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In Vivo EPR Evidence for Free Radical Adducts of Nifedipine

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Nifedipine [3,5-pyridinedicarboxylic acid, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-dimethyl ester] is a calcium channel blocker that has been widely used as a prescription drug for patients with hypertension. After illumination by ordinary light for 24 hr, nifedipine is converted completely to its nitroso analog without further photochemical degradation. Evidence for stable, nitroxyl-like free radical generation in mice was observed 15 min after intramuscular (i.m.) or intraperitoneal (i.p.) injection of illuminated nifedipine as monitored by in vivo L-band electron paramagnetic resonance (EPR) spectrometry. This was confirmed in more detail by ex vivo measurements on excised muscle and liver tissue. The nature of these radicals was surmised by comparing the reaction of illuminated nitroso-nifedipine with polyunsaturated fatty acids. Surprisingly, identical radical spectra were detected from excised liver doped with nonilluminated nifedipine, suggesting that this drug can be enzymatically converted in vivo to its nitroso analog without the requirement for illumination. This is one of the first reports of in vivo EPR evidence for a class of unsaturated fatty acid radical conjugates resulting from the normal metabolism of a common drug. Magn Reson Med 42:691–694, 1999. © **1999 Wiley-Liss, Inc.**

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Nifedipine [3,5-pyridinedicarboxylic acid, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-dimethyl ester] is a drug belonging to the class of pharmacological agents known as calcium channel blockers. This compound is a slow-channel blocker or calcium ion antagonist, which inhibits the transmembrane influx of calcium ion into cardiac and smooth muscle (1). The contractile processes are dependent on the movement of extracellular calcium ions into these cells through specific ion channels. Nifedipine selectively inhibits calcium ion flux across the cell membrane of these tissues without altering serum calcium concentrations.

Nifedipine is extremely light-sensitive and, as we show in this work, can be converted to its nitroso analog under normal room light levels. In some patients, this photolytic event may result in severe skin inflammation and other adverse reactions during extended sun exposure (2). Stasko and coworkers (3,4) presented an interesting series of articles on reactive radical intermediates formed after high-power illumination of nifedipine. Specifically, their experiments, which involved 1 or 2 hr illumination of nifedipine solutions with a 250–500W mercury lamp, gave two general products: a hydronitroxide adduct with solvent and a pseudo Diels-Alder adduct with unsaturated lipid yielding a nitroxyl radical adduct (4). Since previous in vivo electron paramagnetic resonance (EPR) studies on living animals demonstrated that nitrosoaromatic compounds form stable, long-lived radical adducts in fatty tissue with unsaturated lipids (5), we examined whether these radical adducts might be generated biologically in an animal. We also examined the effects of relatively low levels of light intensity on the reactive properties of nifedipine with unsaturated lipids in both in vitro suspensions and whole animals.

MATERIALS AND METHODS

Materials

Nifedipine was purchased as a pure powder from ICN Biomedicals (Aurora, OH) (Lot No. 21500). Oleic acid was from Aldrich Chemical Co. (Milwaukee, WI). Animals (15–20 g male Swiss mice) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), followed by i.m. injection of 100 μL of 100 mM nifedipine (DMSO), which was illuminated under a 15W fluorescent lamp for up to 36 hr.

Absorption Spectra

Visible absorption spectra were measured on a Uvikon model 930 spectrophotometer. The conversion of nifedipine to nitroso-nifedipine was monitored by following the optical absorbance at 380 nm and 775 nm for nifedipine and nitroso-nifedipine, respectively (4,6).

In Vivo EPR

A mouse was placed in a modified bridged loop-gap resonator in an L-band EPR spectrometer described previously (7). Spectrometer conditions were: frequency, 1.2 GHz; incident microwave power, 50 mW; 100 kHz modulation amplitude, 0.5 gauss; sweep width, 100 gauss; scan time, 2 min; response time, 0.3 sec.

X-Band EPR

In vitro samples were measured in quartz aqueous flat cells, while isolated liver or muscle tissue was placed in quartz tissue cells (Wilmad Glass Co.). At approximately 15 min after i.m. nifedipine injection to an anesthetized mouse, the tissue was biopsied and measured repeatedly every 15 min. Alternatively, untreated mice were euthanized with a high dose of pentobarbital and the excised liver was soaked in nifedipine solution (100 mM in DMSO).

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Typical spectrometer conditions were: frequency, 9.5 GHz; incident microwave power, 20 mW; 100 kHz modulation amplitude, 0.5 gauss; sweep width, 100 gauss; sweep rate: 25 gauss/min; time constant: 0.128 sec.

RESULTS

Illuminated Nifedipine: In Vivo EPR

Figure 1 depicts an L-band EPR spectrum of a 15 g anesthetized mouse 15 min after i.m. injection (100 μL) of 100 mM nifedipine in DMSO, which was illuminated under a 15W fluorescent lamp for 24 hr. The animal was placed in the loop-gap resonator localized at the buttocks muscle area. A broad three-line spectrum was observed (Fig. 1a), characterized by a hyperfine splitting of 16.8 ± 0.4 gauss typical of an immobilized nitroxyl radical adduct similar to that found previously with in vitro illuminated nifedipine/heart homogenates (3) or as a result of injecting live mice with nitrosobenzene (5). The resultant EPR spectrum persisted for more than 1 hr. As a control, the mouse was injected i.m. with DMSO alone, where no signal was observed, even at twice the receiver gain (Fig. 1b). In order to obtain even higher EPR sensitivity of these free radical adducts, a buttocks muscle tissue sample was excised 15 min after injection of 100 mM (100 μL) illuminated nifedipine in vivo and subsequently measured at X-band (Fig. 2a). The EPR spectrum of the excised muscle tissue was almost identical to the spectrum observed in vivo (Fig. 1a).

Since nifedipine is known to be metabolized mainly by the liver, we studied reactions of illuminated nifedipine with excised liver tissue. Figure 2b depicts an EPR spectrum of excised liver tissue, which was presoaked in illuminated nifedipine. Although a somewhat broader spectrum than that observed in excised muscle tissue (Fig. 2a), it was nearly identical to the spectrum detected in vivo (Fig. 1a). Furthermore, a spectrum identical to that observed in Fig. 2b was observed from liver tissue excised 30 min after i.p. injection of illuminated nifedipine in vivo (although the dose was lethal after this time period).

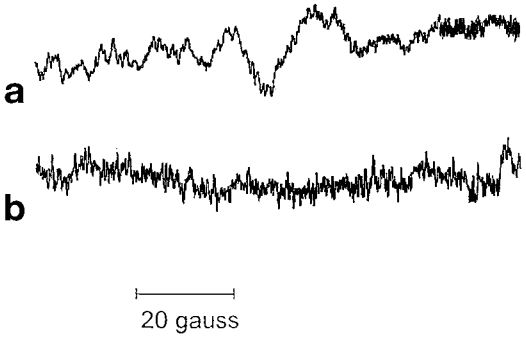

FIG. 1. L-band EPR spectra of an anesthetized (15 g) mouse at room temperature (**a**) 15 min after an i.m. injection of 100 μL of 100 mM illuminated nifedipine in DMSO. (**b**) Same as (**a**) except nifedipine was omitted and the receiver gain was twofold higher to emphasize the lack of any background EPR components. Nifedipine solutions were illuminated by placing samples in a 5 mL (8 mm i.d.) glass test tube situated just above a 15W common household fluorescent tube for 24 hr. All other spectrometer conditions were as noted in Materials and Methods.

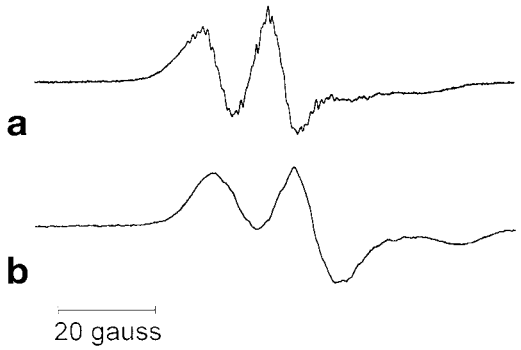

FIG. 2. X-band ex vivo EPR spectra of excised tissue after administration of illuminated nifedipine. (**a**) Buttocks muscle tissue was excised 15 min after i.m. injection of 100 μL illuminated nifedipine (100 mM in DMSO) to a 15 g mouse. (**b**) The liver tissue excised from a control mouse was soaked in 100 mM illuminated nifedipine (in DMSO) for 5 min at room temperature, then measured immediately after wiping excess liquid from the specimen. Spectrometer conditions were as noted in Materials and Methods.

Radical Adducts of Unsaturated Fatty Acids

Several studies have shown that model reactions between nitroso compounds and polyunsaturated fatty acids (PUFA) proceed by a pseudo Diels-Alder reaction mechanism, yielding nitroxide-type free radical adducts (8,9). Figure 3 depicts X-band EPR spectra resulting from mixing illuminated (DMSO or ethanol) solutions of nifedipine with oleic acid. The spectra were very similar to those found previously for nitrosobenzene/oleic acid mixtures, suggesting that the three-line spectrum in Fig. 3 also represents a radical adduct of a nitroso compound across the double bond (5). Figure 4 depicts the time-dependence of the visible absorption spectrum of ''nitroso-nifedipine'' during illumination. The results confirm that nifedipine can be converted almost completely to its nitroso analog within 24 hr by exposure to light intensities similar to that of common daylight. Under these conditions, nifedipine is converted to its nitroso analog, although very little is converted further to the hydronitroxide radical (data not shown), indicating that weak intensity light converts nifedipine to its nitroso analog without further photodegradation products. These results confirm the in vivo generation of free radical adducts from illuminated ''nitroso-nifedipine'' analogs. The spectra of these radical adducts were

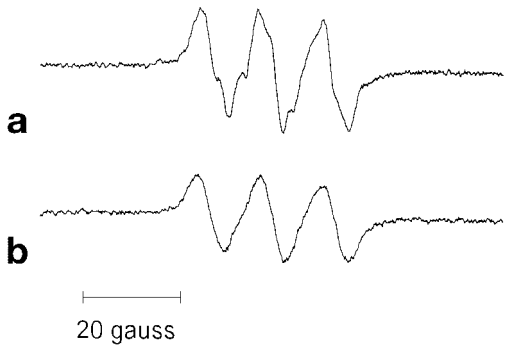

FIG. 3. X-band EPR spectra of the radical adduct of nifedipine and oleic acid. Nifedipine solutions, which were illuminated as described in Figure 1, were mixed at a 1:1 molar ratio of oleic acid in (**a**) DMSO or (**b**) ethanol solvent. Spectrometer conditions were identical to those in Fig. 2.

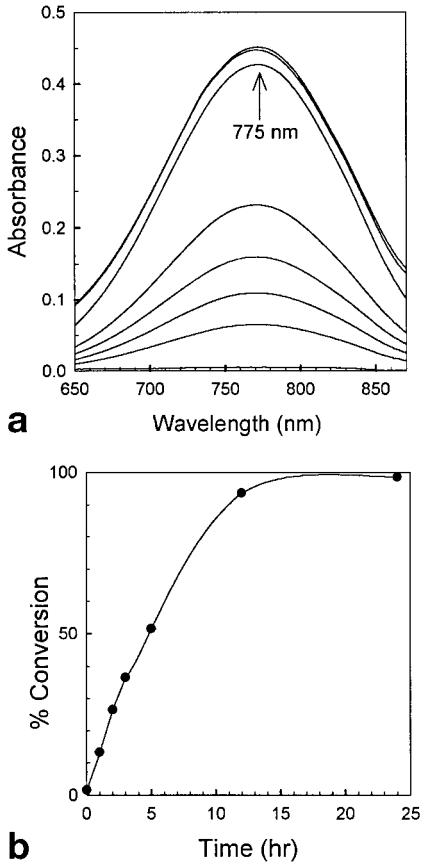

FIG. 4. Conversion of nifedipine to its nitroso analog during illumination with a 15W fluorescent lamp. (**a**) Visible absorption spectra at 0, 1, 2, 3, 5, 12, 24, and 36 hr, respectively. (**b**) Replot of the data in (**a**) as percent conversion (measured from absorbance at 775 nm). Nifedipine (100 mM in DMSO) was illuminated by a fluorescent lamp as described in Fig. 1.

identical to those obtained from mixtures of pure unsaturated fatty acids and nitroso-nifedipine (3).

Illumination Is Not Necessary In Vivo — Reductive Reactions Catalyzed by the Liver

Lastly, we examined whether nonilluminated nifedipine could be converted to the nitroso analog enzymatically in vivo. A freshly prepared nifedipine solution (without direct illumination) was soaked with newly excised liver tissue or liver homogenate. The resultant EPR spectrum generated from nonilluminated nifedipine (Fig. 5b) was superimposable with that from illuminated nifedipine (Fig. 5a), suggesting that nifedipine was converted to its nitroso analog by hepatic enzyme(s). The steady-state concentration of the radical from either illuminated or nonilluminated nifedipine was estimated from the EPR spectra as 43 $\pm$ 5 or 4.5 $\pm$ 0.7 nmol/g of liver, respectively. We also have EPR evidence for the same radical spectrum generated from nisoldipine (H. Fujii and L.J. Berliner, unpublished data).

DISCUSSION

Previous work has shown that nitroso compounds can be converted in vivo to their hydroxylamine derivatives by reductants such as NAD(P)H (11). Subsequently, the hydroxylamine can be reoxidized by oxygen, resulting in the generation of superoxide radicals which persist until all of the oxygen is consumed (11).

Sammartano and Malejka-Giganti (9) presented evidence that nitroxyl radical adducts resulting from the reaction between C-nitroso aromatics and polyunsaturated fatty acids provide a viable route for lipid peroxidation. Both the nitroxyl radical and its hydroxylamine (which can be formed by abstraction of a hydrogen from other unsaturated fatty acids) are potentially capable of reducing trace Fe[III] to Fe[II], with possible initiation of lipid peroxidation (10).

Fatty Acid Free Radical Conjugates

It is rather remarkable that this widely used drug goes through metabolic breakdown processes leading to a new class of unsaturated fatty acid free radical conjugates with spectra long-lived enough to be observed by in vivo EPR spectroscopy. It is well documented that this drug is metabolized principally in the liver. Yet, to the best of our knowledge, although much was known about its metabolic fate, derivatives such as those reported here by in vivo EPR and earlier by Stasko and coworkers (3,4), were neither detected nor suspected in any pharmacological or toxicological studies.

CONCLUSIONS

Overall, the results from this work may be summarized in three important observations:

1) Nifedipine is converted in vitro to its reactive nitroso analog at low levels of light.
2) The nitroso analog reacts with polyunsaturated fatty acids to form a relatively stable, long-lived nitroxyl-like radical adduct(s) that is especially dominant in

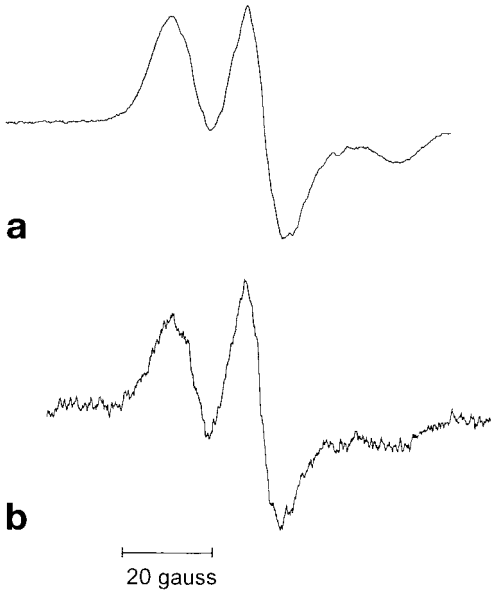

FIG. 5. X-band EPR spectra of liver tissue exposed to (**a**) illuminated and (**b**) nonilluminated nifedipine. Illuminated nifedipine was prepared as described in Fig. 1. Nonilluminated nifedipine solutions were freshly prepared and soaked with liver tissue for 5 min at room temperature in the dark.

the liver after i.p. or i.v. injection or if taken orally. The characteristic EPR spectrum of this adduct is quite similar to that observed earlier for nitrosoaromatic adducts with unsaturated fatty acids.

3) The conversion to nitroso-nifedipine may occur catalytically in the liver (without illumination), leading to the production of unsaturated fatty acid free radical conjugates.

Future work planned involves a more detailed chemical characterization of these fatty acid conjugates as well as both in vivo and in vitro investigations of any secondary chemical reactions that may have occurred as a result of this radical adduct formation.

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