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INHIBITION OF RAT HEART AND LIVER MICROSOMAL LIPID PEROXIDATION BY NIFEDIPINE

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Abstract—Lipid peroxidation in rat heart and liver microsomes was induced by an NADPH-generating system or by ascorbate in the presence of an ADP–iron complex. Microsomal lipid peroxidation, as measured by malonaldehyde formation, was inhibited by nifedipine over a wide range of concentrations (47 μM to 6 mM). Nifedipine also decreased the oxygen consumption of cardiac and hepatic microsomes in a concentration-dependent manner. These results indicate that nifedipine may perturb microsomal electron transport systems. Nifedipine may have the potential to alter the sensitivity of cardiac and hepatic membranes to peroxidative damage.

The calcium channel blocking agent nifedipine [4-(2′ - nitrophenyl) - 2,6 - dimethyl - 3,5 - dimethoxycarbonyl-1,4-dihydropyridine] is effective in the management and control of a variety of cardiac disorders [1–3]. There is experimental evidence to suggest that nifedipine affords myocardial protection during episodes of hypoxia and subsequent reoxygenation [4–7]. The mechanism of such protection is not yet clear. It is known, however, that the post-ischemic damage sustained by cardiac tissue upon reperfusion or reoxygenation involves the formation of free radicals [8–12]. Nifedipine is a reactive chemical with a strong propensity to participate in redox reactions. While the dihydropyridine moiety of nifedipine can undergo biological oxidation to the pyridine structure [13], it is significant that the nitro group in the nitrophenyl portion of the nifedipine structure is a candidate for biological reduction. It is known that metabolic reduction of aromatic nitro compounds can generate free radicals [14–17].

Free radicals are also involved in the lipid peroxidation of microsomes [18, 19], and nifedipine by virtue of its nitroaromatic structure may interact with the cascade of events leading to the peroxidation of microsomal lipids. Since nifedipine may interact with redox systems, it may be expected to alter the rate of oxygen consumption by the microsomal electron transport system. This report examines the effect of nifedipine on the lipid peroxidation process in rat heart and liver microsomes.

MATERIALS AND METHODS

Chemicals. Nifedipine, ADP, NADP, sodium ascorbate, glucose-6-phosphate dehydrogenase, superoxide dismutase (3050 units/mg protein), mannitol, 1,3-dimethylurea, thiourea and bovine serum albumin were obtained from the Sigma Chemical Co. (St. Louis, MO). Desferrioxamine as the methanesulfonate (Desferal) was obtained from the CIBA Pharmceutical Co. (Summit, NJ), and 2-thiobarbituric acid was obtained from Eastman Organic Chemicals (Rochester, NY). All other chemicals were of the highest purity available.

Preparation of microsomes. Male Sprague–Dawley rats, weighing 180–250 g, were maintained on commercial rat chow and starved for 24 hr prior to being killed. The hearts and livers were removed and immediately placed in ice-cold 1.15% KCl buffer. All subsequent steps were carried out at 4°. The hearts and livers were minced and homogenized with 3 vol. of the buffer and fractionated as described previously [20]. The homogenate was centrifuged at 9000 *g* for 20 min, and the resultant supernatant fraction, without the fatty layer at the top, was recentrifuged at 105,000 *g* for 60 min in a Beckman model L8-M preparative ultracentrifuge. The microsomal pellet was washed twice and resuspended in 1.15% KCl buffer. The microsomal protein was determined using an assay kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond CA) based on the protein-dye binding method [21].

Incubation systems. Liver microsomes (1.5 mg protein/ml) and heart microsomes (3 mg protein/ml) were incubated with 0.012 mM $FeCl_3$, 0.4 mM ADP and a freshly prepared NADPH-generating system in 15 mM potassium phosphate buffer (pH 7.4).

The NADPH-generating system was prepared as described earlier [22] and contained, per ml of microsomal incubation system: glucose-6-phosphate, 5 mM; NADP, 0.3 mM; and 0.5 units of D-glucose-6-phosphate dehydrogenase. In some experiments the NADPH system was replaced by 0.5 mM sodium ascorbate. Control systems without the NADPH system or the sodium ascorbate were included. Nifedipine dissolved in methanol (0.25 ml) was added to the incubations in different concentrations (47 μM to 6 mM). The same volume of methanol alone was added to incubation mixtures that did not contain the drug. Zero-time blanks containing all the components of the incubation system were carried out to compensate for any interference by nifedipine. The total incubation volume was 2.15 ml, and all additions are expressed as final concentrations in the

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incubation system. The incubations were carried out at 37° in glass test tubes in an Eberbach water bath shaker under aerobic conditions. Modifications to this basic incubation mixture are noted in the figures.

Assay for lipid peroxidation. Malonaldehyde formation was measured using the 2-thiobarbituric acid method [18]. After 30-min incubations, reactions were stopped by adding 35% trichloroacetic acid (1 ml), and then 1.5% thiobarbituric acid (1 ml) was added. The mixture was heated in a boiling water bath for 15 min. The mixture was allowed to cool after adding 70% trichloroacetic acid (1 ml) and then chloroform (2 ml) was added. The organic and aqueous phases were separated by centrifugation and the optical density of the filtered aqueous layer was measured at 532 nm. Microsomal lipid peroxidation is expressed as nanomoles of malonaldehyde per milligram of protein. Malonaldehyde values were calculated using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ at 532 nm.

Oxygen consumption. This was measured using a Clark type oxygen electrode (Yellow Springs Instrument Co.) at 37°. The incubation conditions were the same as for the induction of lipid peroxidation, and ascorbate or the NADPH-generating system was included. Oxygen uptake was measured in the incubations for both heart and liver microsomal preparations in the presence and absence of different nifedipine concentrations (47 μM to 6 mM). The rate of O_2 consumption was measured from the initial linear part of the curve, and all values were the means of three determinations.

Inhibitors. Desferrioxamine, superoxide dismutase, thiourea, 1,3-dimethylurea and mannitol were dissolved in potassium phosphate buffer (15 mM, pH 7.4) and added to the incubation system which included rat liver microsomes (1.5 mg protein/ml), an ADP–iron complex and an NADPH-generating system. For some experiments ascorbate (0.5 mM) was substituted for the NADPH system. Incubation time was 30 min at 37°. Other incubation conditions and concentrations were as described in the legend for Fig. 2. The experiments with these inhibitors were also repeated for incubation systems containing cardiac microsomes. Ascorbate (0.5 mM) was used to stimulate lipid peroxidation. The incubation conditions were as described in the legend of Fig. 3.

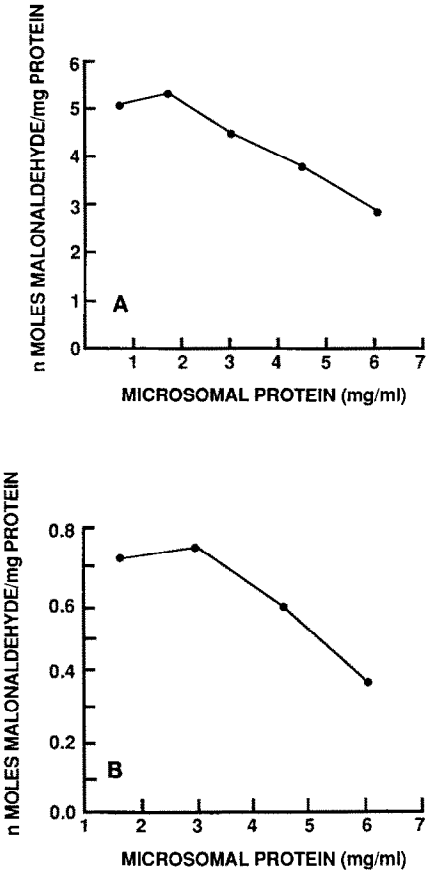

Fig. 1. Effects of varying the microsomal protein concentration of liver microsomes (A) and heart microsomes (B) on lipid peroxidation. In liver microsomes, lipid peroxidation was induced by an NADPH-generating system (glucose-6-phosphate, 5 mM; NADP, 0.3 mM; and 0.5 units of D-glucose-6-phosphate dehydrogenase) in the presence of 0.012 mM $FeCl_3$ and 0.4 mM (ADP–iron complex) in potassium phosphate buffer (15 mM, pH 7.4). The mixture containing the microsomes was incubated for 30 min at 37° in a final volume of 2.15 ml. For heart microsomes, incubation conditions were the same as above, except that ascorbate (0.5 mM) was used instead of the NADPH system. Lipid peroxidation was measured using the 2-thiobarbituric acid assay. The values at each point are the means of three determinations with a single batch of microsomes. The individual values did not differ by more than 5% from the mean value.

RESULTS

Effect of microsomal protein concentration on lipid peroxidation. Under the conditions described, lipid peroxidation was optimal at a microsomal protein concentration of 1.5 mg protein/ml for liver microsomes (Fig. 1A) and 3 mg protein/ml for heart microsomes (Fig. 1B). In both cases, when protein concentration was increased beyond the optimum, the rate of lipid peroxide formation per mg protein decreased.

Effect of nifedipine on NADPH- or ascorbate-induced peroxidation in liver microsomes. The NADPH-generating system induced higher levels of lipid peroxidation in rat liver microsomes (Fig. 2A) as compared to 0.5 mM ascorbate (Fig. 2B). Both the above mentioned systems were inhibited by a wide range of nifedipine concentrations (47 μM to 6 mM) as shown in Fig. 2A and Fig. 2B. After a 30-min incubation period, the lowest concentration of nifedipine tested (47 μM) inhibited NADPH-dependent lipid peroxidation by 12% and ascorbate driven peroxidation by 29%, respectively, relative to their corresponding controls. The highest concentration of nifedipine used (6 mM) inhibited NADPH-dependent peroxidation by 93% and ascorbate-induced lipid peroxidation by 89% after a 30-min incubation period for each. Similar results were observed with nifedipine when these systems were incubated for 15 and 45 min (Fig. 2A and B).

Effect of nifedipine on ascorbate driven lipid peroxidation in cardiac microsomes. Ascorbate

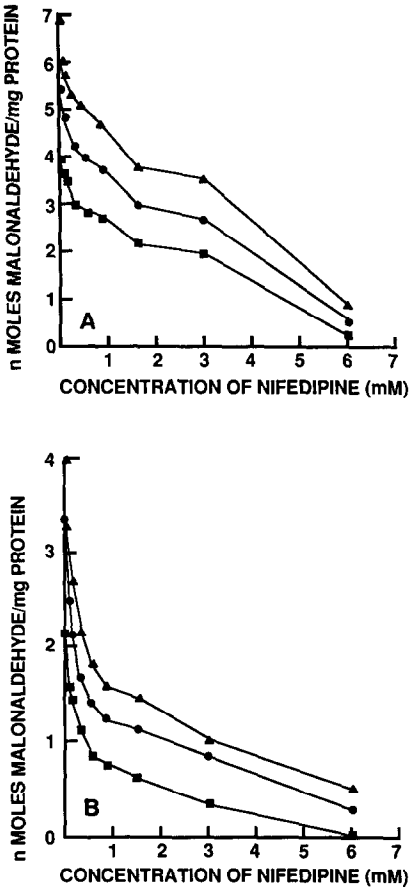

Fig. 2. Effects of varying the concentration of nifedipine on microsoma lipid peroxidation. Hepatic microsomes (1.5 mg protein/ml) were incubated with the NADPH-generating system (A) and an ADP–iron complex in the presence and absence of different concentrations of nifedipine (47 μM to 6 mM). Microsomes were incubated for 15 min (■), 30 min (●) and 45 min (▲) at 37°. All other incubation conditions were as described in the legend for Fig. 1A. The incubations were repeated, using the same incubation conditions as described above except that 0.5 mM ascorbate (B) replaced the NADPH-generating system. The values at each point are the means of three determinations with a single batch of microsomes. The individual values did not differ by more than 5% from the mean value.

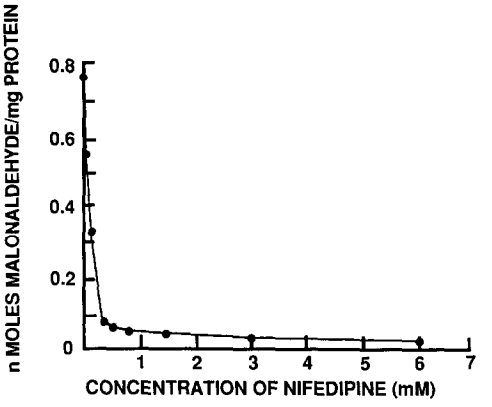

Fig. 3. Influence of varying the concentration of nifedipine on lipid peroxidation in heart microsomes. Lipid peroxidation was induced by 0.5 mM ascorbate in the presence of an ADP–iron complex. Cardiac microsomes (3 mg protein/ml) were incubated for 30 min at 37°, and all other incubation conditions were as described in the legend for Fig. 1B. The values at each point are the means of three determinations with a single batch of microsomes. The individual values did not differ by more than 5% from the mean value.

(0.5 mM) was more efficient than the NADPH-generating system for inducing lipid peroxidation in incubation systems containing cardiac microsomes (3 mg protein/ml). This is in contrast to the cofactor requirements seen for the incubations using liver microsomes (1.5 mg protein/ml) where the NADPH-generating system was more effective in inducing peroxidation. Doubling the concentration of the NADPH-generating system did not result in significantly higher peroxidation levels. We are unable to explain these results. It is usually presumed that cardiac and hepatic microsomes are peroxidized by the same mechanism. It is conceivable that the cardiac and liver microsomal preparations may contain different amounts or even different types of redox enzyme systems. It is also possible that microsomal membranes in cardiac and hepatic microsomes may have different amounts of the components responsible for initiating and/or sustaining lipid peroxidation. That is, the cardiac microsomes may contain lesser amounts of the NADPH-oxidizing enzyme than hepatic microsomes, but more of the factor responsible for iron catalyzed, ascorbate driven peroxidation. Different concentrations of nifedipine (47 μM to 6 mM) inhibited ascorbate driven lipid peroxidation in cardiac microsomes in a concentration-dependent manner (Fig. 3). After a 30-min incubation period, the lowest concentration of nifedipine tested (47 μM) inhibited ascorbate driven lipid peroxidation by 25%, and an 85 μM concentration of nifedipine inhibited lipid peroxidation by 50%. The highest concentration of nifedipine tested (6 mM) virtually abolished lipid peroxidation (>95% inhibition).

Effect of nifedipine on oxygen consumption of liver microsomes. Oxygen uptake increased with increase in the formation of lipid peroxide as determined by malonaldehyde formation, in the presence and absence of different concentrations of nifedipine. As previously reported [23–25], a relatively large amount of oxygen was consumed for each molecule of malonaldehyde formed. This stoichiometry was observed both in the presence and absence of various nifedipine concentrations. Oxygen uptake decreased with increasing concentrations of nifedipine in incubation systems containing liver microsomes and NADPH-generating system (Fig. 4A) or ascorbate (Fig. 4B) and also for incubations containing cardiac microsomes and ascorbate (Fig. 5).

Effects of inhibitors. Microsomal lipid peroxidation is known to be a free radical initiated and sustained event in which non-heme iron plays an important role [26, 27]. Desferrioxamine, an iron chelator and possibly a radical scavenger [28], completely abolished NADPH-induced peroxidation in liver microsomes and partially inhibited ascorbate driven peroxidation, both in the presence and

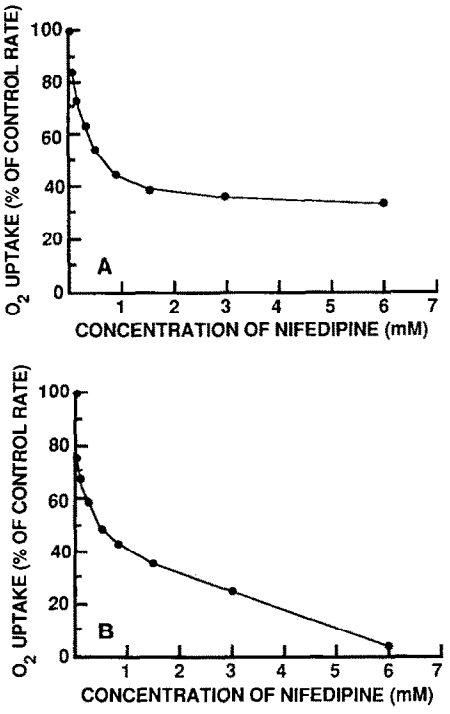

Fig. 4. Inhibition of O_2 consumption of liver microsomes by different concentrations of nifedipine. Oxygen uptake was measured at 37°, in an incubation system containing liver microsomes (1.5 mg protein/ml), ADP–iron complex, an NADPH-generating system (A) or ascorbate (B) in the presence and absence of a range of nifedipine concentrations (47 μM to 6 mM). Other incubation conditions were as described in the legend of Fig. 2. Control values for oxygen consumption were 29.7 and 18.6 nmol per min per mg protein for the NADPH and ascorbate driven systems respectively.

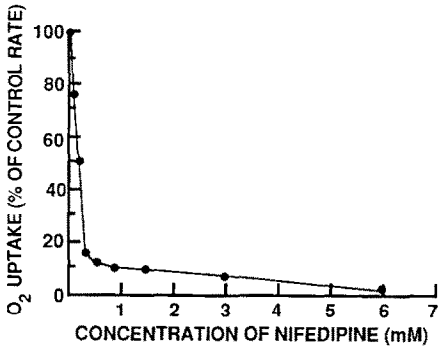

Fig. 5. Inhibition of O_2 consumption of heart microsomes by different nifedipine concentrations. Oxygen consumption was measured at 37° in incubation systems containing cardiac microsomes (3 mg protein/ml), ascorbate and an ADP–iron complex in the presence and absence of a range of nifedipine concentrations (47 μM to 6 mM). All other incubation conditions were as described in the legend of Fig. 3. The control value for oxygen consumption was 11.1 nmol per min per mg protein.

absence of nifedipine (Table 1). Desferrioxamine also led to a marked inhibition of ascorbate-induced lipid peroxidation in incubations containing cardiac microsomes (Table 2).

The superoxide anion is produced in different redox systems and may be a source of free radicals for initiating lipid peroxidation [29, 30]. Superoxide dismutase at a concentration of 3×10^{-5} M failed to inhibit lipid peroxidation in these incubation systems (Tables 1 and 2). It is possible that the superoxide dismutase may not have access to the site of formation of the superoxide anion in the intact microsomal membranes. This is in agreement with the

Table 1. Effect of different inhibitors on rat liver microsomal lipid peroxidation in the presence and absence of nifedipine*

	% Inhibition†			
	Ascorbate system		NADPH system	
Inhibitor (conc)	Control	Nifedipine (1.5 mM)	Control	Nifedipine (1.5 mM)
Desferrioxamine (10^{-3} M)	70 ± 1	24 ± 3	100 ± 0	100 ± 0
Thiourea (2×10^{-2} M)	44 ± 6	21 ± 6	21 ± 2	13 ± 4
1,3-Dimethylurea (10^{-2} M)	47 ± 3	40 ± 5	58 ± 4	60 ± 9
Mannitol (10^{-2} M)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Superoxide dismutase‡ (3×10^{-5} M)	0 ± 0	0 ± 0	0 ± 0	0 ± 0

* Control lipid peroxidation values were 3.3 ± 0.6 and 5.5 ± 1 nmol malonaldehyde/mg protein for the ascorbate and the NADPH-generating systems respectively. In the presence of nifedipine (1.5 mM), lipid peroxidation values were 1.1 ± 0.3 and 3.1 ± 0.9 nmol malonaldehyde/mg protein for the ascorbate and NADPH-generating systems respectively.

† Values are means ± SD, N = 3.

‡ Boiled superoxide dismutase (3×10^{-5} M) and bovine serum albumin equivalent to the protein concentration of 3×10^{-5} M superoxide dismutase were also tested as controls. Both failed to inhibit lipid peroxidation.

Table 2. Effect of different inhibitors on rat heart microsomal lipid peroxidation in the presence and absence of nifedipine*

Inhibitor (conc)	% Inhibition†	
	Control	Nifedipine (85 μM)
Desferrioxamine (10^{-3} M)	52 ± 3	19 ± 2
Thiourea (2×10^{-2} M)	41 ± 4	16 ± 3
1,3-Dimethylurea (10^{-2} M)	48 ± 3	30 ± 4
Mannitol (10^{-2} M)	0 ± 0	0 ± 0
Superoxide dismutase‡ (3×10^{-5} M)	0 ± 0	0 ± 0

* Control lipid peroxidation values were 0.76 ± 0.08 and 0.33 ± 0.06 nmol malonaldehyde/mg protein for incubations in the absence and presence of nifedipine respectively. Ascorbate (0.5 mM) was used to stimulate lipid peroxidation.

† Values are means ± SD, N = 3.

‡ Boiled superoxide dismutase (3×10^{-5} M) and bovine serum albumin equivalent to the protein concentration of 3×10^{-5} M superoxide dismutase were also tested as controls. Both failed to inhibit lipid peroxidation.

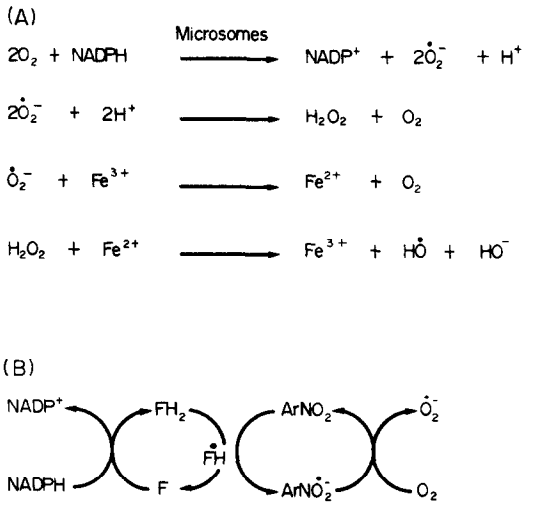

Fig. 6. Scheme for the formation of free radical intermediates in redox systems (top) and the proposed futile metabolic cycle for nitroaromatic compounds (bottom) where the symbol F denotes the oxidized flavoprotein and the $ArNO_2$ symbol denotes the nitroaromatic compound.

results obtained with superoxide dismutase and lipid peroxidation in an earlier study [22]. It must be mentioned, however, that superoxide dismutase would not be expected to inhibit lipid peroxidation if the interaction of hydrogen peroxide with iron is the actual initiator of lipid peroxidation. Superoxide dismutase might facilitate increased formation of hydrogen peroxide [8] which may be followed by the iron catalyzed Haber–Weiss reaction, resulting in the formation of hydroxyl radicals [19]. In such a situation one may even expect superoxide dismutase to stimulate lipid peroxidation. Under our experimental conditions, however, superoxide dismutase had no effect on lipid peroxidation.

Scavengers of the hydroxyl radical (OH·) have often been studied as inhibitors of peroxidation. Three different hydroxyl radical scavengers were included in the incubation systems, and their relative effectiveness as inhibitors of lipid peroxidation was noted. Mannitol, the classical hydroxyl radical scavenger, was an ineffective inhibitor at a concentration of 10^{-2} M in all the systems tested (Tables 1 and 2). The other two hydroxyl radical scavengers, thiourea and 1,3-dimethylurea, were effective inhibitors in systems containing hepatic microsomes (Table 1) and those containing cardiac microsomes (Table 2). These differences in effectiveness of known OH· scavengers cannot be explained solely on their abilities to react with OH· radicals. Perhaps the accessibility of these compounds to specific sites in the microsomal membranes may also play a role in their overall effectiveness as inhibitors of the lipid peroxidation process.

DISCUSSION

The formation of carbon-centered free radicals is an important step in the peroxidation of unsaturated lipids. The reaction of oxygen with these carbon-centered radicals ultimately leads to a series of events that cause peroxidative damage to unsaturated lipids in microsomes. Nitroaromatic compounds can interact with microsomes and an electron source to yield nitroradical anions. These radicals can subsequently react with oxygen to yield superoxide anions which can decompose further to yield hydroxyl free radicals [31]. Redox reactions occurring in microsomal systems involve free radical intermediates (Fig. 6, top). As mentioned earlier, nifedipine, by analogy with other nitroaromatic compounds, can be expected to interact with NADPH and microsomes to generate free radicals that may alter the rate and extent of lipid peroxidation. The results of our experiments clearly showed that nifedipine inhibited both enzymatic (NADPH dependent) and non-enzymatic (ascorbate induced) microsomal lipid peroxidation. It is possible that nifedipine may be diverting electrons away from the path leading to lipid peroxidation in microsomes.

It is known that nitro compounds can form oxygen reactive free radical intermediates [32, 33]. Mason and Holtzman [34] have reported enhanced oxygen consumption in studies with microsomal systems containing certain nitro compounds such as *p*-nitrobenzoate and nitrofurantoin. The scheme (Fig. 6, bottom) depicts the futile metabolic cycle [35] for the apparent oxygen inhibition of nitroreductase activity in microsomes. Under aerobic conditions, the reduction of nitro compounds does not usually proceed beyond the nitro radical anion stage, and the nitro compound is regenerated with concomitant formation of superoxide radicals. While it is true that many nitro compounds increase the rate of oxygen

consumption by microsomal systems containing an appropriate electron source, we have observed the opposite effect for nifedipine (Figs. 4 and 5). Biaglow *et al.* [33] have shown that certain nitrobenzene derivatives, with oxidation–reduction potentials more positive than −0.35 V, stimulated cellular oxygen consumption. The redox characteristics of nifedipine (which contains a nitrobenzene structure linked to an oxidizable dihydropyridine moiety) may be considerably different from the other nitro compounds tested earlier [34], and this may account for its unexpected behavior in our microsomal oxygen consumption studies.

We are currently investigating the effects of verapamil, a calcium channel blocker which is not a nitro compound, on microsomal lipid peroxidation. We are comparing different calcium channel antagonists with known inhibitors of daunomycin-stimulated lipid peroxidation *in vitro*. The results will be reported in the near future.

Free radical formation and consequently lipid peroxidation may lead to membrane alterations and tissue damage [36–40]. We intend to study the effect of nifedipine on heart and liver tissue before and after the ischemia/reperfusion episode *in vivo*.

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