Nitroaryl-1,4-dihydropyridines as antioxidants against rat liver microsomes oxidation induced by iron/ascorbate, Nitrofurantoin and naphthalene

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Abstract

1,4-Dihydropyridines (DHPs) used in the treatment of cardiovascular diseases, are calcium channel antagonists and also antioxidant agents. These drugs are metabolized through cytochrome P_{450} oxidative system, majority localized in the hepatic endoplasmic reticulum. Several lipophilic drugs generate oxidative stress to be metabolized by this cellular system. Thus, DHP antioxidant properties may prevent the oxidative stress associated with hepatic biotransformation of drugs.

In this work, we tested the antioxidant capacity of several synthetic nitro-phenyl-DHPs. These compounds (I–IV) inhibited the microsomal lipid peroxidation, UDPGT oxidative activation and microsomal thiols oxidation; all phenomena induced by Fe^{3+}/ascorbate, a generator system of oxygen free radicals. As the same manner, these compounds inhibited the oxygen consumption induced by Cu^{2+}/ascorbate in the absence of microsomes. Furthermore, compound III (2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-ethyl-dicarboxylate) and compound V (*N*-ethyl-2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-methyl-dicarboxylate) inhibited the microsomal lipid peroxidation induced by Nitrofurantoin and naphthalene in the presence of NADPH. Oxidative stress induced on endoplasmic reticulum may alter the biotransformation of drugs, so, modifying their plasmatic concentrations and therapeutic effects. When drugs which are activated by biotransformation are administered together with antioxidant drugs, such as DHPs, oxidative stress induced in situ may be prevented.

Keywords: Aryl-1,4-dihydropyridines-antioxidant agents; Calcium channel antagonist-oxidative stress; Aryl-1,4-dihydropyridines-ROS

1. Introduction

The nitro-phenyl-1,4-dihydropyridines (DHPs), calcium channel antagonists drugs, are plentifully used in cardiovascular diseases such as ischemia-reperfusion injury, inflammatory vascular disease, arteriosclerosis, and central nervous system (CNS) trauma and stroke. Besides, nitrophenyl-DHPs develop antioxidant protective effects on different biological system: cardiac and liver membranes, brain slices, plasma low density lipoprotein (LDL) (Janero and Burghardt, 1988; Engineer and Sridhar, 1989; Usberti et al., 1991; Díaz-Araya et al., 1998; Napoli et al., 1999; Sevanian et al., 2000; Letelier et al., 2004). Moreover, Nifedipine [4-(2-nitrophenyl)-2,6-dimethyl-3,5-dimethoxicarbonil-1,4-dihydropyridine], a commercial drug, showed

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a considerably lower antioxidant potency than its nitroso-derivative (2,6-dimethyl-4-(2-nitrosophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester) as measured by the inhibition of phosphatidyl choline liposomes peroxidation (Ondrias et al., 1989; Misik et al., 1991). These authors postulated that, a stable nitroxide radical formed by covalent binding to the lipid membrane during the oxidative modification of the liposomes, might explain the DHP antioxidant capacity observed. Furthermore, *N*-ethyl-substituted DHPs showed low reactivity toward alkylperoxyl radicals and ABTS radical cation (ammonium 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonate)) as compared with Trolox (6-hydroxy-2,5,7,8-tetramethylcroman-2-carboxilic acid) or Nisoldipine (López-Alarcón et al., 2003). In all cases, the respective pyridine derivative was detected as the main product of the reaction. Using deuterium kinetic isotope, these authors proved that the hydrogen of the 1-position of the dihydropyridine ring was involved in the proposed antioxidant mechanism. It is necessary to note that when an antioxidant acts by scavenging, it may be advantageous if the derivative radicals can be ''recycled'' to the antioxidant by i.e. ascorbate or GSH. Although the authors propose that DHP may act as hydrogen donator, the final product of this reaction is the stable derivative pyridine, without antioxidant properties.

DHPs, lipophilic drugs, suffers first step oxidative metabolism through cytochrome P_{450} system, which is localized majority in the hepatic endoplasmic reticulum (Drocourt et al., 2001). For example, Nitrofurantoin, an antibiotic which eliminates bacteria that cause urinary tract infections, suffers nitro-reduction generating a nitro anion radical ($NO_2^{\cdot-}$) intermediate; this reaction may be catalyzed by reductases, among them, cytochrome P_{450} reductase. Then, $NO_2^{\cdot-}$ suffers redox-recycling with O_2 generating oxygen free radicals. Thus, when microsomes are incubated with Nitrofurantoin in the presence of NADPH, lipid peroxidation occurs (Letelier et al., 2004). Moreover, UDP-glucuronyltransferase (UDPGT) enzyme which is also localized in the hepatic endoplasmic reticulum, catalyze the conjugation of the hydroxylated metabolites of cytochrome P_{450} system; ROS provoke UDPGT oxidative activation, phenomenon which is totally prevented and reversed by reducing agents such as GSH and DTT (Letelier et al., 2005). These changes in the biotransformation enzymes activities may modify the plasmatic concentration of drugs, so altering their pharmacological effects.

On the other hand, aromatic hydrocarbons, among them naphthalene, are recognized toxic compounds. Naphthalene is oxidized through cytochrome P_{450} system to an epoxide intermediate. This compound reacts with nucleophilic sites on proteins and DNA, but it also may form a conjugate with glutathione (a major route of deactivation and excretion), to be isomerised to yield naphthol, or to be converted into dihydrodiol by epoxide hydrolase. All of the above possibilities are essentially in competition with each other and the nature of the observed metabolites is a function of this competition (Quick and Shuler, 1999). The dihydrodiol metabolite is oxidized to naphthoquinone; reactive oxygen species (ROS) may be generated inducing oxidative stress through this metabolic pathway (Zheng et al., 1997).

It is necessary to note that the DHPs lipophilicity, principal characteristic of cytochrome P_{450} system substrates, also play a role in their calcium channel antagonist's properties (Stengel et al., 1998). Thus, the structure as well as the physical properties of DHPs may influence their concentration in the membrane matrix, developing differential calcium channel antagonist's potency and antioxidant properties.

These data show that hepatic endoplasmic reticulum may be an important target, where DHPs could display their antioxidant properties. Thus, in this work rat liver microsomes put under oxidative stress were used to evaluate the antioxidant capacity of various synthetic nitro-phenyl-DHPs (compounds I–V). Oxygen free radicals were generated through Fe^{3+}/ascorbate and Cu^{2+}/ascorbate systems; iron and copper are transition metals, which generate oxygen free radicals via Haber Weiss and/or Fenton reaction (Halliwell and Gutteridge, 1990; Neuzil et al., 1993; Satoh et al., 1993). Our results showed that the incubation of rat liver microsomes (experimental cellular fraction enriched in endoplasmic reticulum) with the nitro-phenyl-DHPs (compounds I–IV) inhibited the microsomal lipid peroxidation, the UDP-glucuronyltransferase (UDPGT) oxidative activation and the microsomal thiol oxidation, all phenomena induced by Fe^{3+}/ascorbate. As the same manner, these DHPs inhibited the oxygen consumption induced by Cu^{2+}/ascorbate in the absence of microsomes. Furthermore, the compound III (2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-ethyl-dicarboxylate) and the compound V (*N*-ethyl-2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-methyl-dicarboxylate) inhibited the microsomal lipid peroxidation and microsomal thiol oxidation, induced by Nitrofurantoin nitro-reduction and naphthalene oxidation. Nevertheless, the antioxidant effects of compound V, nitro-phenyl-DHP in which, the hydrogen of the 1-position of the dihydropyridine ring was replaced by ethyl group, were significantly lower than those of compound III. Our results show that DHPs may prevent the oxidative stress induced by the biotransformation of some drugs, such as Nitrofurantoin, an antibiotic used in urinary tract infections. Because the oxidative stress activates the UDPGT and may inhibited the cytochrome P_{450} system activity, in this condition the pharmacokinetic of drugs as well as the pharmacodynamic of them may be altered. We discussed the relation between the DHPs structure and their antioxidant properties and their effects on the pharmacotherapy.

2. Material and methods

2.1. Chemicals

Ascorbic acid, dimethylsulphoxide (DMSO), thiobarbituric acid (TBA); potassium dihydrogen phosphate

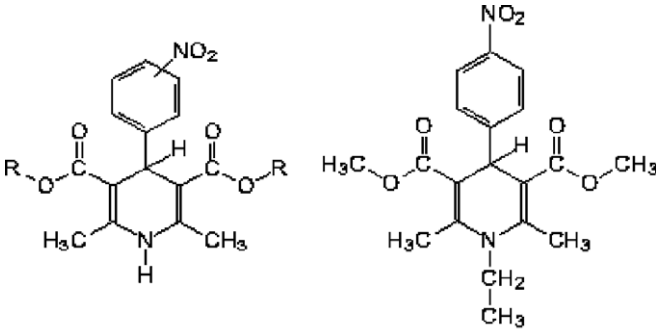
(KH_2PO_4), copper sulphate ($CuSO_4 \times 5H_2O$), $FeCl_3$, and trichloroacetic acid (TCA) were obtained from Merck Chile. Tris-(hydroxymethyl)-amino methane (TRIS), 5,5′-dithio-bis(2-nitrobenzoic) acid (Ellman's reagent, DTNB); *p*-nitrophenol (PNP), UDP-glucuronic acid (UDPGA) (ammonium salt), bovine albumin fraction IV, Folin–Ciocalteau-phenol, Glucose-6-phosphate, NADP and Glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

All the 1,4-dihydropyridines derivatives were synthesized in the Laboratory of Bioelectrochemistry from the Pharmaceutical Sciences School, University of Chile:

- 2,6-Dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridin-3,5-ethyl dicarboxylate (I).
- 2,6-Dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridin-3,5-(2-isopropyl)-dicarboxylate (II).
- 2,6-Dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-ethyl-dicarboxylate (III).
- 2,6-Dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-(2-isopropyl)-dicarboxylate (IV).
- *N*-ethyl-2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-methyl-dicarboxylate (V).

Dimethyl-sulphoxide (DMSO) was used to dissolve all these drugs; the final concentration of DMSO used in the experimental mixture was not higher than 1% v/v, concentration that did not affect the control values of any performed experiments.

All the other chemicals were of analytical grade.



Compounds I–IV
R: CH_3CH_2- ; $(CH_3)_2CH$-

Compound V

2.2. Animals

Adult male Sprague Dawley rats (200–250 g), derived from a stock maintained at the University of Chile, were used. They had free access to pelleted food, maintained with controlled temperature (22 °C) and photoperiod (lights on from 07:00 to 19:00 h). Protocols approved by the Institutional Ethical Committee of the Faculty of Chemical and Pharmaceutical Sciences, University of Chile were performed in all animals' procedures.

2.3. Microsomal preparation

Animals were fasted for 15 h with water ad libitum, and sacrificed by decapitation. Then, four volumes of 25 ml 0.9% w/v NaCl were used to perfuse in situ the livers. At the end of this process, homogenates of the livers excised and devoid of connective and vascular tissue were prepared with five volumes of 0.154 M KCl, with eight strokes in a Dounce Wheaton B homogenizer. Homogenates were centrifuged at 9000*g* for 15 min, and sediments were discarded. Then supernatants were centrifuged at 105,000*g* for 60 min; sediments (microsomes, enriched in endoplasmic reticulum) were stored at −80 °C until use. All homogenization and fractionation procedures were performed at 4 °C using either a Suprafuge 22 Heraeus centrifuge or an XL-90 Beckmann ultracentrifuge. Protein determinations were performed according to Lowry et al. (1951).

2.4. Microsomal lipid peroxidation

2.4.1. Microsomal lipid peroxidation induced by Fe^{3+}/ascorbate

The extent of microsomal lipid peroxidation following Fe^{3+}/ascorbate preincubation was estimated by determining TBARS concentrations, according to Letelier et al. (2004). Microsomes (0.2 mg of protein) suspended in 50 mM phosphate pH 7.4 were or not preincubated with DHPs for 5 min at 37 °C before to add 600 μM $FeCl_3$ and 1 mM sodium ascorbate. Then, mixtures were incubated for 20 min to 37 °C. Blanks did not contain microsomes. At the end of this period, 0.5 ml of 20% TCA was added to all mixtures; later, mixtures were centrifuged at 9000*g* for 10 min at 4 °C in a Suprafuge 22 Heraeus centrifuge. Supernatants Aliquots of 0.5 ml and 0.5 ml of 1% TBA were incubated for 60 min to 50 °C. At the end of this period, mixtures were cooled and lipid peroxidation was estimated by recording TBARS absorbance in a UV3 Unicam UV–VIS spectrophotometer ($\xi_{532\,nm} = 153\,mM^{-1}\,cm^{-1}$). Reaction rates were determined at conditions where product formation were linearly dependent to time and protein concentration. Induced lipid peroxidation was estimated calculating the difference between microsomes incubated without and with 600 μM $FeCl_3$ and 1 mM sodium ascorbate.

2.4.2. Microsomal lipid peroxidation induced by biotransformation of Nitrofurantoin and naphthalene

Microsomes (2 mg of protein) suspended in 50 mM phosphate pH 7.4 were preincubated without or with DHPs for 5 min at 37 °C before to add Nitrofurantoin (10 μM) or naphthalene (100 μM) and NADPH generator system (10 mM glucose-6-phosphate, 1 mM NADP and 5 U of glucose-6-phosphate dehydrogenase) Blanks did not contain microsomes. All mixtures were incubated for 15 min at 37 °C. Then, 0.5 ml of 20% TCA was added and mixtures were centrifuged at 9000*g* for 10 min at 4 °C in a Suprafuge 22 Heraeus centrifuge. Supernatants (0.5 ml) and 0.5 ml of 1% TBA were incubated during

60 min to 50 °C. At the end of this period, lipid peroxidation was recording by TBARS absorbance in a UV3 Unicam UV–VIS spectrophotometer ($\xi_{532\,nm} = 153\ mM^{-1}\ cm^{-1}$). Reaction rates were determined at conditions where product formation were linearly dependent to time and protein concentration.

2.5. Oxygen consumption

Oxygen consumption extent was continuously polarographically determined during 10 min with a Clark electrode No. 5331 (Yellows Springs instrument) in a Gilson 5/6 oxygraph. Reaction mixture contained 50 μM Cu^{2+}/1 mM ascorbate dissolved in 50 mM phosphate pH 7.4.

2.6. UDP-glucuronyltransferase (UDPGT) activity

p-Nitro phenol (PNP) conjugation was studied essentially as described in Letelier et al. (2005). Microsomes (2 mg of protein) suspended in 50 mM phosphate pH 7.4 were preincubated without or with DHPs for 5 min at 37 °C. Then, 600 μM $FeCl_3$ and 1 mM sodium ascorbate were added and mixtures were incubated for 20 min at 37 °C. At the end of this period 0.5 mM PNP; 2 mM UDPGA, 100 mM Tris HCl, pH 8.5, 4 mM $MgCl_2$ were added and the mixtures were incubated for 15 min at 37 °C. Control samples were performed in absence of UDPGA. Reactions were stopped by adding trichloroacetic acid (5% final concentration); samples were then centrifuged at 10,000*g* for 10 min in a Suprafuge 22 Heraeus centrifuge and NaOH was added to the mixture in order to achieve 0.5 M NaOH final concentration. Remaining PNP was determined at 410 nm in a UV3 Unicam UV–VIS spectrophotometer, using control samples of known PNP initial concentration as standard. Reaction rates were determined at conditions where product formation were linearly dependent to time and protein concentration.

2.7. Microsomal thiol content

Microsomal thiols were titrated with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) as described by Letelier et al. (2004). Thiol concentration was estimated by the extinction coefficient of 5-thio-2-nitrobenzoic-acid ($\xi_{410} = 13{,}600\ M^{-1}\ cm^{-1}$).

2.7.1. Microsomal thiol oxidation induced by Fe^{3+}/ascorbate

Microsomes (1 mg of protein) suspended in 50 mM phosphate pH 7.4 were or not incubated with DHPs for 5 min at 37 °C before to add Fe^{3+}/ascorbate at 37 °C for 20 min. Blanks did not contain microsomes. Then 0.6 mM DTNB was added to all the mixtures, and they were incubated for 60 min at 37 °C. At the end of this period, protein was separated by centrifugation to 9000*g* for 10 min at 4 °C. The absorbance of sample supernatants was recorded at 410 nm in a UV3 Unicam UV–VIS spectrophotometer. The microsomal thiol concentration was calculated using the $\xi_{410} = 13{,}600\ M^{-1}\ cm^{-1}$. Reaction rates were determined at conditions where product formation were linearly dependent to time and protein concentration.

2.7.2. Microsomal thiol oxidation induced by biotransformation of Nitrofurantoin

Microsomes (1 mg of protein) suspended in 50 mM phosphate pH 7.4 were or not incubated with DHPs for 5 min at 37 °C before to add 10 μM Nitrofurantoin and NADPH generator system. Then, mixtures were incubated at 37 °C for 90 min. Blanks did not contain microsomes. At the end of this period, 0.6 mM DTNB was added to all the mixtures, and they were incubated for 60 min at 37 °C. Protein was separated by centrifugation to 9000*g* for 10 min at 4 °C in a Suprafuge 22 Heraeus centrifuge. The absorbance of samples supernatant was recorded at 412 nm in a UV3 Unicam UV–VIS spectrophotometer. The microsomal thiol concentration was calculated using the $\xi_{410} = 13{,}600\ M^{-1}\ cm^{-1}$.

2.8. Statistical analysis

Data presented in this work correspond to the arithmetical mean of at least four independent experiments ± SD values. Statistical significance (Student's *t*-test) and regression analyses were performed using the Origin 7.0 Software. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Microsomal lipid peroxidation provoked by Fe^{3+}/ascorbate

The effect of nitro-phenyl-DHPs I and II upon microsomal lipid peroxidation induced by Fe^{3+}/ascorbate is shown in Fig. 1A and nitro-phenyl-DHP III and IV, in Fig. 1B. All nitro-phenyl-DHP tested inhibited the microsomal lipid peroxidation in a concentration-dependent manner. However, no significant differences between the anti-lipoperoxidative effects of ethyl and isopropyl *m*-nitro-phenyl-DHP (compounds I and II) as well as between those of ethyl and isopropyl *p*-nitro-phenyl-DHP (compounds III and IV) were observed ($p > 0.05$). The maximum anti-lipoperoxidative effect developed by all DHPs was 70% and it was reached to an approximately 25 μM concentration.

The lipid peroxidation IC_{50} value of nitro-phenyl-DHPs were obtained by plotting log[DHP] vs the inhibition percentages; these results are shown in Table 1. The IC_{50} value represent the DHP concentration which inhibited 50% the microsomal lipid peroxidation induced by Fe^{3+}/ascorbate. No significant differences in the IC_{50} values of *m*-and *p*-nitro-phenyl-DHPs as well as of ethyl and isopropyl-nitro-phenyl-DHPs were observed ($p > 0.05$).

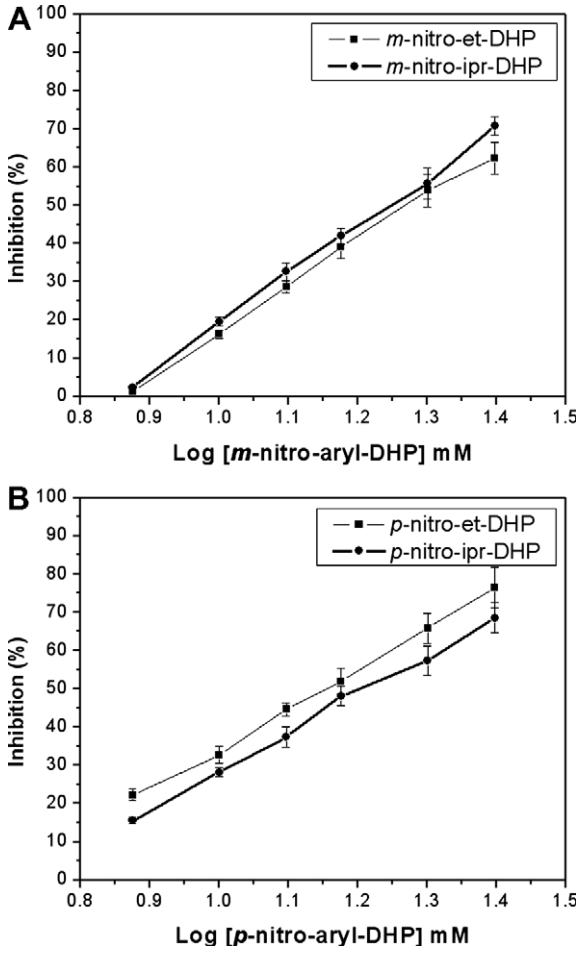


Fig. 1. Effect of compounds I–IV on microsomal lipid peroxidation induced by Fe^{3+}/ascorbate. Inhibition percentages were calculated considering as 100% the value of lipid peroxidation measured in the absence of DHP. Assay conditions are described in Section 2. All values represent the mean of at least four independent experiments ± SD.

3.2. Microsomal lipid peroxidation induced by Nitrofurantoin and naphthalene in the presence of NADPH

The incubation of microsomes with Nitrofurantoin and the generator system of NADPH provoked microsomal lipid peroxidation. The preincubation of microsomes with compound III (2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-ethyl-dicarboxylate) and compound V (*N*-ethyl-2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-methyl-dicarboxylate) for 10 min before adding 10 μM Nitrofurantoin and NADPH generator system, inhibited the microsomal lipid peroxidation 56.6% and 18.4%, respectively (Fig. 2A).

As the same manner, the incubation of microsomes with 10 μM naphthalene and the generator system of NADPH provoked microsomal lipid peroxidation. When microsomes were preincubated for 10 min with compound III (2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-ethyl-dicarboxylate), lipid peroxidation was inhibited 36.3%; however, in the same conditions, compound V (*N*-ethyl-2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-methyl-dicarboxylate) did not inhibited this phenomenon (Fig. 2B).

Table 1
Microsomal lipid peroxidation induced by Fe^{3+}/ascorbate: DHPs IC_{50} values

Compounds	TBARS (nmoles of/min/mg of microsomal protein)	IC_{50} (μM)
Fe^{3+}/ascorbate	1.34 ± 0.08	–
m-Nitro-ethyl-DHP: Compound I	0.69 ± 0.04	18.4*
m-Nitro-isopropyl-DHP: Compound II	0.69 ± 0.05	16.6*
p-Nitro-ethyl-DHP: Compound III	0.68 ± 0.03	15.3*
p-Nitro-isopropyl-DHP: Compound IV	0.72 ± 0.04	15.9*

IC_{50} values represent the DHP concentration which inhibited 50% the microsomal lipid peroxidation induced by Fe^{3+}/ascorbate. Assay conditions are described in Section 2. All values represent the mean of at least four independent experiments ± SD.

* Values not significantly different ($p > 0.05$).

3.3. Oxygen consumption

With the purpose to evaluate the capacity of DHPs to scavenge ROS, we assayed the oxygen consumption induced by Cu^{2+}/ascorbate in the absence of microsomes. In these experiments, we used the polarographic technique described in Section 2. The DHP concentrations used in these experiments corresponded to IC_{50} obtained in the assays of lipid peroxidation induced by Fe^{3+}/ascorbate (Table 1). Fig. 3 shows the effect of compound II (2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridin-3,5-(2-isopropyl)-dicarboxylate) upon the oxygen consumption as an example of the experimental layout; this experimental condition was analogue to those developed for the rest of nitro-phenyl-DHPs tested. Table 2 shows the oxygen consumption slope values in the presence of all DHPs assayed. The oxygen consumption induced by Cu^{2+}/ascorbate (trace a) was inhibited ~50% by all nitro-aryl-DHPs (compounds I–IV) tested (trace b).

3.4. Oxidative activation of UDPGT

The effects of DHP upon the UDPGT oxidative activation induced by Fe^{3+}/ascorbate are shown in Table 3. Preincubation of microsomes with Fe^{3+}/ascorbate increased the microsomal UDPGT activity approximately 10-fold. Interestingly, the IC_{50} concentrations obtained from lipid peroxidation assays of compounds I–IV, inhibited 50% the UDPGT oxidative activation induced by Fe^{3+}/ascorbate.

3.5. Microsomal thiol content reduction induced by Fe^{3+}/ascorbate

The biological activities of several proteins depend of the redox state of their cysteine residues. An example is

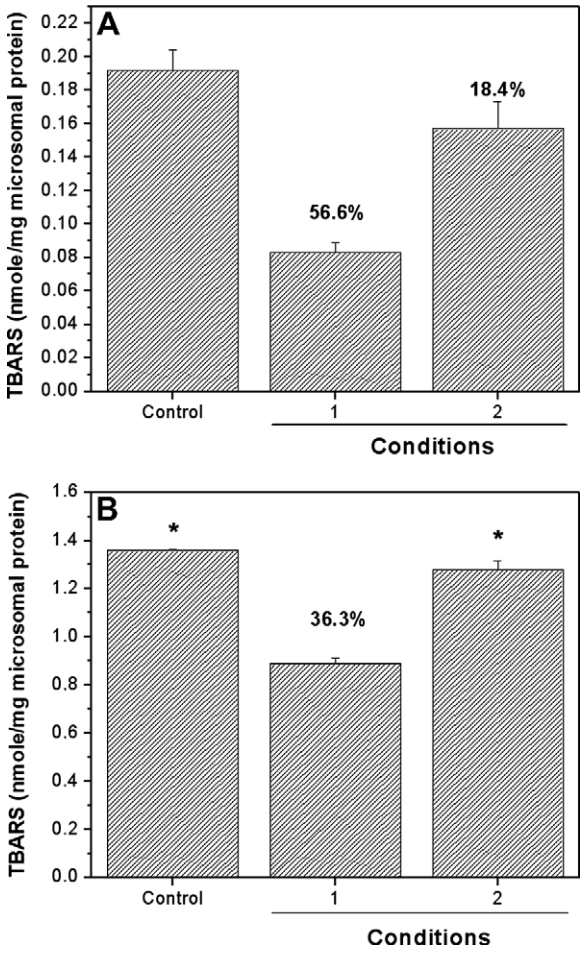


Fig. 2. Effect of compounds III and V on microsomal lipid peroxidation induced by biotransformation of Nitrofurantoin (A) and naphthalene (B) in the presence of NADPH. [Nitrofurantoin]: 10 μM; [Naphthalene]: 100 μM. Control: microsomes in the presence of Nitrofurantoin and NADPH (A) or naphthalene and NADPH (B). Condition 1: microsomes preincubated with 100 μM compound III before inducing microsomal lipid peroxidation. Condition 2: microsomes preincubated with 100 μM compound V before adding Nitrofurantoin or naphthalene and NADPH to induce microsomal lipid peroxidation. Values on the bars represent the microsomal lipid peroxidation's inhibition; they were calculated considering as 100% the TBARS produced in the control. Assay conditions are described in Section 2. (★) Values not significantly different ($p > 0.05$).

UDPGT microsomal enzyme, which is activated by oxidative stress; this oxidative activation is prevented and reversed by thiol reducing agents, so maintaining its basal activity (Letelier et al., 2005). Table 4 shows the effect of nitro-phenyl-DHPs (compounds I–IV) upon the reduction of microsomal thiol content induced by Fe^{3+}/ascorbate. The incubation of microsomes with this oxidative system diminished the microsomal thiol content 45.7%. However, the preincubation of microsomes with nitro-phenyl-DHPs at IC_{50} concentrations obtained from lipid peroxidation assays, decreased the microsomal thiol content only 21%; this value corresponds approximately to the half of the diminishing percentage obtained in the presence of only Fe^{3+}/ascorbate (45.7%).

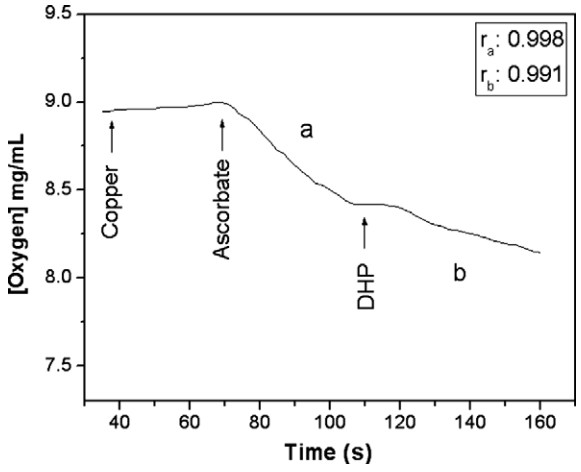


Fig. 3. Polarographical trace of oxygen consumption induced by Cu^{+2}/ascorbate. Effect of Compound II. [Compound II]: 16.6 μM. Assay conditions are described in Section 2. *r*: represent the linear regression of slope *a* and *b*.

Table 2
Effect of DHPs on the oxygen consumption induced by Cu^{2+}/ascorbate

Compounds	$(m \pm SD) \times 10^3$	Inhibition %
Cu^{2+}/ascorbate	15.0 ± 0.72	–
m-*N*-ethyl-DHP · Compound I	7.3 ± 0.36	49.4*
m-Nitro-isopropyl-DHP · Compound II	7.4 ± 0.28	9.3*
p-Nitro-ethyl-DHP · Compound III	7.7 ± 0.32	50.4*
p-Nitro-isopropyl-DHP · Compound IV	7.5 ± 0.22	50.0*

[DHP]: IC_{50} obtained for the microsomal lipid peroxidation induced by Fe^{3+}/ascorbate (Table 1). *m*: slope of the oxygen consumption. To calculate the inhibition %, the slope value of oxygen consumption induced by Cu^{2+}/ascorbate in the absence of DHP was considered as 100%. Assay conditions are described in Section 2. All slope values represent the mean of at least four independent experiments ± SD.

* Values not significantly different ($p > 0.05$).

Table 3
Effect of DHPs on the UDPGT activation induced by Fe^{3+}/ascorbate

Compounds	UDPGT activity	
Control	0.21 ± 0.015	
Fe^{3+}/ascorbate	2.07 ± 0.166	
	Δ Activation	Activation %
Fe^{3+}/ascorbate	1.86 ± 0.165	100
m-Nitro-ethyl-DHP: Compound I	0.94 ± 0.071	50.5*
m-Nitro-isopropyl–DHP: Compound II	0.97 ± 0.061	52.1*
p-Nitro-ethyl-DHP: Compound III	0.95 ± 0.083	51.1*
p-Nitro-isopropyl–DHP: Compound IV	0.93 ± 0.051	50.0*

[DHP]: IC_{50} obtained for the microsomal lipid peroxidation (Table 1). UDPGT activity is expressed as nmoles of *p*-nitrophenol conjugated/min/mg of microsomal protein. Δ Activation values correspond to the difference between the UDPGT activity in the presence and in the absence of Fe^{3+}/ascorbate. Assay conditions are described in Section 2. All UDPGT activities values represent the mean of at least four independent experiments ± SD.

* Values not significantly different ($p > 0.05$).

Table 4
Microsomal thiol in the presence of Fe^{+3}/ascorbate

Conditions	Thiols (nmoles/mg of microsomal protein)	Decreasing %
Control	73.0 ± 0.78	–
Fe^{3+}/ascorbate	39.7 ± 1.21	45.7
	Δ Decrease	
Fe^{3+}/ascorbate	33.3 ± 0.95	100
m-Nitro-ethyl-DHP · Compound I	16.4 ± 1.17	49.2*
m-Nitro-isopropyl-DHP · Compound II	16.1 ± 0.62	48.3*
p-Nitro-ethyl-DHP · Compound III	16.3 ± 1.86	48.9*
p-Nitro-isopropyl-DHP · Compound IV	16.0 ± 0.60	48.0*

Effect of Compounds I–IV. [DHP]: IC_{50}obtained for the microsomal lipid peroxidation (Table 1). Δ Diminution values represent the differences between nmoles of thiol in the absence and presence of Fe^{3+}/ascorbate and DHP. Diminution % were calculated considering control thiol value as 100%. Assay conditions are described in Section 2. All thiol values represent the mean of at least four independent experiments ± SD.
* Values not significantly different ($p > 0.05$).

3.6. Microsomal thiol reduction induced by Nitrofurantoin in the presence of NADPH

These results are shown in Fig. 4. The incubation with Nitrofurantoin (10 μM) and NADPH provoked a reduction of microsomal thiol (30.8%). When microsomes were preincubated with 100 μM compound III (2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-ethyl-dicarboxylate) for 10 min, this decline went only to 15.6%. Nevertheless, in the same conditions, compound V (*N*-ethyl-2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-methyl-dicarboxylate) did not modify the diminishing of microsomal thiol induced by Nitrofurantoin (Fig. 4).

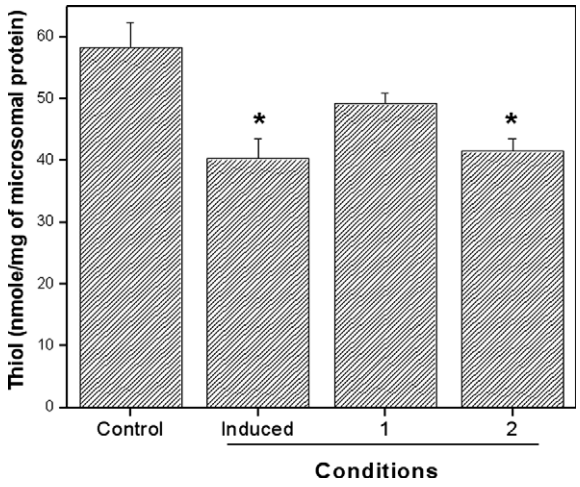


Fig. 4. Effect of compounds III and V on microsomal thiol oxidation induced by biotransformation of Nitrofurantoin in the presence of NADPH. [Nitrofurantoin]: 10 μM. Induced: microsomes not preincubated with DHPs. Condition 1: microsomes preincubated with 100 μM compound III and Condition 2: microsomes preincubated with 100 μM compound V before to induce microsomal thiol oxidation in the presence of Nitrofurantoin and NADPH. Values on the bars represent the inhibition of microsomal thiol oxidation; they were calculated considering as 100% the control thiols. Assay conditions are described in Section 2. (★) Values not significantly different ($p > 0.05$).

4. Discussion

Data reported about the antioxidant properties of commercial nitro-phenyl-1,4-DHPs, have been mainly related with the prevention of lipids and LDL oxidation (Janero and Burghardt, 1988; Engineer and Sridhar, 1989; Díaz-Araya et al., 1998; Sevanian et al., 2000; Letelier et al., 2004). DHPs are lipophilic drugs, characteristic which seem relevant in their therapeutic effects. Since DHPs are xenobiotics, they must be biotransformed to polar compounds to be eliminated through urine. Thus, DHPs suffer metabolism through the cytochrome P_{450} oxidative system, which is the principal pathway through lipophilic compounds are biotransformed (Drocourt et al., 2001). Nevertheless, through this biotransformation pathway several drugs are activated, so generating oxidative stress. The principal localization of cytochrome P_{450} oxidative system is the hepatic endoplasmic reticulum. Thus, this subcellular organelle may be a target where DHPs may develop their antioxidant properties. In this work, we evaluated the antioxidant capacities of several synthetic DHPs using rat liver microsomes (experimental preparation enriched in endoplasmic reticulum) and Fe^{3+}/ascorbate and Cu^{2+}/ascorbate as generator systems of oxygen free radicals. As expected, the synthetic nitro-aryl DHPs tested (compounds I–IV) inhibited the microsomal lipid peroxidation induced by Fe^{3+}/ascorbate (Fig. 1 and Table 1). Nevertheless, the anti-lipoperoxidative effects of all the DHP isomeric forms tested, (*m*- and *p*-NO_2 as also those of ethyl- and isopropyl-DHPs) were not significantly different. Data reported show that the DHP concentration in the lipid matrix of the membranes depends of their lipophilicity (Stengel et al., 1998). As the same manner, the prevention of the membrane lipid peroxidation also seems to depend of the DHP concentration in the lipid matrix (Mason and Trumbore, 1996). Probably, the differences in the lipophilicity of synthetic DHPs tested were not sufficient to provoke a significant variation in their microsomal membrane concentration, which did not allow observing differences in their anti-lipid peroxidant potency.

As expected, Nitrofurantoin and naphthalene in the presence of NADPH induced microsomal lipid peroxidation (Fig. 2). Moreover, the preincubation of microsomes with 2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-ethyl-dicarboxylate (III) diminished this phenomenon and *N*-ethyl-2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridin-3,5-methyl-dicarboxylate (V) had no effect. Several mechanisms are involved in the cellular antioxidant capacity; between them the scavenging of oxygen free radicals and the metal chelating properties of different molecules.

The chemical structures of DHPs are reminiscent of NADH where NAD-linked dehydrogenases donate hydrogen atoms to substrates. One hydrogen is transferred from NADH as a hydride ion (H^-) and another is taken as H^+ from the medium (Lehninger, 1982). Thus, it is tempting to speculate that DHPs can react analogously, transferring one hydrogen atom of 4-position (H^-) to anion superoxide and another of the 1-position (H^+) by way of a cationic radical intermediate to generate pyridine derivatives and water. Because electron withdrawal groups negatively affect the stability of the intermediates, this predicts that nitro groups in the phenyl ring would diminish their reactivity (Ortiz et al., 2003; Carraway et al., 2004). This postulate could explain the differences in the anti-lipoperoxidative potencies between nitro-aryl-DHP and their nitroso-derivatives described (Ondrias et al., 1989; Misik et al., 1991).

On the other hand, López-Alarcón et al. (2003) using deuterium kinetic isotope proved that the hydrogen of the 1-position of the dihydropyridine ring was involved in the proposed antioxidant mechanism; in all cases, the respective pyridine derivative was detected as the main product of the reaction. Nevertheless, compound V, DHP bearing an ethyl group in 1-position inhibited the microsomal lipid peroxidation induced by Nitrofurantoin and NADPH only 18.4% and that induced by naphthalene and NADPH was not affected (Fig. 2). Probably, DHP bearing an ethyl group in 1-position transfer the hydrogen in 4-position of the dihydropyridine ring as a hydride ion (H^-) and H^+ might be taken from the medium. This postulate could explain the lower antioxidant potency of DHP bearing an alkyl group and that bearing hydrogen in 1-position.

It is necessary to note that thiol groups of several proteins are involved in their biological functions. Interestingly, DHPs (compounds I–IV) IC_{50} obtained from microsomal lipid peroxidation assays, decreased to the same extent as the microsomal thiols oxidation provoked by Fe^{3+}/ascorbate (Table 4). In the same manner, compound III inhibited the reduction of microsomal thiol induced by Nitrofurantoin in the presence of NADPH and compound V had not effect (Fig. 4). These results are in agreement with those of microsomal lipid peroxidation, thus confirming that the hydrogen of 1-position plays a role in the DHPs antioxidant properties.

UDPGT, enzyme specially involved in drugs biotransformation is a thiol protein. Recently we proved that Fe^{3+}/ascorbate activate significantly this enzyme and reducing agents such as DTT and GSH prevent and reverse this activation (Letelier et al., 2005). Moreover, several commercial DHP used in the cardiovascular diseases developed similar reducing activity on UDPGT oxidative activation (Letelier et al., 2004). Synthetic DHPs tested (compounds I–IV) prevented the UDPGT oxidative activation induced by Fe^{3+}/ascorbate. Moreover, DHPs IC_{50} concentration obtained from microsomal lipid peroxidation assays also inhibited ~50% the UDPGT oxidative activation, so corroborating the protective effects that nitro-aryl-DHPs develop on the thiol protein oxidation (Table 3).

It is necessary to note that the first step in the metabolism of classical DHP drugs is an oxidative process catalyzed by cytochrome P_{450} system, leading to the corresponding pyridines with complete loss of activity (Drocourt et al., 2001). In the presence of air and daylight, also fast oxidation of the dihydropyridine group to pyridine ring occur (Díaz-Araya et al., 1998). This phenomenon provokes the subsequent loss of the secondary nature of the nitrogen of the 1-position and so, the loss of the hydrogen bound to this nitrogen. Different author have postulated that these chemical changes may alter the DHP calcium channel antagonism (Ondrias et al., 1989, 1994; Hayase et al., 1995; Díaz-Araya et al., 1998). Thus, the resistance of DHPs towards oxidation may explain the prolonged duration of antihypertensive activity as compared to classical DHP drugs of the Nifedipine-type (Kappe, 1998). T-type Ca^{2+} channel currents in the neuroblastoma-glioma cell line NG108-15 were inhibited with decreasing potency in the order Niguldipine > Nicardipine > Nimodipine > Nifedipine (IC_{50}-values 244 nM, 2.5 μM, 9.8 μM, 39 μM) (Stengel et al., 1998). These data contribute to support the idea that the –NH– dihydropyridine ring group could be involved in calcium channel antagonism and antioxidant properties of DHPs; moreover, both phenomena could occur simultaneously, cancelling each other. This postulate has to be confirmed and new studies are in progress.

In the biological point of view, an antioxidant is a drug capable to display their cellular antioxidant effects to low concentrations. The microsomal antioxidant effects were displayed to μM concentrations of DHPs tested, so, DHPs may be considered as biological antioxidants. Because DHPs suffer first step hepatic metabolism, endoplasmic reticulum may represent a target in which they may exert their antioxidant properties. For example, Nitrofurantoin, an antibiotic used in urinary infections, provokes several adverse effects derived from its reductive metabolism that induce oxidative stress. Cardiovascular diseases affect principally third age patients who currently also suffer this type of urinary infections. Thus, the simultaneous administration of DHPs and Nitrofurantoin may reduce the adverse effects of Nitrofurantoin in these patients. However, oxidative stress may also elicit a fast oxidation of the dihydropyridine group to pyridine and this chemical change may alter the DHP calcium channel antagonism (Ondrias et al., 1989; Ondrias et al., 1994; Hayase et al., 1995; Díaz-Araya et al., 1998). The extension of both phenomena will be dependent of the oxidative stress extension provoked by the Nitrofurantoin nitro-reduction. Moreover, several cytochrome P_{450} system metabolites are pharmacologically active and in general, they are UDP-glucuronyltransferase substrates. Glucuronides are hydrosoluble and pharmacological inactive metabolites. Since oxidative stress activates the UDP-glucuronyltransferase, in this

condition, the elimination rate of drugs may be increased, and diminishing the plasmatic concentration of drugs, and decreasing their pharmacological effects. These observations open a wide field in the investigation of hepatic drug biotransformation associated with oxidative stress, and new studies are in process in our laboratory.

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