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ABSENCE OF ANTIOXIDANT EFFECTS OF NIFEDIPINE AND DILTIAZEM ON MYOCARDIAL MEMBRANE LIPID PEROXIDATION IN CONTRAST WITH THOSE OF NISOLDIPINE AND PROPRANOLOL

HITOSHI SUGAWARA,* KATSUYUKI TOBISE and SOKICHI ONODERA
First Department of Internal Medicine, Asahikawa Medical College, Asahikawa 078, Japan

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Abstract—Both the production of active oxygen species and cellular damage due to concurrent lipid peroxidation are believed to be important factors in the pathogenesis of cardiovascular diseases and the ageing process. Since cardiovascular drugs are often administered over a long term, it might be advantageous if they reduced lipid peroxidation. There have been conflicting reports concerning the antiperoxidant effect of nifedipine. Therefore, we investigated whether nifedipine could inhibit lipid peroxidation in a nonenzymatic active oxygen-generating system, utilizing rat crude myocardial membranes, and compared its effect with those of propranolol, nisoldipine, and diltiazem. Nifedipine and diltiazem had no inhibitory effects on the lipid peroxidation of myocardial membranes. In contrast, nisoldipine and propranolol had a concentration-dependent antiperoxidant effect, with IC_{50} values of 28.2 and 50.1 μM, respectively. In addition, nisoldipine appeared to possess dual antiperoxidant mechanisms, involving both preventive and chain-breaking properties.

Key words: nisoldipine; nifedipine; myocardial membrane lipid peroxidation; antiperoxidation; calcium antagonists; TBA test

Cellular damage due to lipid peroxidation caused by the production of active oxygen species has a role in various disease states, such as ischemia–reperfusion injury [1], coronary arteriosclerosis [2], and diabetes mellitus [1], as well as contributing to "normal" ageing [3]. Calcium antagonists are a major class of therapeutic agents for hypertension and angina pectoris. When these agents are administered to treat such diseases over a long period of time, it might be advantageous if they were also effective against lipid peroxidation due to oxygen radicals.

Janero *et al.* [4] were the first to study the direct effect of calcium antagonists on the lipid peroxidation of cardiac membranes. They found that bepridil and prenylamine were the most potent antiperoxidants in the calcium antagonists tested. However, there are contradictory reports published with regard to nifedipine, one of the most popular drugs in this class. Janero and Burghardt [5] found that nisoldipine was the most potent dihydropyridine antiperoxidant and that nifedipine had little effect when compared with the antiperoxidant activities of seven dihydropyridine calcium antagonists, using a xanthine oxidase-dependent active oxygen-generating system and liposomes derived from cardiac membrane phospholipids. In contrast, when Mak and Weglicki [6] compared the antiperoxidant activities of four calcium antagonists with that of propranolol by using an active oxygen-generating system involving DHF† auto-oxidation and sarcolemma isolated by density gradient centrifugation, they found that nifedipine had the most potent antiperoxidant effect.

The purpose of the present study was to determine whether nifedipine could inhibit lipid peroxidation in a nonenzymatic active oxygen-generating system (DHF/$FeCl_3$-ADP) and to compare the antiperoxidant effect of nifedipine with those of nisoldipine, diltiazem, and propranolol in an *in vitro* study using crude myocardial membranes.

MATERIALS AND METHODS

Animals

Seven-week-old male Sprague–Dawley rats were purchased from Charles River Laboratories, Inc. (Tokyo, Japan) and housed for 1 week at the animal laboratory of Asahikawa Medical College before use in this study.

Materials

DHF was purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). ADP, nifedipine, DL-propranolol, bovine erythrocyte SOD (S-2515; 3570 U/mg), and bovine liver catalase (C-10; 2000 U/mg) were all purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Diltiazem was

* Corresponding author: Hitoshi Sugawara, M.D. Present address: Rishiri Island Central Hospital, 11 Aza-Midorimachi, Kutsugata, Rishiri-cho, Rishiri-gun, Hokkaido 097-04, Japan. Tel. 81-1638-4-2626; FAX 81-1638-4-2640.

Permanent address: First Department of Internal Medicine, Asahikawa Medical College, 4-5-3 Nishikagura, Asahikawa 078, Japan. Tel. 81-166-65-2111; FAX 81-166-65-9473.

† Abbreviations: DHF, dihydroxyfumarate; SOD, superoxide dismutase; BHT, butylated hydroxytoluene; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; and NBT, nitroblue tetrazolium.

provided by Tanabe Seiyaku Co. Ltd. (Osaka, Japan) and nisoldipine by Bayer Yakuhin Ltd. (Osaka Japan). The other agents used were purchased from Nacalai Tesque Inc. (Tokyo, Japan). Concentrated stock solutions of nifedipine, DL-propranolol, diltiazem, or nisoldipine were diluted with ethanol before use, so that the final solvent concentration in the reaction system did not affect lipid peroxidation. Water was purified with a Milli-Q Plus filter (Millipore, Tokyo, Japan) before use.

Preparation of crude myocardial membranes

Eight-week-old rats were decapitated, and their hearts were isolated and washed with physiological saline. The aorta, atria, and fatty tissue were removed, and the ventricles were frozen in liquid nitrogen and stored at −80°. All subsequent procedures were conducted at 4°. Using the method of Wagner *et al.*, as modified by Kaneko *et al.* [17], the crude myocardial membrane fraction was prepared from the heart tissue without utilizing chelating agents such as EDTA or a sucrose gradient. Briefly, the myocardium was minced in 50 mM Tris–HCl buffer (pH 7.4), homogenized for 20 sec with a Polytron PTA 10S homogenizer (Brinkmann Instruments Inc.) on setting No. 5, and then centrifuged at 1000 *g* for 10 min. The supernatant obtained was centrifuged at 48,000 *g* for 25 min. The resultant supernatant was discarded, and the pellet was resuspended in the same buffer and centrifuged again at 48,000 *g* for 25 min. This procedure was repeated twice, after which the final pellet was resuspended in 10 mM Tris–HCl buffer (pH 7.4) and adjusted to a protein concentration of 1–2 mg/mL before being stored at −80°. Protein concentrations were determined according to the method of Lowry *et al.* [18].

Active oxygen-generating system

A nonenzymatic active oxygen-generating system involving the auto-oxidation of DHF/Fe^{3+}-ADP was used, according to the method of Mak *et al.* [9]. Following the auto-oxidation of DHF, the resultant superoxide anions produced hydroxyl radicals in a reaction catalyzed by Fe^{3+}-ADP and initiated the lipid peroxidation chain reaction [10].

Experimental protocols

Experiment 1. Under amber lighting provided by a sodium lamp, 100 μg of myocardial membrane protein was incubated and shaken in 10 mM Tris–HCl buffer (pH 7.4) for 30 min at 37° in a warm water bath with each agent at a final concentration of 1 mM. After adding 6.66 mM DHF, 1 mM ADP, and 0.1 mM $FeCl_3$ (in a total volume of 0.5 mL), the mixtures were incubated and shaken in a warm water bath at 37° for a further 120 min, and the extent of lipid peroxidation was determined. The IC_{50} value was determined for all agents that inhibited lipid peroxide production by over 80% when compared with the control.

In addition, to confirm that well-known antioxidants inhibited lipid peroxidation in this experimental system, we tested SOD, catalase, α-tocopherol, 1 mM DL-histidine, 10 mM D-mannitol, and 20 mM DMSO in the same manner.

Experiment 2. Using the agents that inhibited lipid peroxidation by over 80% when compared with the control, this subsequent experiment was performed. Under the same conditions as in Expt. 1, 100 μg of membrane protein in 10 mM Tris–HCl buffer (pH 7.4) was preincubated and shaken in a warm water bath at 37° for 30 min, following which 6.66 mM DHF and 0.1 mM $FeCl_3$-1 mM ADP were added. After 30 min, the test agent (1 mM) was added in a total volume of 0.5 mL, the mixture was incubated and shaken in a warm water bath at 37° for a further 90 min, and the extent of lipid peroxidation was measured.

Experiment 3. The effect of our incubation buffer (10 mM Tris–HCl, pH 7.4) and Mak's reaction buffer (120 mM KCl, 50 mM sucrose, and 10 mM potassium phosphate, pH 7.2) on lipid peroxidation was examined under the experimental conditions of Mak and Weglicki [6]. Each buffer was preincubated for 10 min without crude myocardial membranes, after which 0.83 mM DHF and 0.025 mM $FeCl_3$-0.25 mM ADP were added. Following incubation for a further 20 min, the extent of lipid peroxidation was measured.

Determination of lipid peroxidation

Lipid peroxide production was measured as TBARS, using the improved TBA test of Ogura *et al.* [11]. Although the relationship of this test to actual lipid peroxide production is not necessarily direct [12], we defined TBARS (nmol) as the malondialdehyde (MDA) equivalent of lipid peroxide production based on data obtained with a standard solution of 1,1,3,3-tetraethoxypropane (9.487 nmol/mL). After the last incubation in the warm water bath, 10 μL of 2% (w/v) BHT in methanol was immediately added to stop the reaction. Then 200 μL of 10% (w/v) SDS and 3 mL of the TBA reagent were added. The TBA reagent was a mixture of equal volumes of 0.67% (w/v) TBA aqueous solution and glacial acetic acid. Next, the mixture was heated for 30 min at 90° and subsequently cooled in ice water for 10 min, after which 3 mL of chloroform was added while stirring. Following centrifugation at 1650 *g*, the absorbance of the supernatant was determined at 532 nm using a UV-160 Shimadzu ultraviolet-visible light spectrophotometer (Kyoto, Japan).

Determination of DHF auto-oxidation

The auto-oxidation rate of DHF was determined by the NBT method [10] for well-known antioxidants and all agents that inhibited lipid peroxidation production by over 80% compared with the control. NBT (50 μM), the test agent, and 3.33 mM DHF were added to 10 mM Tris–HCl buffer (pH 7.4) in a total reaction volume of 2 mL, and the change in absorbance at 560 nm was determined using a Hitachi 557 double-wavelength spectrophotometer (Tokyo, Japan). The DHF auto-oxidation rate was then calculated from the rate of NBT reduction using the Lambert-Beer formula and a molecular absorbance coefficient of $17.9 \times 10^3\,M^{-1}\cdot cm^{-1}$ [10]. For statistical analysis, the outlier test, analysis of variance, or multiple comparisons were used as was appropriate.

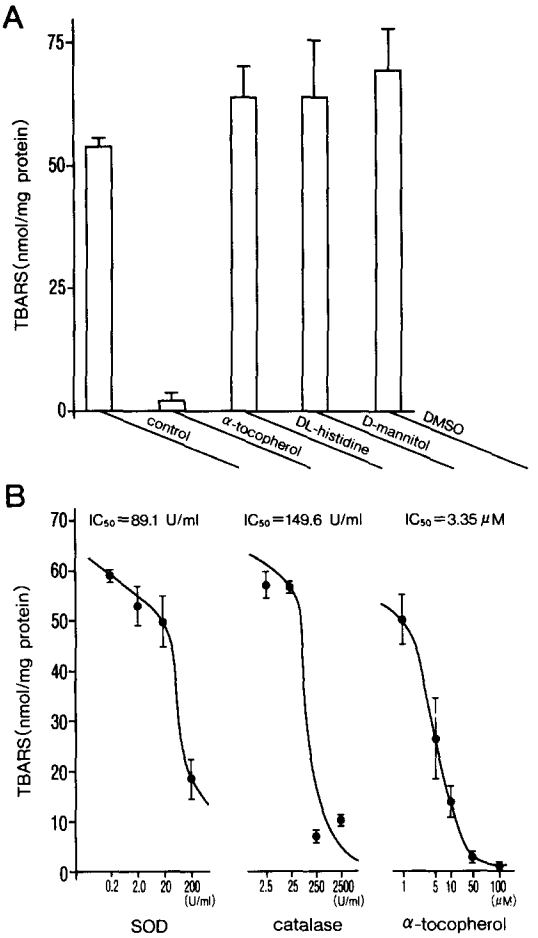

Fig. 1. Effects of well-known antioxidants on lipid peroxidation. (A) Comparative effect of α-tocopherol (1 mM), DL-histidine (1 mM), D-mannitol (10 mM), and DMSO (20 mM) on the lipid peroxidation of myocardial membranes. Crude myocardial membranes (100 μg) were preincubated and shaken in 10 mM Tris–HCl buffer (pH 7.4) for 30 min at 37° in a warm water bath with each agent, and then 6.66 mM DHF and 0.1 mM $FeCl_3$-1 mM ADP were added. After a further 120 min, the extent of lipid peroxidation was measured. Values are the means ± SD of 5–11 samples. (B) Concentration-dependent antiperoxidant effects of SOD, catalase, and α-tocopherol. Values are the means ± SEM of 4–8 samples.

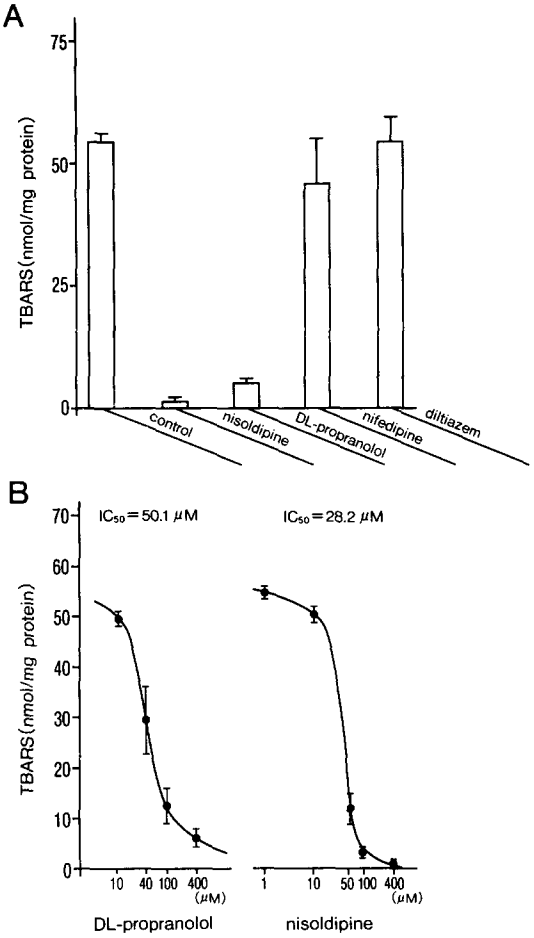

Fig. 2. Effects of various agents on lipid peroxidation. (A) Comparative effects of nisoldipine, DL-propranolol, nifedipine, and diltiazem on the lipid peroxidation of myocardial membranes. The methods were the same as given in the legend of Fig. 1. Values are the means ± SD of 5–11 samples. (B) Concentration-dependent antiperoxidant effects of DL-propranolol and nisoldipine. Values are the means ± SEM of 4–8 samples.

RESULTS

Effect of well-known antioxidants on lipid peroxidation

In this experimental system, a quenching agent (DL-histidine) and hydroxyl radical scavengers (D-mannitol and DMSO) did not inhibit lipid peroxidation (Fig. 1A). In contrast, lipid peroxidation was inhibited concentration dependently by SOD, catalase, and α-tocopherol, with respective IC_{50} values of 89.1 U/mL, 149.6 U/mL, and 3.35 μM (Fig. 1B).

Effects of the various agents on lipid peroxidation

The extent of lipid peroxidation (nmol/mg protein) in the presence of each agent (1 mM) was as follows: control, 53.4 ± 3.21; nisoldipine, 1.57 ± 0.62; DL-propranolol, 4.96 ± 1.07; nifedipine, 46.3 ± 8.98; and diltiazem, 54.9 ± 4.98 (Fig. 2A). DL-Propranolol and nisoldipine inhibited lipid peroxidation by over 80%, and their IC_{50} values were 50.1 and 28.2 μM, respectively (Fig. 2B). In contrast, nifedipine and diltiazem had no inhibitory effect on lipid peroxidation.

Effects of the various agents on DHF auto-oxidation

The DHF auto-oxidation rate (nmol/min) in the presence of each agent tested was as follows; control, 3.39; SOD (50 U/mL), 0.60; catalase (250 U/mL), 0.54; D-mannitol (10 mM), 2.57; DMSO (20 mM), 2.85; DL-histidine (1 mM), 3.10; α-tocopherol (1 mM), 2.91; nisoldipine (1 mM), 1.76; nifedipine (1 mM), 2.46; and DL-propranolol (1 mM), 3.06 (Fig.

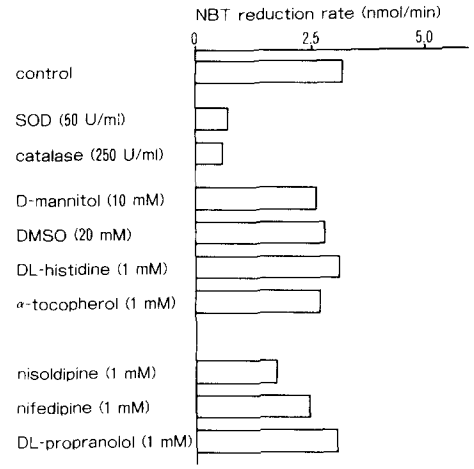

Fig. 3. Effects of oxygen radical scavengers and various agents on DHF auto-oxidation. The auto-oxidation of DHF was monitored spectrophotometrically at 560 nm as the rate of NBT reduction. The extinction coefficient used for NBT was $17.9 \times 10^3\,M^{-1} \cdot cm^{-1}$. Values shown are the means of two experiments.

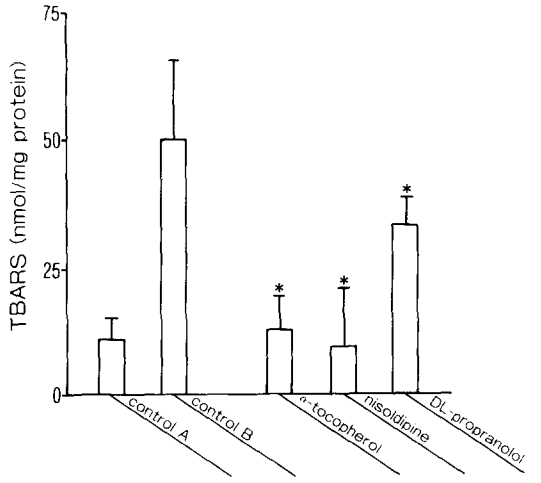

Fig. 4. Antiperoxidant effect of various agents applied to the crude myocardial membrane fractions at 30 min after the addition of DHF/Fe^{3+}-ADP. Crude myocardial membranes (100 μg) were preincubated and shaken for 30 min in 10 mM Tris–HCl buffer (37°, pH 7.4), following which 6.66 mM DHF and 0.1 mM $FeCl_3$-1 mM ADP were added. After 30 min, the tested agent (1 mM) was added, incubation was continued for 90 min under the same conditions, and the extent of lipid peroxidation was measured. Control A: incubation for 30 min after the addition of DHF/$FeCl_3$-ADP. Control B: incubation for 120 min after DHF/$FeCl_3$-ADP addition, as previously shown in Fig. 1. Values are the means ± SD of 5–10 samples. Key: (*) $P < 0.05$ vs control B (analysis of variance and multiple comparison).

3). The DHF auto-oxidation was reduced by SOD, catalase, and nisoldipine, but not by D-mannitol, DMSO, DL-histidine, α-tocopherol, nifedipine, or DL-propranolol.

Lipid peroxidation following application of each agent at 30 min after DHF/Fe^{3+}-ADP addition

When each agent was applied to the crude myocardial membrane fraction at 30 min after the addition of DHF/Fe^{3+}-ADP, the extent of lipid peroxidation (nmol/mg protein) was as follows; control A (total incubation time of 30 min), 10.7 ± 4.42; control B (total incubation time of 120 min), 49.9 ± 14.7; α-tocopherol (1 mM), 13.0 ± 6.55; nisoldipine (1 mM), 9.39 ± 11.2; and DL-propranolol (1 mM), 36.5 ± 6.00 (Fig. 4). Lipid peroxidation was inhibited significantly by α-tocopherol, nisoldipine and DL-propranolol when compared with control B ($P < 0.05$).

Spectrophotometric profiles of the TBA test

When DHF/Fe^{3+}-ADP was added to Mak's reaction buffer under Mak's experimental conditions without any membrane fraction, an absorbance peak was identified at 532 nm and artificial products were detected (Fig. 5B). In contrast, our incubation buffer did not produce a peak at 532 nm (Fig. 5C).

DISCUSSION

This study showed that nifedipine and diltiazem did not inhibit lipid peroxidation in crude myocardial membranes, whereas nisoldipine and propranolol had a concentration-dependent antiperoxidant effect on our nonenzymatic active oxygen-generating system (Fig. 2). In addition, nisoldipine slightly decreased the DHF auto-oxidation rate, whereas propranolol had no effect on this parameter (Fig. 3).

In the present experimental system, lipid peroxidation was also inhibited by SOD, catalase, and α-tocopherol (Fig. 1B). The DHF auto-oxidation rate was decreased by SOD and catalase, whereas α-tocopherol had no effect (Fig. 3). Further, when α-tocopherol, nisoldipine or propranolol was added to the crude myocardial membranes at 30 min after DHF/Fe^{3+}-ADP addition, lipid peroxidation was inhibited significantly (Fig. 4). These findings indicate that nisoldipine had both a preventive antiperoxidant effect [5, 13], like that of SOD or catalase, and a chain-breaking effect [5, 13], like that of α-tocopherol. Thus, our findings support the results of Janero and Burghardt [5] who suggested that the antiperoxidant effect of dihydropyridine calcium antagonists was related to their ability to block free radical formation, thereby preventing the propagation of lipid peroxidation. In contrast, propranolol had only a chain-breaking antiperoxidant effect [14].

Janero *et al.* reported that there was no absolute correlation between the antiperoxidant potency of calcium antagonists and their lipophilicity as expressed by the partition coefficient. They suggested that lipophilicity *per se* may be only one factor contributing to the antiperoxidant effect of these agents [4, 5]. Similarly, our experiments showed

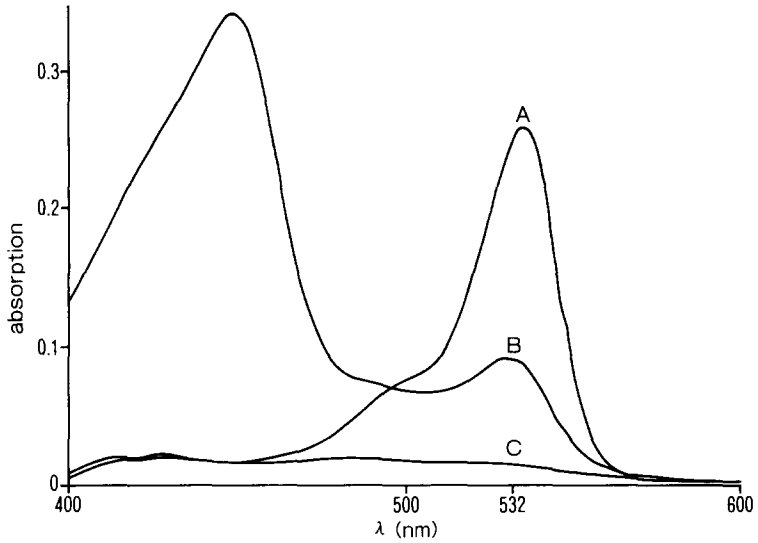

Fig. 5. Spectrophotometric profiles obtained under Mak's conditions. Each incubation buffer was preincubated and shaken for 10 min without crude myocardial membranes, after which 0.83 mM DHF and 0.025 mM $FeCl_3$-0.25 mM ADP were added. Following incubation for a further 20 min, the TBA test was performed. (A) standard (1,1,3,3-tetraethoxypropane); (B) Mak's incubation buffer consisting of 120 mM KCl, 50 mM sucrose, and 10 mM potassium phosphate (pH 7.2); and (C) the present assay buffer consisting of 10 mM Tris–HCl (pH 7.4).

that nifedipine and nisoldipine had different antiperoxidant effects, although both drugs have relatively high partition coefficients into biological membrane [15, 16]. These findings suggest that the antiperoxidant effect of calcium antagonists is not necessarily determined by lipid solubility [4, 5].

The present study was compared with the results of Mak and Weglicki [6] and Janero and Burghardt† [5]. In contrast to the methods of Mak and Weglicki [6], we did not use density gradient centrifugation in the presence of a chelating agent and sucrose during membrane purification, and reaction buffer containing sucrose. Chelating agents are reported to affect iron ion-dependent lipid peroxidation [12, 17], and the presence of sucrose affects the reaction of TBA [12, 18]. In fact, Mak and Weglicki isolated sarcolemma by density gradient centrifugation in the presence of sucrose, chelators, and other agents that can affect either lipid peroxidation and/or the TBA test [12, 17]. In addition, when DHF/Fe^{3+}-ADP was added to Mak's reaction buffer without any cardiac membrane fraction, an absorbance peak was still identified at 532 nm and artificial products were detected (Fig. 5B). This indicates that the membrane peroxidation reaction mixture used by Mak and Weglicki [6] may be unsuitable due to the generation of *sucrose*-derived TBARS rather than *lipid*-derived TBARS. It may influence some of its other constituents on the TBA test [12].

In summary, our present results support the findings of Janero and Burghardt [5], who reported that nifedipine had no inhibitory effect on lipid peroxidation. In contrast, nisoldipine not only inhibits xanthine oxidase [5], but possesses a dual antiperoxidant effect that could, perhaps, play a beneficial role in clinical practice.

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