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Antioxidant Properties of Dihydropyridines in Isolated Rat Hearts

COMPARISON OF NISOLDIPINE, NISOLDIPINE ENANTIOMERS, AND NIFEDIPINE

William F. Kauder and John A. Watts†‡*

*Department of Biology, University of North Carolina at Charlotte, Charlotte, NC 28223; and †Emergency Medical Research, Carolinas Medical Center, Charlotte, NC 28232, U.S.A.

ABSTRACT. Isolated Sprague-Dawley rat hearts were perfused under constant flow conditions. Hearts were treated with vehicle or treatment buffers, including nisoldipine, nifedipine, or the optical isomers (+)- or (−)-nisoldipine. H_2O_2 (500–600 μM) was then added to the treatment buffer for 12 min. H_2O_2 was removed and perfusion continued with treatment buffers (10 min) followed by control buffer (20 min). Contractile function decreased following perfusion with H_2O_2. Contractile function was protected in a concentration-dependent manner (nisoldipine = 19, 26, 50, 63, and 78%; nifedipine = 23, 37, 55, 61, and 63% of pre-peroxide function, 0, 0.1, 0.5, 1.0, and 5 nM, respectively). There were no significant differences between equal concentrations of nisoldipine and nifedipine. Contractile function was equally protected by both (+)- and (−)-nisoldipine compared with vehicle-treated hearts (56, 67, and 16% of pre-peroxide function, respectively). Biochemical analyses indicated that H_2O_2 damaged plasma membranes (increased lactate dehydrogenase leak) and caused lipid peroxidation (elevated tissue thiobarbituric acid reactive substances). Biochemical changes were equally reduced by nisoldipine and nifedipine treatments and by (+)- and (−)-nisoldipine. The treatment groups have widely differing IC_{50} values as calcium channel antagonists, yet they had equal effects in reducing oxidative injury, suggesting that this beneficial effect is not mediated by calcium antagonism. BIOCHEM PHARMACOL 51;6:811–819, 1996.

KEY WORDS. nisoldipine; calcium antagonist; dihydropyridine; peroxide; free radicals; antioxidant

During the first few minutes of reperfusion following ischemia, there is an elevation of free radical production including primary superoxide and hydroxyl radicals [1–3] and carbon-centered or alkoxyl radicals [4]. Phospholipids in plasma membrane and mitochondrial membranes are susceptible to peroxidation by these radicals [5]. Accumulation of excess intracellular calcium activates phospholipases and proteolytic enzymes [6], which also damage the components of membranes. Increased cytosolic calcium also contributes to free radical production [7], and it is unclear if free radicals and calcium contribute independent damage to hearts, act synergistically, or if one effect precedes and triggers the other effect. Loss of membrane integrity has long been known to be a major factor associated with the pathology of ischemia/reperfusion injury [8].

Calcium antagonists are beneficial when given prior to experimentally induced ischemia, but not when given only during reperfusion [9–11]. Protective effects observed with nisoldipine do not require preischemic cardiodepression [9, 12, 13], indicating that blockade of myocardial calcium channels is not required for this effect. Calcium antagonists also reduce oxidative injury in isolated membrane vesicles [14] and endothelial cells [15]. Nisoldipine is particularly effective in reducing the injury to endothelial cell function following peroxidative injury, while nifedipine, verapamil, and diltiazem do not provide this protection [16, 17]. Thus, there may either be fundamental differences in the ability of these drugs to reduce oxidative injury, or the experimental conditions may mask beneficial effects of the other calcium antagonists upon endothelial cell injury. Other studies focusing on myocardial function indicate that nisoldipine [9, 18–21] and nifedipine [18] have protective abilities against free radical mediated damage.

Dihydropyridines have a high partition coefficient [22] and therefore accumulate in the lipid bilayer at concentrations that are well above the plasma concentration of the drug. Dihydropyridine calcium channel blockers reduce the damage that occurs to myocardial membrane liposomes exposed to free radicals [14]; however, several aspects of the protective capabilities seen by dihydropyridines remain to be determined. First, it is not clear if the antioxidant effects of these compounds occur in a physiologically relevant range of concentrations. Second, it is uncertain if the benefits obtained by dihydropyridines during oxidative injury are a result of calcium channel blockade or non-calcium channel mediated mechanisms. In addition, it has not been determined if the benefits observed in models of ischemia and reperfusion accrue from calcium channel blockade or reduction of oxidative stress.

‡ Corresponding author: John A. Watts, Ph.D, Emergency Medicine Research, Carolinas Medical Center, P.O. Box 32861, Charlotte, NC 28232-2861. Tel: (704) 355-2678; FAX (704) 355-2845.

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The present experiments used isolated rat hearts that were perfused with H_2O_2 to induce oxidative injury. The effects of clinically relevant concentrations of nisoldipine and nifedipine upon the oxidative injury were first compared to determine if both dihydropyridine compounds were effective in reducing oxidative injury. Two optical isomers of nisoldipine were also tested for their ability to protect rat hearts from H_2O_2-induced damage to assess the role of calcium channel blockade in oxidative injury. The IC_{50} value for the (−) isomer is 100 times higher than the IC_{50} value of the (+) isoform, which acts on calcium channels in concentrations similar to those of the racemic mixture [23]. Therefore, the degree of Ca^{2+} blockade should be different in hearts treated with equal concentrations of the (+) and the (−) isomer; however, the interaction of these enantiomers with the lipids should be similar.

MATERIALS AND METHODS

Heart Perfusion

Male Sprague-Dawley rats (250–400 g) were heparinized (2.5 mg/0.25 mL saline = 1500 U/kg, i.p. injection) and anesthetized with sodium pentobarbital (50 mg/kg, i.p. injection). Hearts were excised and placed in ice-cold saline. The hearts were then attached to the Langendorff perfusion apparatus and perfused with non-recirculating buffer. An incision was made in the pulmonary artery, and the left ventricle was perforated to allow drainage of Thebesian venous flow. A fluid-filled (0.9% NaCl) balloon was inserted into the left ventricle through the mitral valve. The balloon volume was large enough to accommodate 200 μL of fluid without a change in pressure. This balloon was connected to a pressure transducer and monitored by a Grass polygraph, so that pressures within the left ventricle and heart rate could be recorded.

The perfusion buffer was a modified KHB* buffer consisting of NaCl (118 mM), KCl (4.7 mM), KH_2PO_4 (1.2 mM), $MgSO_4 \cdot 7H_2O$ (1.2 mM), $CaCl_2 \cdot 2H_2O$ (1.25 mM), dextrose (15 mM), and $NaHCO_3$ (25 mM). It was oxygenated with 95% O_2:5% CO_2 (pH 7.40). The temperature of the perfusate was held at 37°, and all buffers were heated in a column for at least 5 min before being used. Nisoldipine (Bayer USA, West Haven, CT) and nifedipine (Sigma, St. Louis, MO) were dissolved in PEG (Sigma) to form 1.0 mM stock solutions. These solutions were then diluted into a solution that was 50% PEG and 50% saline (0.9% NaCl) to yield 0.1 mM solutions, and further dilutions were made with 0.9% NaCl. An aliquot was added to the perfusate to achieve the final concentration indicated for each experiment. Control experiments were conducted with perfusate containing the highest concentration of PEG to determine the effect of the vehicle upon function of the heart. Experiments were carried out under sodium lights to avoid photodegradation of nisoldipine or nifedipine.

Oxidative injury was induced by perfusing hearts with H_2O_2 (Sigma). H_2O_2 was highly labile and decomposed in control buffer over time. To ensure consistent treatments in all experiments, H_2O_2 was added to 250 mL of modified KHB buffer 7.5 min prior to initiation of perfusion through the heart. The buffer containing H_2O_2 was placed in the perfusion system 2.5 min later, allowing 5 min of warming before perfusing the heart with this solution. The hearts were then perfused with H_2O_2 for 12 min (see below). Thus, the H_2O_2 was present in the KHB buffer for a total of 19.5 min.

Experimental Protocol

Unpaced hearts (N = 7–12 in each group) were allowed to stabilize for a 15-min period during which time coronary perfusion pressure was held constant at 50 mm Hg, and balloon volume was adjusted to the point where diastolic pressure remained slightly above zero and systolic pressure was no lower than 65 mm Hg. After the stabilization period, neither coronary flow nor balloon volume was adjusted so changes in the indices of function reflect the effects of drug and H_2O_2 treatments. At this time, the hearts were perfused with buffer containing either nifedipine, nisoldipine, or one of the isomers of nisoldipine for 25 min. After 25 min of drug treatment, the hearts were perfused with H_2O_2 for 12 min. Alternatively, hearts were perfused with control buffer (time-matched controls). Upon the completion of H_2O_2 treatment, hearts were perfused with drug treatment buffer for 10 min. Finally, all hearts were switched back to control buffer for a 20-min wash. At that time, hearts were frozen with liquid nitrogen cooled tongs and stored at −80° for biochemical analysis. Indices of function were measured at each transition time in the experiment. Also, measurements were taken every 5 min during the 30-min perfusion period following the H_2O_2 treatment.

The initial experiment evaluated the effects of the time of pretreatment with nisoldipine upon the damage to contractile function caused by H_2O_2 (600 μM, 12 min). Hearts were pretreated with no nisoldipine, or with 5 nM nisoldipine for 5 or 25 min before the addition of H_2O_2.

Concentration-response curves for both nisoldipine and nifedipine were then constructed, using 25 min of pretreatment time, to compare the ability of each treatment to reduce the functional damage that occurred when hearts were exposed to H_2O_2 (600 μM, 12 min). Hearts, (N = 7–12 in each group) were perfused using the protocol described above. Concentrations of 0.0, 0.1, 0.5, 1.0, and 5.0 nM were utilized to determine concentration-response curves for each drug. Additional hearts (N = 5–7 in each group) were perfused with either 5 nM nisoldipine or 5 nM nifedipine for performance of the biochemical analysis.

Hearts were then perfused with 5 nM solutions of either (+)- or (−)-nisoldipine to assess the role of calcium channels in the reduction of oxidative injury (500 μM H_2O_2, 12 min). At this concentration, the (−) isomer has demonstrated no effect upon calcium channels in K^+-depolarized rings from coronary

* *Abbreviations:* HR, heart rate; KHB, Krebs-Henseleit bicarbonate; LDH, lactate dehydrogenase; LVEDP, left ventricular end diastolic pressure; LVGP, left ventricular generated pressure; LVPSP, left ventricular peak systolic pressure; MDA, malondialdehyde; PEG, polyethylene glycol; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; and TCA, trichloroacetic acid.

vessels or isolated ventricular myocytes, and does not show significant binding to calcium channels in rat brain[23]. The (+) isomer, on the other hand, shows significant inhibition in the same systems (IC_{50} = 100 fold lower than the (−) isomer [23]).

A group of hearts was perfused with neither H_2O_2 nor drug to determine effects of the vehicle (5.5×10^{-3}% by vol. PEG) and time of perfusion on the indices of function. These groups were labeled in the figures as time controls. A second group of hearts was perfused with PEG (5.5×10^{-3}% by vol.) as above, but H_2O_2 was used to determine if the vehicle exerts antioxidant effects. These groups were labeled in the figures as peroxide control.

Indices of Heart Function

The intraventricular balloon was attached to a pressure transducer (Statham P23) that allowed measurements of pressure changes within the ventricle. HR, LVPSP, and LVEDP were measured directly by output from this transducer. LVGP was calculated as the difference between LVPSP and LVEDP. The product of HR and LVGP was then calculated as an overall index of ventricular function.

Biochemical Assays

Coronary effluent was analyzed for the presence of LDH to assess the damage to the integrity of plasma membranes. LDH was measured by monitoring the conversion of pyruvate to lactate in the presence of NADH by the method of Bergmeyer and Bernt [24]. Activity was expressed as milliunits released per gram wet weight per minute (1 mU = 1 nmol/min of assay).

Levels of TBARS were determined by the method of Buege and Aust [25] as an index of the extent of lipid peroxidation. Briefly, frozen hearts were homogenized in a KCl (0.16 M):Tris (0.2 M) buffer (pH 7.4). One milliliter of homogenate was added to 1.5 mL of an extraction/reaction mixture containing TCA (0.8 M), HCl (0.85 N), and TBA (0.023 M). The reaction mixture was then boiled to extract MDA and to increase the reaction with TBA. After being cooled to room temperature, 1.0 mL of 70% TCA was added to the reaction solution and this mixture was incubated for 20 min. Samples were then centrifuged at 17,590 g_{ave} for 15 min. Finally, the optical density (532 nm) of the supernatant was determined and compared to standards of MDA ranging from 0 to 5 nmol. Standard curves of MDA were prepared daily.

H_2O_2 was assayed in normal, heated buffer solution and compared to buffer solutions containing 5 nM nisoldipine or nifedipine. Buffer samples were collected every 4 min over a 20-min period. This was the span of time between the initial preparation of H_2O_2 buffer and the cessation of peroxide treatment in heart perfusions. The assay used 200 μL of perfusion buffer, 11.5 μL of horseradish peroxidase (Sigma, 10 mg/mL, 250 U/mg), and 800 μL of a solution containing aminoantipyrene (3.0 mM) and phenol (220 mM) [26]. Optical density was measured spectrophotometrically (510 nm) after a 2-min incubation period in the reaction vessel. H_2O_2 concentrations were determined using the molecular extinction coefficient of 6.58 mM^{-1} cm^{-1}.

Statistical Analysis

The initial values of contractile function were compared in all treatment groups by one-way ANOVA. Changes in contractile function over time (see Figs. 1, 2 and 4) were analyzed by repeated measures ANOVA or by Friedman repeated measures ANOVA when the data did not meet tests of normality (Kolmorogov-Smirnov test) or homogeneity of variance (Levene Median test). Comparisons of individual time points within a line were made by the Student-Newman-Keuls method. The final recoveries of contractile function in the different treatment groups (42-min values) were compared by one-way ANOVA followed by the Student-Newman-Keuls method. Final values of contractile function were also compared in nisoldipine and nifedipine treatments using Student's *t*-test (see Fig. 2C) when either raw or transformed data passed normality and equality of variance tests. Values of HR × LVGP (mm Hg/min) obtained in the comparison of the 0.5 nM and the 1.0 nM groups failed the equal variance test; therefore, the non-parametric Mann-Whitney rank sum test was utilized. A value of $P < 0.05$ was used for statistical significance in all comparisons.

Results from the biochemical assays (peak of LDH release and level of TBARS) were compared by one-way ANOVA, followed by Student-Newman-Keuls tests using raw data or transformed data. In the (+)- and (−)-nisoldipine comparison, LDH values failed normality tests; therefore, a non-parametric comparison of all groups to control (Dunn's method) was utilized to determine significance. A value of $P < 0.05$ was used for statistical significance in all comparisons.

RESULTS

Baseline Values of Contractile Function

The baseline value of HR, determined after the 15-min stabilization period of perfusion in all hearts in the study, was 277.3 ± 3.1 bpm, while LVGP was 74.9 ± 1.1 mm Hg (N = 133). The overall baseline contractile function (HR × LVGP) for all of the hearts was 20,439 ± 280 mm Hg/min (N = 133). There were no significant differences in the baseline contractile function among any of the treatment groups (P = 0.427, 18 treatment groups).

Effect of Pretreatment Time upon Contractile Function

Hearts that were perfused with H_2O_2 in the presence of vehicle showed a significant reduction in contractile function following 12 min of perfusion with H_2O_2 (baseline = 19,558 ± 1,119; 12 min H_2O_2 = 5,261 ± 1,175 mm Hg/min) (Fig. 1). Reduced contractile function persisted throughout the rest of the perfusion with control buffer (final value = 4,395 ± 1,377 mm Hg/min). When hearts were pretreated for 5 min with 5 nM nisoldipine, the recovery of contractile function was improved significantly (final value = 10,386 ± 1,416 mm Hg/

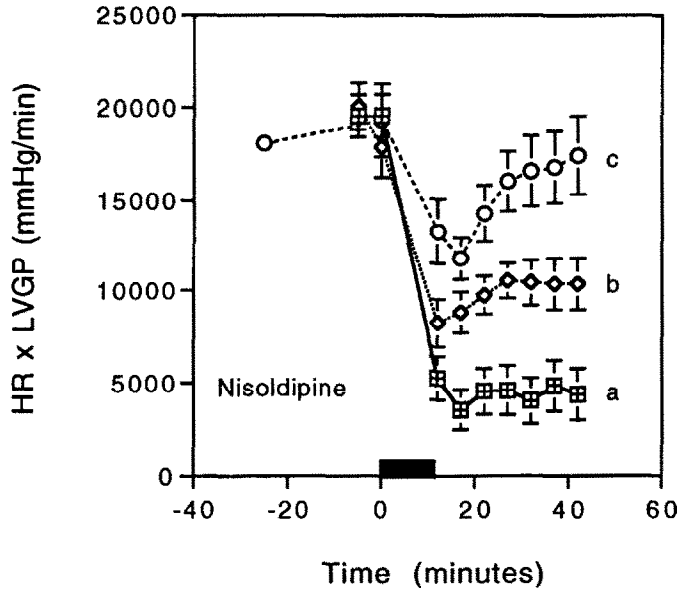

FIG. 1. Effects of H_2O_2 upon contractile function (HR × LVGP, mmHg/min) in hearts that were pretreated with vehicle (⊞), 5 min of 5 nM nisoldipine (◇), or 25 min of 5 nM nisoldipine (○). H_2O_2 (600 μM) was added between time 0 and 12 min (indicated by the solid bar on the time axis). Values are means ± SEM, N = 9. Values with different letters were significantly different ($P < 0.05$) at the end of perfusion.

min). Pretreatment for 25 min with 5 nM nisoldipine resulted in recovery of contractile function (final value = 17,436 ± 2,097 mm Hg/min) that was significantly greater than 5 min of pretreatment (Fig. 1). Subsequent experiments were then performed with 25 min of pretreatment with the drugs being examined, and the control hearts received an equivalent period of perfusion with vehicle.

Comparison of Effects of Nisoldipine and Nifedipine

Values of HR × LVGP dropped from 21,450 ± 1,332 (baseline) to 4,063 ± 1,714 at the end of the protocol in hearts that received no nisoldipine (Fig. 2A), and from 20,370 ± 827 to 4,764 ± 2,637 mm Hg/min in hearts that received no nifedipine (Fig. 2B). Treatment of hearts with nisoldipine for 25 min resulted in no reduction of function prior to initiation of H_2O_2 perfusion (Fig. 2A). Individual components of this index, HR or LVGP, were also not changed significantly by the addition of nisoldipine before the initiation of H_2O_2 perfusion (data not shown). While hearts that were treated with 0, 0.1, and 0.5 nM nifedipine showed no significant decrease in contractile function prior to the addition of H_2O_2, hearts treated with 1.0 and 5.0 nM nifedipine showed a small (22 and 16%, respectively), but statistically significant, decrease in function before the addition of H_2O_2 (Fig. 2B).

Treatment with nisoldipine produced a concentration-dependent increase in recovery of contractile function following H_2O_2 treatment (Fig. 2A). Treatment with 1.0 and 5.0 nM significantly improved recovery of function to 14,287 ± 2,447 and 15,821 ± 1,488 mm Hg/min, respectively, compared with

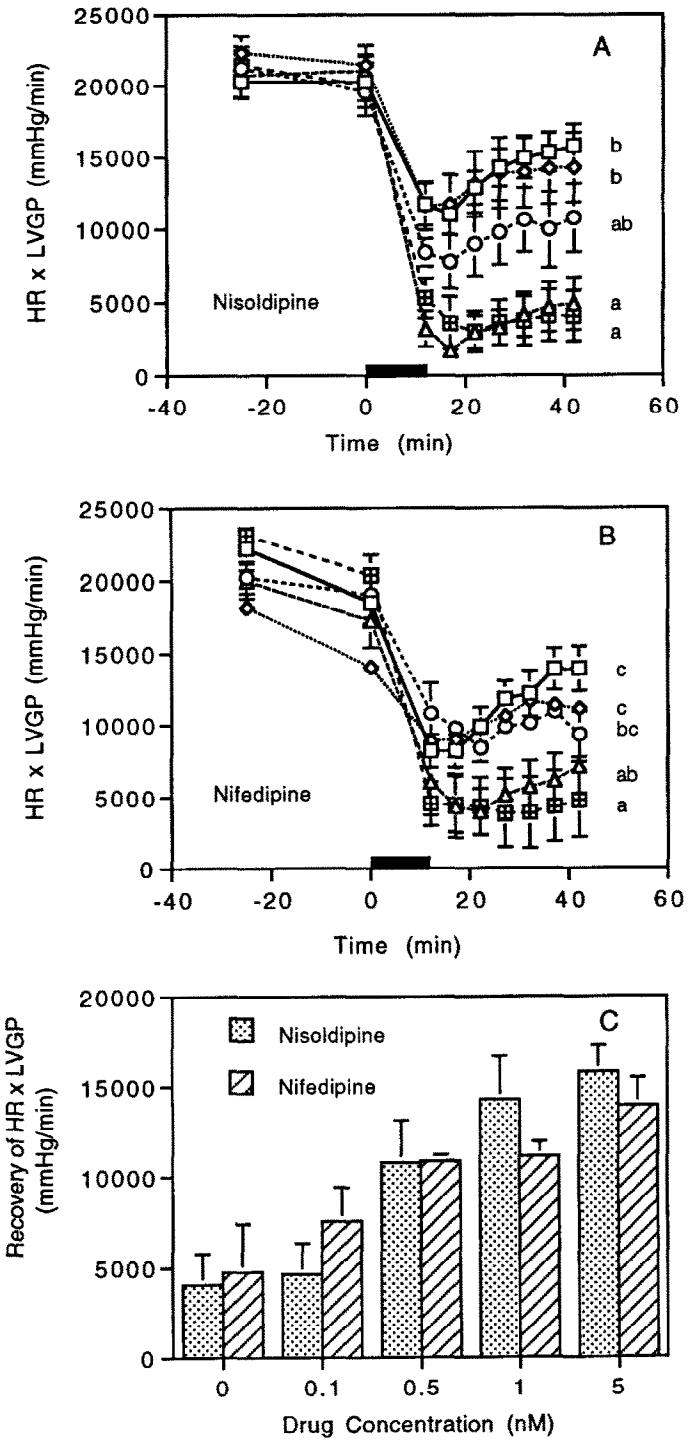

FIG. 2. Effects of H_2O_2 upon contractile function. H_2O_2 (600 μM) was added between time 0 and 12 min (indicated by the solid bar on the time axis). (A) Effects of 25 min of pretreatment with nisoldipine [(⊞) 0 nM, (△) 0.1 nM, (○) 0.5 nM, (◇) 1 nM, (□) 5 nM]. (B) Effects of 25 min of pretreatment with nifedipine [(⊞) 0 nM, (△) 0.1 nM, (○) 0.5 nM, (◇) 1 nM, (□) 5 nM]. (C) Comparison of the effects of nisoldipine and nifedipine upon the recovery of contractile function. Values are means ± SEM, N = 7–12. Values with different letters were significantly different ($P < 0.05$) at the end of perfusion.

hearts receiving no nisoldipine ($P < 0.05$). Treatment with nifedipine also showed a concentration-dependent increase in recovery of contractile function following H_2O_2 treatment with statistical significance at a concentration of 0.5, 1, and 5 nM (Fig. 2B). Comparisons of the effects of nisoldipine and nifedipine upon recovery of contractile function at the end of the experiment indicated that there were no significant differences between values of HR × LVGP (Fig. 2C) at any concentration (5.0 nM, $P = 0.369$; 1.0 nM, $P = 0.069$; 0.5 nM, $P > 0.1$; 0.1 nM, $P = 0.271$).

Samples of coronary venous effluent were tested for the presence of LDH during the perfusion. There was very little release of LDH from hearts that were perfused with vehicle only (54 ± 26 mU/g wet wt/min) (Fig. 3A). Perfusion with H_2O_2 in the presence of vehicle alone caused an increase in the leak of LDH that peaked 10 min following the treatment with H_2O_2 (2,463 ± 1,132 mU/g wet weight/min). Treatment with nisoldipine (5 nM) or nifedipine (5 nM) resulted in a significant reduction of the peak of release of LDH (217 ± 97 and 307 ± 76 mU/g wet weight/min, respectively) compared with hearts treated with vehicle and H_2O_2. The values for hearts treated with nisoldipine and nifedipine were significantly greater than those of time-matched controls, but were not statistically different from each other (Fig. 3A).

Frozen hearts were analyzed for TBARS (Fig. 3B) as an index of lipid peroxidation. Treatment with H_2O_2 produced a significant increase in tissue TBARS (26.8 ± 4.1 nmol/g wet weight) compared with vehicle control hearts (11.3 ± 1.5 nmol/g wet weight, control). Both nisoldipine and nifedipine significantly ($P < 0.05$ vs peroxide control) reduced the rise in TBARS (13.9 ± 2.5 and 10.3 ± 1.6 nmol/g wet weight, respectively). The values obtained in hearts treated with nisoldipine and nifedipine were not significantly different from each other.

The similarity between the effectiveness of nisoldipine and nifedipine was surprising in relation to the differences in potency of these substances as calcium antagonists. A comparison of optical isomers of nisoldipine was undertaken to determine if calcium channels were involved in the protective mechanism.

Comparison of (+)- and (−)-Nisoldipine

There were no significant changes in contractile function from −25 min to 37 min of perfusion of hearts with control buffer (time controls). A small (16%), but statistically significant decrease was observed in this group of hearts at 42 min of the protocol compared with baseline contractile function. Therefore, hearts treated with no H_2O_2 (time controls) maintained high values of contractile function throughout the experiment (Fig. 4). In contrast, a significant decrease in contractile function was observed following treatment with H_2O_2, in the absence of either isomer of nisoldipine (peroxide controls). This reduced contractile function was maintained throughout the rest of the perfusion (initial HR × LVGP = 20,840 ± 1,258; final = 3,004 ± 1,424 mm Hg/min) (Fig. 4).

There was no significant drop in contractile function after 25 min of treatment with 5 nM (+)-nisoldipine (19,640 ± 950 vs 19,480 ± 1,177 mm Hg/min, $P = 0.868$) or with 5 nM (−)-nisoldipine (19,600 ± 920 vs 18,790 ± 1,119 mm Hg/min, $P = 0.164$) before the addition of H_2O_2 (t = −25 min vs t = 0 min) (Fig. 4). There were also no significant changes in the individual components (HR or LVGP) with the addition of (+)- or (−)-nisoldipine before the addition of H_2O_2 (data not shown). Treatment with (+)- or (−)-nisoldipine significantly increased final values of contractile function (11,040 ± 1,330 and 13,210 ± 1,615 mm Hg/min, respectively) compared with those hearts that did not receive nisoldipine treatment (3,004

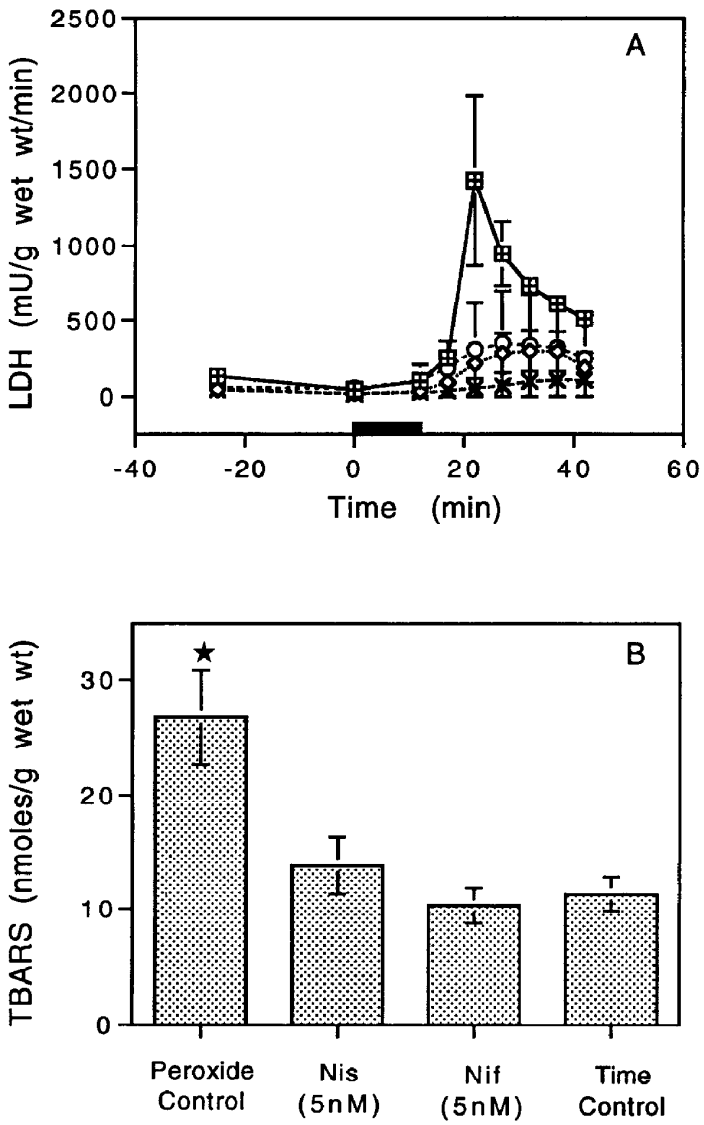

FIG. 3. Effects of H_2O_2 upon release of LDH activity into the perfusate (A) in hearts that were pretreated with vehicle (⊞), 5 nM nisoldipine (◇), 5 nM nifedipine (○), or in hearts that received vehicle with no H_2O_2 (X). H_2O_2 (600 μM) was added between time 0 and 12 min (indicated by the solid bar on the time axis). Values are means ± SEM, N = 5. (B) Tissue levels of TBARS in hearts at the end of perfusion. Hearts were treated with H_2O_2 (600 μM) after pretreatment with vehicle (peroxide control), 5 nM nisoldipine, 5 nM nifedipine, or received vehicle with no H_2O_2 (time control). Values are means ± SEM, N = 6–7. Value with star was significantly greater ($P < 0.05$) than other values.

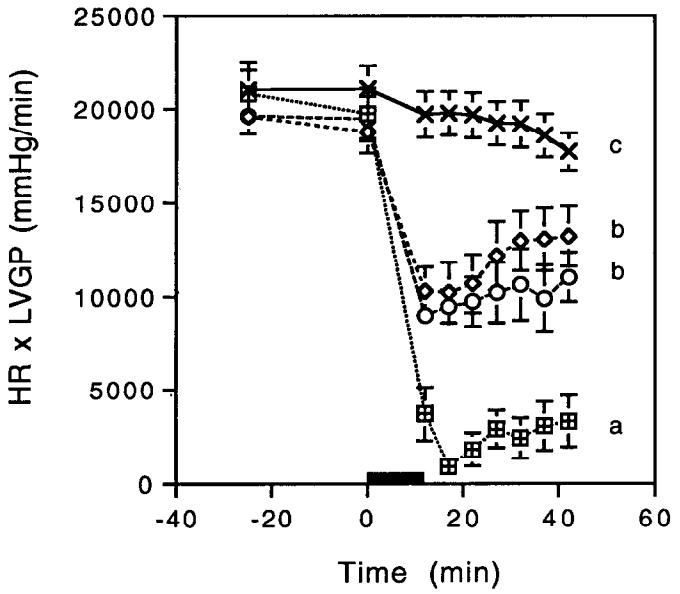

FIG. 4. Values of contractile function (HR × LVGP) in hearts that were treated with vehicle only (time controls, X) or received H_2O_2 (500 μM) from time 0 to 12 min (indicated by the solid bar on the time axis), following pretreatment with vehicle (peroxide controls, ⊞), 5 nM (+)-nisoldipine (○) or 5 nM (−)-nisoldipine (◇). Values as means ± SEM, N = 7–9. Values with different letters were significantly different ($P < 0.05$) at the end of perfusion.

± 1,424 mm Hg/min) (Fig. 4). The values obtained for contractile function in hearts treated with (+)-nisoldipine and (−)-nisoldipine were not statistically different from each other, but the protection was not complete, since the values obtained with (+)-nisoldipine and (−)-nisoldipine were significantly less than the time controls.

Coronary perfusate was tested for the presence of LDH activity (Fig. 5A). Treatment with H_2O_2 in the absence of either isomer of nisoldipine produced a peak release of 6,671 ± 1,025 mU/g wet weight, which was significantly greater than the release of LDH in control hearts (36 ± 9 mU/g wet weight). This release of LDH began at the end of the H_2O_2 perfusion and peaked 10 min after the cessation of H_2O_2. Treatment with both the (+) and the (−) isomers resulted in a significant reduction of this peak release of LDH (465 ± 219 and 217 ± 36 mU/g wet weight, respectively). The release of LDH in hearts treated with (+)- or (−)-nisoldipine was significantly ($P < 0.05$) greater than time-matched controls, but was not statistically different from each other (Fig. 5A).

Hearts treated with H_2O_2 in the absence of either isomer of nisoldipine had significantly elevated TBARS levels (31.4 ± 4.36 nmol/g wet wt) compared with control hearts that received vehicle only (9.75 ± 2.86 nmol/g wet weight) (Fig. 5B). The isomers of nisoldipine significantly reduced the TBARS values [11.4 ± 1.7 nmol/g wet wt for (+)-nisoldipine and 15.1 ± 1.4 nmol/g wet wt for (−)-nisoldipine] compared with hearts that received H_2O_2 in the absence of nisoldipine. There was no significant difference in the TBARS values observed in hearts treated with either isomer of nisoldipine (Fig. 5B).

H_2O_2 Assay

The concentration of H_2O_2 decreased from 456 ± 22 to 206 ± 19 μM after 20 min of incubation in control KHB buffer at 37°, indicating that H_2O_2 was not stable in the perfusate under these conditions. Perfusion of hearts with H_2O_2 actually began 7.5 min after preparation of the H_2O_2 solution where the concentration of H_2O_2 would have decreased by about 30%. Perfusion continued until 19.5 min during which time the concentration of H_2O_2 would have decreased further. The presence of nisoldipine or nifedipine had no significant effect on the degradation of H_2O_2 in buffer (Fig. 6). Thus, while the

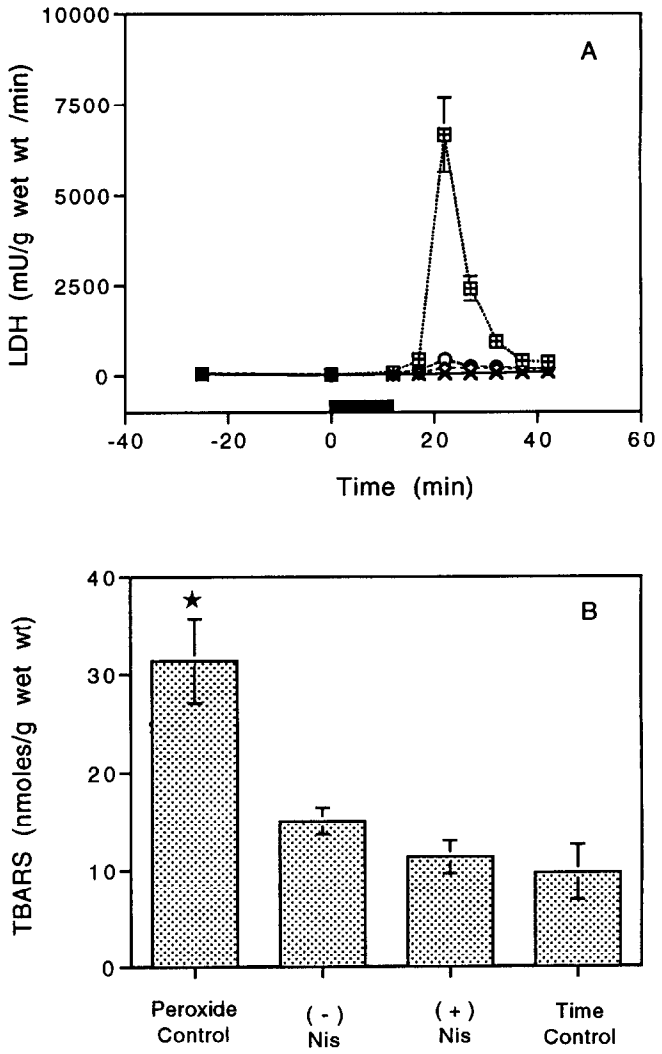

FIG. 5. Effects of H_2O_2 upon release of LDH activity into the perfusate (A) in hearts that were pretreated with vehicle (⊞), 5 nM (+)-nisoldipine (○), 5 nM (−)-nisoldipine (◇), or in hearts that received vehicle with no H_2O_2 (X). H_2O_2 (500 μM) was added between time 0 and 12 min (indicated by the solid bar on the time axis). Values are means ± SEM, N = 6–8. (B) Tissue levels of TBARS in hearts at the end of perfusion. Hearts were treated with H_2O_2 after pretreatment with vehicle (peroxide control), 5 nM (+)-nisoldipine, 5 nM (−)-nisoldipine, or received vehicle with no H_2O_2 (time control). Values are means ± SEM, N = 5–7. Value with star was significantly greater ($P < 0.05$) than other values.

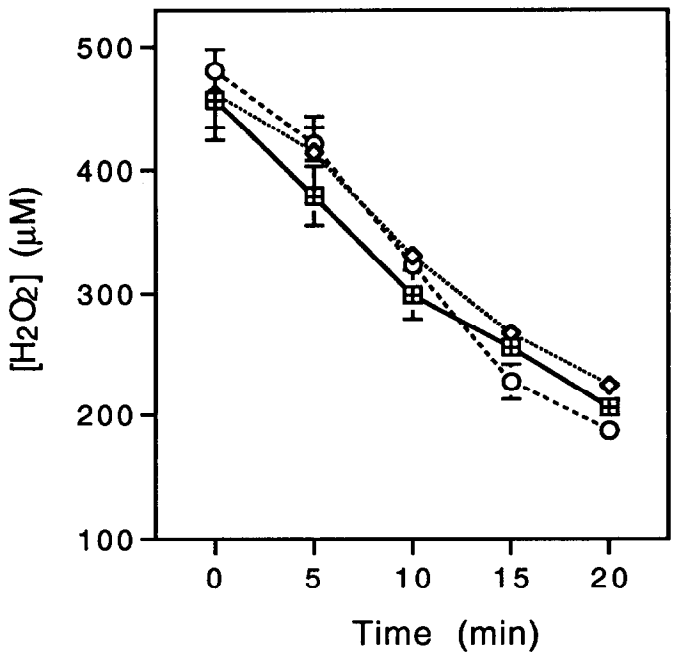

FIG. 6. H_2O_2 concentrations determined in heated (37°), oxygenated modified KRB buffer, with vehicle (⊞), 5 nM nisoldipine (◇), or 5 nM nifedipine (○). Values are means ± SEM, N = 3. Comparisons indicate that the three lines were not significantly different.

H_2O_2 concentration decreased with time of perfusion, the degree of free radical attack was the same in all treatment groups.

DISCUSSION

Calcium Channel Dependence vs Independence

Treatment of isolated rat hearts with H_2O_2 produced a decrease in overall contractile function that followed the addition of H_2O_2 and persisted throughout the subsequent perfusion. Biochemical analyses indicated that the plasma membranes of these hearts were damaged since there was a massive leak of LDH into the perfusate and an increase in lipid peroxidation products (TBARS) observed in the tissue. There were no differences in protection afforded by equal concentrations of nisoldipine and nifedipine, which was surprising since they differ by 20-fold in their binding affinity for calcium channels [27]. To examine the role of Ca^{2+} channels in this protective mechanism, the enantiomers of nisoldipine (+ and −) were compared. These enantiomers differ by 100-fold in their ability to act on Ca^{2+} channels [K_i = 0.17 nM (+)-nisoldipine, 22.6 nM (−)-nisoldipine, and 0.24 nM (±)-nisoldipine [23]], yet they were equipotent in providing both biochemical and functional protection to the hearts. Furthermore, the concentrations of nisoldipine used in the experiments did not have negative inotropic effects. These findings indicated that nisoldipine reduced oxidative injury without acting upon calcium channels.

Calcium antagonists have been utilized experimentally to reduce the damage to hearts from ischemia and reperfusion. Early studies with various calcium antagonists indicated that the protection provided by these compounds against injury from ischemia and reperfusion was in proportion to the degree of cardiodepression induced before the onset of ischemia due to the effects of the calcium antagonists upon the calcium channels of the myocardium [10, 28, 29]. Later studies employed nanomolar concentrations of nisoldipine to determine if protection against ischemia and reperfusion could be obtained without cardiodepression and with a clinically relevant concentration of nisoldipine. It was shown that nisoldipine (1 nM) reduces the injury from ischemia and reperfusion in isolated rat hearts [12, 13] and rabbit hearts [9, 30]. These studies concluded that the beneficial effects required pretreatment with nisoldipine, occurred without cardiac depression before ischemia, and were associated with improved coronary flow. Recent studies also demonstrated that the balance between constrictor responses to endothelin [31] and endothelium-dependent dilator responses [17] was improved by nisoldipine treatment; however, the mechanism of this protection has not yet been elucidated. One interpretation of these findings was that nisoldipine was protecting by calcium antagonist activity, which was selective to the vasculature; however, calcium antagonists may also prevent the free radical damage associated with ischemia and reperfusion. It is not clear if the antioxidant abilities of these drugs is a result of calcium antagonism or an independent interaction with the cellular membranes. Most studies of the effects of dihydropyridines upon oxidative injury employed extremely high concentrations of these compounds (10^{-6}–10^{-3} M). These concentrations reduce the peroxidation of isolated membranes by free radicals [14, 15, 32, 33]. Nisoldipine (50 nM) also reduces oxidative injury to contractile function in rat hearts induced by H_2O_2 [20], and prevents arrhythmias induced by superoxide ($O_2^{\cdot -}$) damage in Langendorff-perfused rat hearts [34]. At this concentration, nisoldipine would act as a calcium antagonist as well as an antioxidant. Concentrations between 0.1 and 5.0 nM were selected for the present study. The results indicated that nisoldipine reduced oxidative injury without acting upon calcium channels, although the interaction between nisoldipine and calcium channels could be altered by oxidative injury. Binding of nisoldipine to calcium channels is greater in the open channel state [35]. Free radical damage likely depolarizes cells, which may increase the affinity of nisoldipine for calcium channels [27]. The binding studies indicating the 100-fold difference in affinity of the (+) and (−) enantiomers of nisoldipine were performed using membrane vesicles and arterial rings, both of which were depolarized. For this reason, it is unlikely that (−)-nisoldipine (5 nM) changes affinity for calcium channels due to depolarization in the damaged heart. Oxidative injury may also alter the calcium channel and increase the affinity of the antagonist for the receptor; however, Sasaki and Okabe [36] observed that nisoldipine (0.01 to 1.0 nM) inhibits both KCl-induced and norepinephrine-induced contraction of smooth muscle when administered 90 min after damage from hydroxyl ($OH^{\cdot}$) radicals. This indicates that the drugs still bind to the vascular calcium channels with normal affinity after free radical formation.

Membrane Interactions

The mechanism by which nisoldipine protects the heart from free radical damage remains to be identified definitively. A common findings throughout the literature is that nisoldipine must be administered well before the free radicals are either added or produced. Dihydropyridins are highly lipophilic, and the protection from free radical damage may depend upon accumulation of the drug in membranes. Nisoldipine has a partition coefficient in biological membranes that is at least twice that of nifedipine (6,000–27,000 vs 2,900, respectively) [27]. The dihydropyridines accumulate within the bilayer and reach a concentration within the membrane that is at least three orders of magnitude greater than in the aqueous phase surrounding the membrane [37]. Dihydropyridines with a relatively low partition coefficient may simply require more time to accumulate in the membrane. While the final concentration of compounds in the membrane may be different, the time of pretreatment may allow the drugs to reach an adequate level for antioxidant protection against the same degree of free radical attack. This would explain why, when hearts were treated for 25 min with the drugs prior to free radical damage, nisoldipine and nifedipine were equipotent. Other studies in which hearts were treated with the drugs for less time claimed that nisoldipine, but not nifedipine, was able to prevent free radical damage [17, 34].

There does not appear to be a simple correlation between antioxidant capabilities and partition coefficient [38]. The position of the drug in the membrane and the structure of the drug may also be important. X-ray diffraction studies show that the phenyl ring of nisoldipine lies at a depth in the membrane which positions it near the 9–10 double bond of unsaturated lipids [39]. The 9–10 bond is highly susceptible to peroxidation since that is where most of the double bonds are located. Janero and Burghardt [14] noted that asymmetry between the groups attached to C-3 and C-5 on the dihydropyridine ring was characteristic of effective antioxidants among dihydropyridines. In particular, the presence of a methyl-ester group at C-3 in association with a hydrophobic long-chain ester at C-5 marked strong dihydropyridine antioxidants. Another structural factor that may play a role is stearic hinderance between the group at C-5 and the $-NO_2$ substituent of the phenyl ring [18]. The two groups are as close together as possible in nisoldipine and nifedipine, maximizing stearic hindrance and indicating the potential for these compounds to reduce the propagation of lipid peroxidation. Therefore, optimal structure and positioning may allow nisoldipine and nifedipine to serve as chain-breaking antioxidants. Electron spin resonance studies indicate that free radical reactions lead to nifedipine-stable radicals in liposomes, suggesting that nifedipine may scavenge free radicals within the lipid environment [40]. Lacidipine, another dihydropyridine, acts similar to vitamin E by reacting with both alkoxyl ($RO^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals that are intermediates of the lipid peroxidation chain reaction [41]. Reduction of membrane peroxidation may reduce the fragility of either the plasmalemmal or mitochondrial membranes to osmotic stretch imposed during ischemia and reperfusion. Ferrari *et al.* [9] postulated this explanation since the leak of creatine kinase during reperfusion was reduced by nisoldipine. Results from the current data support this claim, since nisoldipine and nifedipine prevent the leak of LDH from hearts injured with H_2O_2.

Nifedipine is light sensitive. Studies comparing illuminated to non-illuminated nifedipine show that the illuminated form (NTP) has antioxidant capabilities, whereas the original form of nifedipine has less antioxidant capacity [40, 42]. NTP serves as a spin trap that will directly scavenge free radicals. The scavenging studies measure the direct reaction of these drugs with generated free radicals in environments outside of a biological system (i.e. in a buffer). The present results show that neither nisoldipine nor nifedipine are able to scavenge H_2O_2 in heated, oxygenated KHB buffer. Other studies found similar results when $OH^{\cdot}$ or $O_2^{\cdot}$ scavenging was measured [18]. These drugs do not directly scavenge free radicals under these conditions, but it is possible that they become modified by interaction with components of the cells, and thus become scavengers.

In summary, clinically relevant concentrations of nisoldipine and nifedipine dramatically protect isolated rat hearts from free radicals mediated damage. The present experiments also compared isomers of nisoldipine that differ widely in their abilities as calcium channel blockers, yet these results showed that they were equipotent in their ability to prevent free radical damage, suggesting that the beneficial antioxidant effects were not mediated by the calcium channels. It is likely that these drugs have a quality that is independent of their calcium channel-blocking properties, but reflects their ability to insert and accumulate within the lipid bilayer, and position the appropriate portion of the molecular near the position of the double bonds that are most likely to be attacked by the free radicals. It is not yet clear whether calcium antagonism or protection of lipid structure is the most important effect in ischemia and reperfusion.

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