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Antiperoxidant effects of dihydropyridine calcium antagonists

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While sharing the ability to impede calcium (Ca^{2+}) entry into the heart-muscle cell (myocyte) via the voltage-dependent slow Ca^{2+} channel [1], Ca^{2+} antagonists may also possess "cytoprotective" properties independent of Ca^{2+}-channel blockade [2, 3]. Other than myocyte Ca^{2+} overload [4], superoxide (O_2^-)- and iron-dependent membrane phospholipid peroxidation, with xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) (XOD) as a potential O_2^- source, may promote myocardial ischemic and reperfusion injury [5, 6]. A previous investigation from this laboratory [7] demonstrated that some Ca^{2+} antagonists reduce the susceptibility of cardiac-membrane phospholipid to free radical-induced peroxidation. In that study, the dihydropyridine class, represented by nifedipine and nitrendipine [8], was the only one of the four major chemical classes of Ca^{2+} antagonists which did not demonstrate at least some antiperoxidant efficacy. Since the prototype nifedipine is among the most potent Ca^{2+} antagonists known [1, 9], and a number of structurally-related dihydropyridines hold promise for the clinical management of cardiovascular [2] and cerebral [10] disorders, we have investigated the antiperoxidant potential of five other dihydropyridine Ca^{2+} antagonists: niludipine, nimodipine, nisoldipine, nicardipine, and felodipine. Our findings show that dihydropyridine Ca^{2+} antagonists have a wide range of antioxidant potencies and may indeed protect myocardial-membrane phospholipid from peroxidative injury. One dihydropyridine, nisoldipine, was found to inhibit an enzymatic O_2^- source, XOD.

Materials and methods

Materials. All reagents and buffers were from commercial sources at the highest available grade, as specified [7, 11]. Ca^{2+} antagonists were obtained from their manufacturers: felodipine (H 154/82; A.B. Hässle, Mölndal, Sweden); nicardipine (YC-93; Yamanouchi Pharmaceutical Co. Ltd, Tokyo, Japan); and niludipine (Bay 7168), nimodipine (Bay 9736), and nisoldipine (Bay K5552) (Bayer AG, Wuppertal, F.R.G.). Concentrated stock solutions of Ca^{2+} antagonists were made in ethanol such that the final solvent concentration in the peroxidation reaction system did not affect lipid peroxidation. XOD (analytical preparation from bovine milk; 1 unit/mg) and superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1; analytical preparation from bovine erythrocytes; 5000 units/mg) (SOD) were from Boehringer-Mannheim (Indianapolis, IN). Water was purified with a Milli-Q system (Millipore, Bedford, MA). All experiments were carried out under amber lighting.

Peroxidation reaction system. Liposomes were prepared from purified rat myocardial-membrane phospholipid, as detailed [11]. The phospholiposomes were subjected to O_2^-- and iron-dependent peroxidation at 37° in a reaction system containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–0.145 M KCl, pH 7.4; 0.1 mM Fe^{3+}–1.0 mM ADP complex; 1.0 mM hypoxanthine; 10 mUnits XOD/ml; and 125 μg cardiac phospholipid/ml. These conditions are optimal for initial linear rates of O_2^- production and O_2^--dependent peroxidation [11, 12]. After 60 min, peroxidation was terminated by acidifying each milliliter of peroxidation reaction with 0.15 ml of ice-cold 76% (w/v) trichloroacetic acid in 2.3 N HCl.

Quantification of lipid peroxidation. Lipid peroxide formation was measured as thiobarbituric acid (TBA)-reactive material [11]. Under these conditions, TBA-reactivity largely reflects malondialdehyde (MDA) produced from cardiac phospholipid peroxidation [11]. The Ca^{2+} antagonists studied did not influence the TBA-reactivity of MDA standard and were not themselves TBA-reactive. Concentration–response curves were generated with the assistance of RS/1 software (BBN Corp., Cambridge, MA).

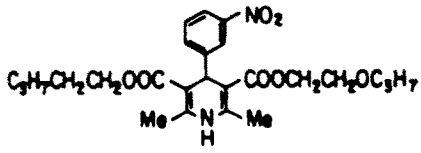
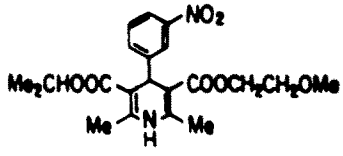
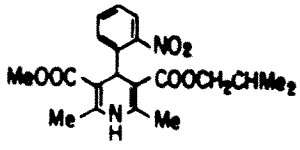
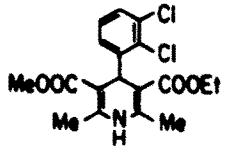
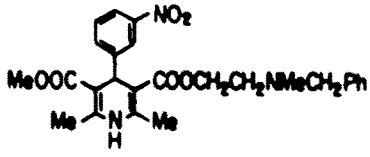
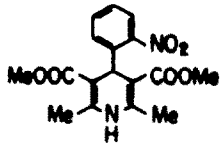
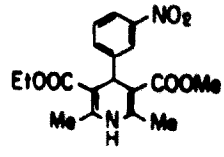
Assessment of O_2^- trapping. The O_2^--scavenging potential of test substances (i.e. Ca^{2+} antagonists) was assayed as prevention of the SOD-inhibitable reduction of ferricytochrome *c* at 22° [13]. The assay contained: 0.25 mM potassium-phosphate buffer, pH 8.6; 10^{-4} M EDTA; 2 mM NaOH; air-saturated dimethyl sulfoxide (DMSO) containing 0.55 M water, and 76 μM ferricytochrome *c*. The linear rate of SOD-inhibitable cytochrome *c* reduction was monitored at 550 nm, and attentuation of this rate was considered evidence of the O_2^--trapping ability of a substance. The extinction coefficient 21 mM^{-1} cm^{-1} was used to calculate the amount of O_2^- produced.

XOD activity. XOD was assayed spectrophotometrically by monitoring the conversion of xanthine substrate to uric acid at 25° [14]. The standard assay mixture contained: 50 mM potassium-phosphate buffer, pH 7.8; 10 μM EDTA; 4.2×10^{-9} M catalytically flavin-active XOD; and 0.5 mM xanthine. Some incubations included a Ca^{2+} antagonist at concentrations specified in the text.

Results

Antiperoxidant effect of dihydropyridine Ca^{2+} antagonists. Five dihydropyridine Ca^{2+} antagonists were tested for their ability to inhibit O_2^-- and iron-dependent myocardial-membrane phospholipid peroxidation (Table 1). Niludipine could not protect the phospholipid from oxidative injury, even at a 500 μM final concentration. Nimodipine, at 500 μM, inhibited peroxidation by 15%; solubility limits prevented its being tested at higher concentrations. In contrast, nisoldipine, felodipine, and nicardipine effectively protected myocardial-membrane phospholipid from oxidative injury at low micromolar concentrations. The concentration–response curves of the Ca^{2+} antagonist-antiperoxidants allowed determination of their respective antiperoxidant IC_{50} values (i.e. the concentration of Ca^{2+} antagonist at which peroxidation was inhibited by 50%). Nisoldipine, the most potent dihydropyridine Ca^{2+} antagonist-antiperoxidant identified with an antiperoxidant IC_{50} of 80 μM, was able to prevent completely myocardial phospholipid peroxidation at a concentration of ~200 μM. Felodipine and nicardipine, with respective IC_{50} values of 110 and 150 μM, were somewhat less potent than nisoldipine and inhibited peroxidation by ~75% at 500 μM; higher concentrations were not attainable without the need

Table 1. Inhibition of myocardial-membrane phospholipid peroxidation by dihydropyridine Ca^{2+} antagonists

Ca^{2+} antagonist	Structure	Concentration (μM) inhibiting peroxidation by: 50%	100%
Niludipine		>500	>500
Nimodipine		>500	>500
Nisoldipine		80 ± 6*	225 ± 16
Felodipine		110 ± 8	>500
Nicardipine		150 ± 10	>500
Nifedipine		>500	>500
Nitrendipine		>500	>500

Myocardial-membrane phospholipid was exposed to peroxidative injury stimulus (XOD + hypoxanthine + Fe^{3+} · ADP) for 60 min either in the absence (control) or presence of various concentrations of each listed Ca^{2+} antagonist. Inhibition of peroxidation (evaluated as TBA-reactivity) was then assessed relative to 0% inhibition of the control reaction. Values were obtained from four independent experiments and for nisoldipine, felodipine and nicardipine are given as means ± SD. For comparative purposes, data on nifedipine and nitrendipine are included from a previous publication [7].

for an organic vehicle at levels which adversely affected the peroxidation reaction. As detailed previously [7], neither 500 μM nifedipine nor 500 μM nitrendipine affected peroxidation. Nisoldipine, felodipine, or nicardipine could not scavenge O_2^- at concentrations which inhibited O_2^--dependent, iron-promoted lipid peroxidation by at least 75% (Table 2).

Inhibition of XOD by dihydropyridine Ca^{2+} *antagonist-antiperoxidants.* The antiperoxidant data presented prompted investigation as to whether nisoldipine, felodipine, or nicardipine could act as a XOD inhibitor. To this intent, XOD activity was assayed directly with xanthine as substrate in the absence or presence of various concentrations of each dihydropyridine Ca^{2+} antagonist-antiperoxidant. Under the XOD assay conditions employed, allopurinol, a competitive (with respect to xanthine) XOD inhibitor which is converted by the enzyme to the actual, tight-binding inhibitor, oxypurinol [15], inhibits XOD by 50% at a concentration of 8.2 μM [7]. Neither felodipine nor nicardipine affected XOD activity at a concentration (500 μM) that inhibited XOD-dependent cardiac phospholipid peroxidation by some 75%. However, XOD was inhibited by nisoldipine in a concentration-dependent manner (Fig. 1). A steady-state enzymological analysis was carried out by varying systematically the concentrations of xanthine and nisoldipine. The results, expressed as a Lineweaver–Burk [16] plot (Fig. 1A), demonstrate that nisoldipine acted as a noncompetitive inhibitor with respect to reducing substrate, for the lines at different nisoldipine concentrations converged very near the x-axis. The apparent K_m of ~2 μM in the absence of nisoldipine approximates literature K_m values with xanthine substrate [17]. A plot of the y-axis Lineweaver–Burk intercept versus nisoldipine concentration (Fig. 1B) defined an apparent K_i of 210 μM.

To characterize further the mode of XOD inhibition by nisoldipine, additional studies were carried out. In the first, XOD which had been incubated with 210 μM nisoldipine for 60 min at 25° prior to its enzyme activity being assayed was found to display 50% of its activity, whereas XOD preincubated with 8.2 μM allopurinol was inactive. This difference indicates that nisoldipine acted as a simple, reversible XOD inhibitor in marked contrast to allopurinol which, when converted by the enzyme to oxypurinol, dissociates from the enzyme very slowly [15]. Also in contrast to allopurinol, an alternative XOD substrate [18], nisoldipine was not oxidized by the enzyme, for mixtures of nisoldipine and XOD did not reduce electron acceptors such as ferricytochrome *c* (data not shown). Nisoldipine itself could not directly oxidize either xanthine or hypoxanthine (data not shown).

Table 2. Dihydropyridine Ca^{2+} antagonists as O_2^- scavengers

Ca^{2+} antagonist	Concentration (μM)	O_2^- production (nmol/min)
None		4.2 ± 0.2
Niludipine	500	4.1 ± 0.2
Nimodipine	500	4.4 ± 0.3
Nisoldipine	250	4.2 ± 0.3
Felodipine	500	3.9 ± 0.3
Nicardipine	500	4.2 ± 0.3

O_2^-, generated as described [13], was detected as ferricytochrome *c* reduction in the absence or presence of each listed Ca^{2+} antagonist at the final concentrations tabulated. The mean rates of O_2^- production were calculated from the extinction coefficient 21/mM/cm and are given as means ± SD (N = 4).

Discussion

The results presented, along with a previous investigation [7], demonstrate that members of the dihydropyridine class of Ca^{2+} antagonists differ widely in their abilities to inhibit myocardial phospholipid peroxidation. Nifedipine, nitrendipine, and niludipine were devoid of antiperoxidant properties, at least to a 500 μM concentration. Nimodipine was

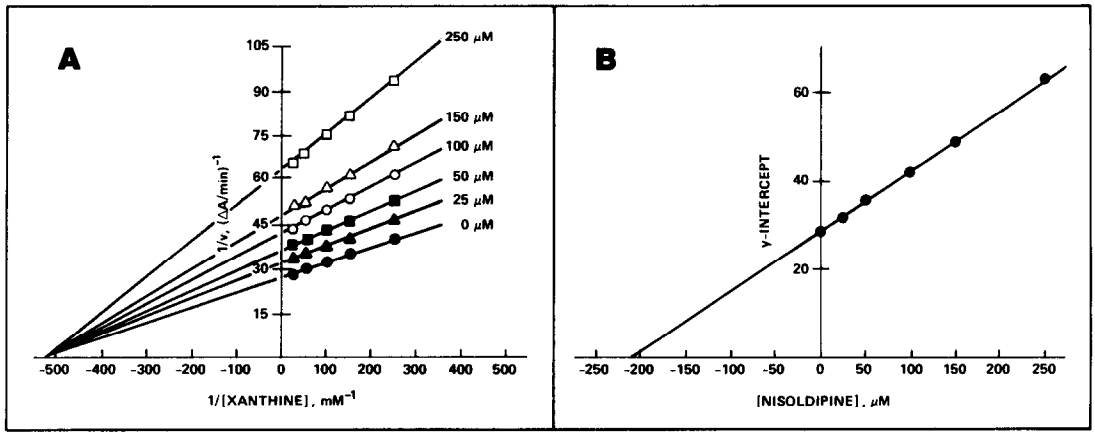


Fig. 1. Steady-state analysis of XOD inhibition by nisoldipine. Panel A: XOD (4.2×10^{-9} M catalytically flavin-active) was assayed spectrophotometrically [14], and the substrate (xanthine) concentration was systematically varied at each of the six fixed nisoldipine concentrations indicated (0 to 250 μM). The results, given in a Lineweaver–Burk plot, were used in a secondary plot (panel B) of y-intercept versus inhibitor concentration to determine the K_i value for the inhibition of XOD by nisoldipine. This K_i value, obtained as the x-intercept of the line in Panel B, was 210 μM nisoldipine. The plots shown are representative of three independent experiments.

a weak antiperoxidant. However, nisoldipine, felodipine, and nicardipine reduced the susceptibility of myocardial-membrane phospholipid to free radical attack in a concentration-dependent manner, and 225 μM nisoldipine completely prevented the peroxidative lipid injury.

Although the antiperoxidant IC_{50} values of the three most potent dihydropyridine Ca^{2+} antagonist-antiperoxidants were significantly greater than their IC_{50} values for the receptor-mediated depression of the slow action potential of heart-muscle cells [1, 19], these compounds can accumulate intracellularly to reach concentrations several-thousand- to several-hundred-thousand-fold higher than their extracellular, aqueous concentrations [20–22]. The marked tendency for dihydropyridine Ca^{2+} antagonists to partition into myocardial membranes would localize these compounds strategically within the prime targets of cellular oxidative injury and accumulate them there to high micromolar–low millimolar intramembranous concentrations in the face of circulating therapeutic levels (in humans) of around 0.2 μM [21, 23]. With the limited data available at present on dihydropyridine partition coefficients, an absolute correlation cannot be made between antiperoxidant potency and lipophilicity. However, since both nisoldipine and nimodipine have similar membrane partition coefficients [21], but very different antiperoxidant efficacies, lipophilicity *per se* may be only one contributor to dihydropyridine antiperoxidant action. It is not known, for example, whether the various dihydropyridine Ca^{2+} antagonists have different intramembranous orientations [22, 24] which might influence their relative antiperoxidant activities.

The precise structural requirements for the selectivity of antiperoxidant activity among the dihydropyridines remain to be established. Nevertheless, it is noteworthy that the three most potent dihydropyridine antiperoxidants (nisoldipine, felodipine, and nicardipine) have a methyl-ester at C-3 of the dihydropyridine ring and a hydrophobic, long-chain ester at C-5 (Table 1). Dihydropyridines with long-chain esters at both C-3 and C-5 (niludipine and nimodipine) or methyl-esters at C-3 and C-5 (nifedipine) were ineffective as antiperoxidants. Structural comparison between nitrendipine and felodipine suggests that substitutions on the phenyl ring may also act as determinants of antiperoxidant potency.

No dihydropyridine Ca^{2+} antagonist-antiperoxidant could scavenge O_2^-. A comparison of the antiperoxidant activities of the dihydropyridines with the antiperoxidant profiles of known iron-chelators and antioxidants in our peroxidative membrane-injury model, as described [7, 11, 12], would indicate that the antiperoxidant efficacy of some dihydropyridine Ca^{2+} antagonists rests with their ability to block lipid free radical formation and thereby prevent the propagation of lipid peroxidation [6, 11]. A direct inhibitory effect of nisoldipine on XOD, the enzymatic O_2^- generator, may influence its antiperoxidative potency, although it is unlikely that XOD inhibition by nisoldipine fully accounts for the protective effects of nisoldipine against XOD-dependent lipid injury: the K_i for XOD inhibition by nisoldipine was 210 μM, and at virtually the same concentration lipid peroxidation was prevented completely. This reasoning is supported by the finding that 225 μM nisoldipine prevented myocardial lipid peroxidation under conditions whereby O_2^- was generated non-enzymatically (data not shown). Since nimodipine can be readily washed out of cardiac membranes [25], it is possible that nisoldipine, having a similar membrane partition coefficient [21, 22], is likewise located near the lipid bilayer/cytosol interface and could thereby interact with cytosolic constituents such as XOD.

In summary, of five dihydropyridine Ca^{2+} antagonists evaluated as antiperoxidants, nisoldipine, felodipine, and nicardipine inhibited O_2^--, iron-dependent injury to myocardial membrane phospholipid in a concentration-dependent manner, but did not directly scavenge O_2^-. Nisoldipine acted as a simple, reversible, non-competitive XOD inhibitor, but XOD inhibition was not a requirement for dihydropyridine antiperoxidant activity and appeared not to be critical even in the case of nisoldipine. The high membrane partition coefficients of these agents may help localize them within the membrane target of oxidative damage. Comparison with other antiperoxidative agents whose mechanism of action is known suggests that the dihydropyridine Ca^{2+} antagonist-antiperoxidants prevent the formation of lipid-radical intermediates critical to the propagation of peroxidation.

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