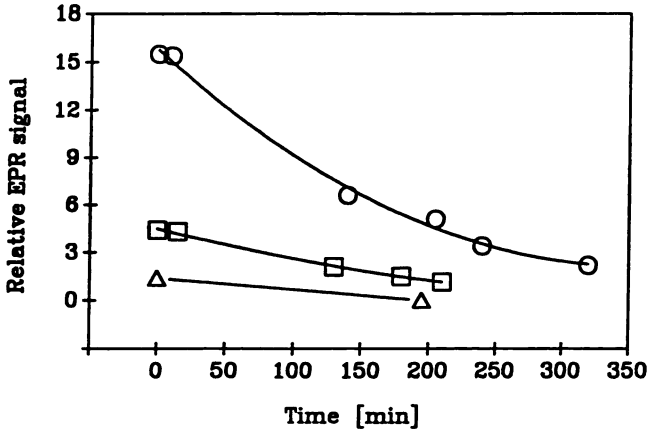

Fig. 4. Time dependence of the relative EPR signal intensity of the NTP-adduct in the homogenate without (○) (spectrum shown in Fig. 2B) and with 0.2 mM $FeSO_4$ and 0.2 mM tBuOOH, incubated for 10 min (□) and for 20 min (△). Temperature, 25°.

EPR spectra were partly asymmetric, due to low molecular motion of the radicals even at 60°, we evaluated only the splittings between the first two lines in the lower field, with a_N = 1.32 ± 0.03 mT for DOPC and a_N = 1.24 ± 0.04 mT for the homogenate. The same EPR spectra were obtained when the DOPC to NTP molar ratio was 3:1 or 50:1, but in the latter case the signal intensity was lower. This indicates that the shape of the EPR spectra was not influenced by spin-spin interactions.

Spin-trapping properties of NTP. In some model reactions, the spin-trapping properties of NTP were investigated. Free radicals were formed either by thermal or photoinduced decomposition of AIBN or by photolysis of DTBP. The observed EPR spectra, along with their simulation and the assignment of their splitting constants, are shown in Fig. 6. The splitting constants of the NTP-adduct in DTBP (Fig. 6A) were $a_N(NO)$ = 1.44 mT, $a_H(o,p)$ = 0.29 mT, $a_H(m,m)$ = 0.1 mT, and g = 2.0049. The half-lifetime of its decay ($t_{1/2}$) was about 200 sec. The EPR spectrum of the NTP-adduct obtained in the thermal decomposition of AIBN at 60° is shown in Fig. 6B. The splitting constants obtained by simulation were $a_N(NO)$ =

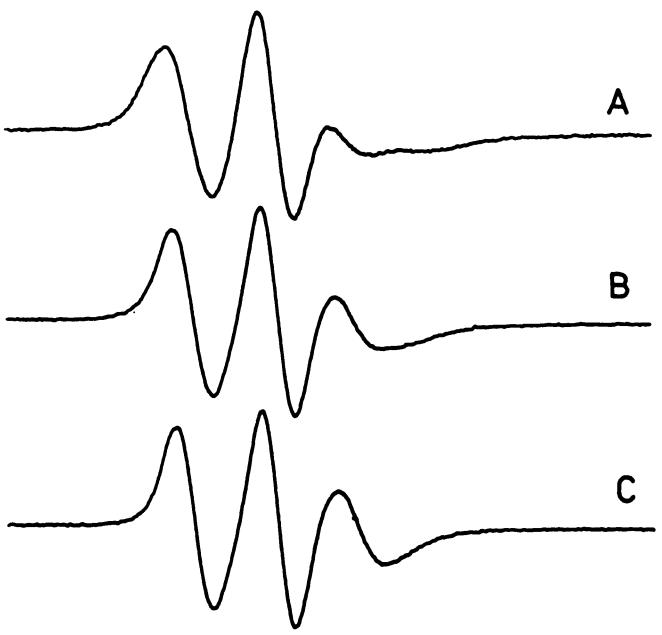

Fig. 5. EPR spectra found in DOPC in the presence of NTP (19.3 mM) at the temperatures 37° (A), 50° (B), and 60° (C). Spectrum width, 10 mT; gain, 5×10^4.

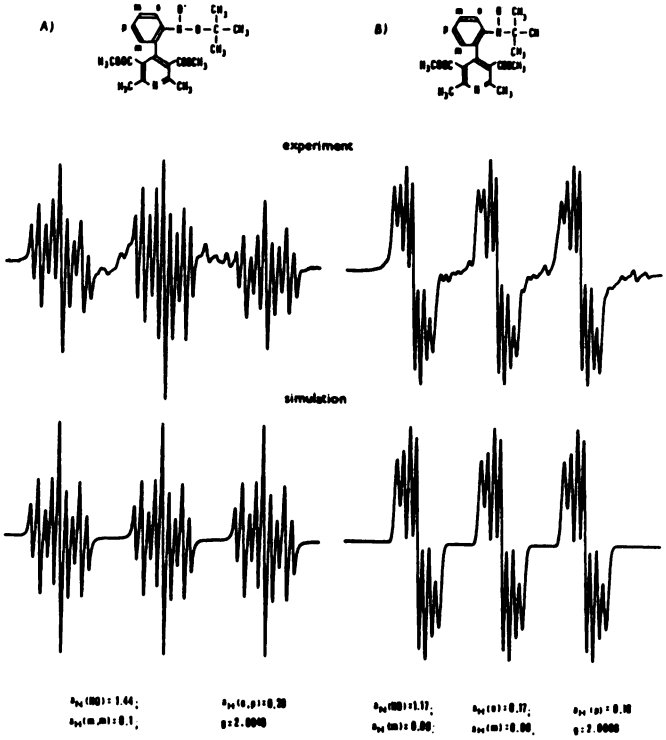

Fig. 6. Experimental and simulated EPR spectra of NTP-adducts found on photochemical decomposition of DTBP at 25° (A) and on thermal decomposition of AIBN at 60° (B). a_H and a_N are splitting constants, and g represents g values. Spectral width, 4.4 mT.

1.17 mT, $a_H(p) = 0.19$ mT, $a_H(o) = 0.17$ mT, $a_H(m) = 0.09$ mT, $a_H(m) = 0.08$ mT, and $g = 2.0060$. The radical was relatively stable, with $t_{1/2} > 60$ min.

The obtained splitting constants and their assignment, as well as the g values, are in good agreement with those found for similar structures using nitrosobenzene as the spin trap (13). The NTP spin trap itself shows a minor EPR signal, as evident from the difference between the experimental and simulated spectra in Fig. 6. The minor radical product has not be identified so far.

Discussion

Because the solubility of nifedipine and nimodipine in water is very low, we used DHPC liposomes as a delivery system for the drugs. The DHPC liposomes prepared with the drug at a 3:1 molar ratio were found to be a very effective system for delivery of the drugs into the membranes of the rat heart homogenate, as confirmed by observation of the NTP-adduct radical in the homogenate.

Antioxidant properties. The spin-trapping and antioxidant properties of illuminated nifedipine (NTP) result from the conversion of nifedipine to NTP due to radiation-induced loss of a molecule of water, as found in Ref. 12, according to the scheme in Fig. 7.

The higher antioxidant potency of NTP versus nonilluminated nifedipine, as found in the homogenate, was also reported in phosphatidylcholine liposomes (8). This indicates that the NTP effect is probably associated with NTP-lipid interactions in the homogenate. From the broad nonsymmetric spectra of the NTP-adduct radical in the homogenate, it is evident that the density of unpaired electrons was mostly localized on the nitrogen atom, and the motion of the NTP-adduct radical was restricted. This indicates that the radical was located in the membrane of the homogenate and not in the buffer system. Similar EPR spectra of illuminated nifedipine were found in phosphatidylcholine liposomes (8).

The similarity between the EPR spectra of the NTP-adduct radical in the homogenate and in DOPC (Figs. 2, 3, and 5) suggests that NTP forms radicals mainly by interaction of its nitroso group with the unsaturated bonds of lipids. Similar reactions for other compounds have already been reported in the literature (14–17). Sullivan (14) described the reaction of nitrosobenzene and its derivatives with 2,3-dimethyl-2-butene, in which the nitroso compound reacted with the alkene to form a hydroxylamine derivative that was readily oxidized to a nitroxyl radical. 2-Nitrosofluorene also reacted similarly with lipids (15) and 2-nitrosopropane with arachidonic acid (16). We suggest that NTP undergoes reaction with the unsaturated lipids (Fig. 8) in a pseudo-Diels-Alder reaction, as described in Refs. 14–17 for other nitroso compounds.

The formation of the nitroxide radical resulting from NTP-

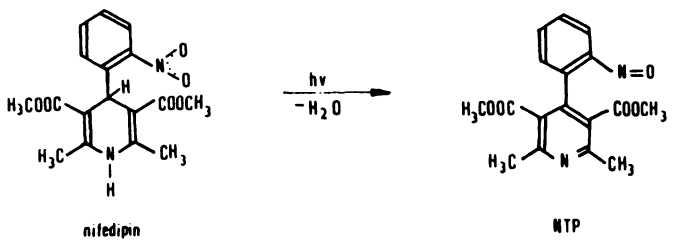

Fig. 7. Scheme for conversion of nifedipine to NTP.

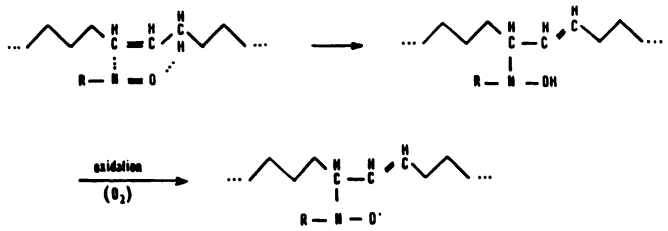

Fig. 8. Pseudo-Diels-Alder reaction of NTP with unsaturated lipids.

lipid interaction is assumed to contribute to the antioxidant properties of NTP, because nitroxide radicals were found to inhibit lipid peroxidation (18). The EPR signal of the nitroxide radicals was decreased in the samples with increased lipid peroxidation (18). A similar tendency was found in our study for the dependence of EPR signal intensity of the NTP-adduct radical on the peroxidation of the homogenate (Fig. 4).

NTP as a spin trap. The spin-trapping properties of NTP are similar to those found for other nitroso compounds, such as the spin trap 2-methyl-2-nitrosopropane (for review, see Refs. 13 and 19). The spin-trapping properties of NTP contribute to its antioxidant activities. It is noteworthy that compounds with spin-trapping properties can improve the functional recovery of the heart after ischemia and reperfusion, as found for the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (20).

The differences in antioxidant properties of illuminated and nonilluminated nifedipine have to be considered in experiments in which free radicals are involved, e.g., in hypoxia and reoxygenation of tissue. Nifedipine inhibits slow calcium channels, whereas NTP does not. NTP and nifedipine might be used to distinguish the extent to which free radical trapping and slow calcium channel inhibition contribute to the beneficial effect of nifedipine in hypoxia or reoxidation.

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