Scavenging of the One-Electron Reduction Product from Nisoldipine with Relevant Thiols: Electrochemical and EPR Spectroscopic Evidences

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Purpose. To determine the formation of the one-electron reduction product from nisoldipine and its reactivity with relevant thiols in mixed medium.

Methods. Cyclic voltammetry (CV) and electron paramagnetic resonance (EPR) techniques were used to determine the one-electron reduction product corresponding to the nitro radical anion. CV was employed to assess both the rate constants corresponding to the decay of the radicals and its interaction with relevant thiols.

Results. The nisoldipine radical anion follows second order kinetics, with an association rate constant of 283 ± 16 $lmol^{-1} sec^{-1}$. Nitro radical anion from nisoldipine significantly reacted with thiol compounds. This reactivity was significantly higher than the natural decay of the radical in mixed medium. EPR spectra recorded *in situ* using DMF/ 0.1 N NaOH (pH 13) confirmed the formation of the nitro radical anion, giving a well-resolved spectra in 35 lines using 0.1 G modulation.

Conclusions. Electrochemical and EPR data indicated that all the tested thiols scavenged the nitro radical anion from nisoldipine. The following tentative order of reactivity towards the thiols can be proposed: cysteamine ~ glutathione > N-acetylcysteine > captopril > penicillamine.

KEY WORDS: nitro radical anion; nisoldipine; cyclic voltammetry; EPR spectroscopy; thiol; scavenging.

INTRODUCTION

1,4-dihydropyridine derivatives are rapidly and extensively metabolized in rat, dog, and man (1–4). There are also no important species differences regarding the metabolic patterns. Most metabolites have been identified. However, some common biotransformation reactions, such as dehydrogenation of the 1,4-dihydropyridine system, oxidative ester cleavage, oxidative O-demethylation, and subsequent oxidation of the resulting primary alcohol to the carboxylic acid have to be considered (3,4). Another biotransformation route of nisoldipine is the reduction of its nitro group (5), yielding amino metabolites. This pathway accounts for about 1% and 3% of the dose for the studied 1,4-dihydropyridine derivative (5). In spite of these data, the significance of the reduction intermediates of nisoldipine has not yet been established.

The electrochemistry of 4-(nitrophenyl) substituted 1,4-dihydropyridines has been extensively studied in the last few years. Some of these studies have been devoted to the electrooxidation of the dihydropyridine moiety (6–8). Most of them have involved the electroreduction of the nitroaromatic group present in these molecules (9–11). The electrochemical reduction of nitro aryl 1,4-DHP derivatives in aqueous media follows the general pattern of nitroaromatic compounds involving a single 4-electron step producing the hydroxylamine derivative (12–13). On the other hand, the electrochemical reduction of these derivatives is dramatically affected in mixed media (14–17). Thus, the isolation of the 1-electron reduction product, the nitro radical anion, is possible. Finally, the electrochemical reduction of a series of 4-nitrophenyl-substituted 1,4-dihydropyridines in aprotic media that leads to a free radical formation, both of nitro and nitrous nature has been reported (18).

Electrochemical techniques can detect and quantify the interaction between a reduction product and its target (e.g., DNA or its individual bases). Consequently, most works dealing with the interaction of nitrocompounds and their intermediates with DNA bases have been reported (19–22). Particularly, we have studied the feasibility of nitro radical formation from some nitro aryl 1,4-dihydropyridines using cyclic voltammetry (23,24). More recently (25), we have proved the *in vitro* reactivity of the one-electron reduction product from nifedipine with some relevant biological targets, such as, glutathione, cysteamine and the nucleic acid bases, adenine and uracil.

This paper reports a study by both EPR spectroscopy and cyclic voltammetry on the formation of the one-electron reduction product from nisoldipine in mixed media. In addition to its electrochemical and EPR characterization, the reactivity of the radical towards thiol compounds was also assessed. The reactivity was quantitatively determined by cyclic voltammetry through the calculation of the respective interaction rate constants.

MATERIALS AND METHODS

Drug

Nisoldipine

1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid, methyl 2-methyl-propyl ester (Sanitas Laboratories, Santiago, Chile) (Fig. 1). Dimethylformamide (DMF), spectroscopic grade, tetrabutylammonium iodide (TBAI), glutathione, cysteamine, were purchased from Merck. Tetrabutylammonium perchlorate was purchased from Fluka.

N-acetylcysteine, penicillamine and captopril were kindly provided by Chile Laboratories, Santiago, Chile.

Cyclic Voltammetry

Experiments were carried out in an INELECSA assembly PDC 1212, containing generator/potentiostat with an A/D converter interface attached to a 12-bit microprocessor and suitable software for totally automatic control of the experiments and data acquisition. An DTK 486 SX microcomputer was used for data control, acquisition, and treatment.

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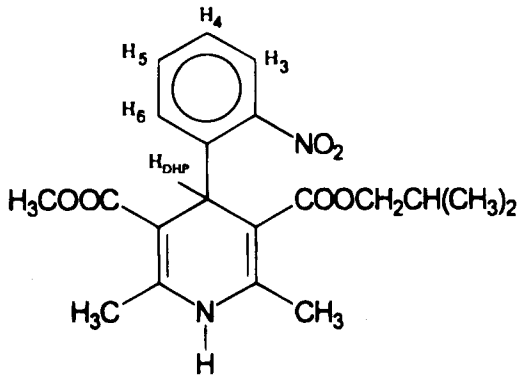

Fig. 1. Chemical structure of nisoldipine.

Electrodes

A Metrohm h.m.d.e. with a drop surface of 1.92 mm^2 was used as the working electrode and a platinum wire as a counter electrode. All potentials were measured against an SCE.

Methods

The experimental anodic peak current (I_{pa})/cathodic peak current (I_{pa}) ratios (I_{pa}/I_{pc}) were calculated according to Nicholson's procedure, using individual cyclic voltammograms (26). Furthermore, the switching potential (E_λ)was selected to reduce the influence of the second cathodic peak. Fifteen runs with E_λ varying between −1195 and −1285 mV *versus* SCE did not show a significative variation in I_{pa}/I_{pc} values (coefficient of variation = 2.2%). Kinetic reaction orders for the nitro radical anion were quantitatively assessed for first and second-order coupled reactions according to previous studies (27,28).

To ensure that changes in the voltammetric parameters (I_{pa}/I_{pc} ratio, E_{pc}, etc.) of the drug by the addition of the thiol compounds were due to reaction between electrochemically generated radical and not due to an electrode adsorption phenomenon, 100 μl of cyclohexanol was added to mixed media (21,22).

To quantitatively estimate the interaction rate constant (k_i) for the reaction between the nisoldipine radical anion generated and the thiols, we used a method previously developed in our laboratory (24,25).

All cyclic voltammograms were carried out at a constant temperature of 25°C and the solutions were purged with pure nitrogen for 10 minutes before the voltammetric runs. The return-to-forward peak current ratio, I_{pa}/I_{pc}, for the reversible first electron transfer (the $Ar\text{-}NO_2/Ar\text{-}NO_2^{\bullet-}$ couple) was measured, varying the scan rate from 0.1 Vs^{-1} up to 5.0 Vs^{-1}.

Mixed Media

To obtain the optimal mixed media conditions, different supporting electrolytes such as KCl, LiCl, tetrabutyl ammonium iodide (TBAI) and tetrabutyl ammonium perchlorate (TBAP) were tested. On the other hand, citrate, camphor and hexamethylphosphotriamide (HMPA) were tested as active-surface substances. The concentrations of these substances varied between 0.01 M and 0.1 M. Percentages of DMF varied from 40% to 80% (v/v). From these experiments, the following optimum composition was selected: 0.015 M aqueous citrate/DMF: 40/60, pH 9.0, 0.1 M TBAI and 0.3 M KCl. For the studies conducted at pH 7.4, the same substances as that of the medium at pH 9.0 was used.

pH in Mixed Media

pH measurements were corrected according to the following expression (29): $pH^* - B = \log U_H^0$ where pH* equals $-\log a_H$ in the mixed solvent, B is the pH meter reading and the term $\log U_H^0$ is the correction factor for the glass electrode, which was calculated for the different mixtures of DMF and aqueous solution, according to a previously reported procedure (30).

Drug Solution

Stock solutions of 10 mM nisoldipine in DMF was prepared and protected from daylight to avoid photodecomposition. A routine drug concentration of 5 mM for all the experiments was used. Fifty mM stock solutions of all the tested thiol compounds in citrate, pH 9.0, were prepared to obtain solutions with concentrations ranging from 0.1 mM to 5 mM.

EPR Measurements

The nitro radical anion from nisoldipine was generated *in situ* by electrochemical reduction at room temperature. A 5 mM solution of nisoldipine containing 0.1 M TBAP in DMF/aqueous 0.1 N NaOH pH 13, 99/1, was degassed with nitrogen for 10 minutes, reduced and immediately its EPR spectrum was recorded in the microwave band X (9.85 GHz) in a Brucker ECS 106 spectrometer, using a rectangular mode cavity with a 50 kHz field modulation. Hyperfine splitting constants were estimated to be accurate within 0.05 G.

For spin-trapping experiments radical generation was similar to that previously described and 100 mM DMPO (Aldrich) was added to the mixture reaction shortly before the addition of 20 mM GSH.

RESULTS

The main goal of this paper was to establish the formation of the nitro radical anion from nisoldipine by both cyclic voltammetry and EPR spectroscopy and its reactivity with thiols of biological relevance.

Cyclic voltammetry of nisoldipine in protic media (ethanol/0.12 mM aqueous citrate, 20/80) revealed that this drug was irreversibly reduced, with no indication of return peaks, irrespective of the sweep rate. Thus, in this medium the reduction occurs via a 4-electron transfer step to give the hydroxylamine derivative (data not shown). Furthermore, the addition of an aprotic solvent to an aqueous citrate buffered solution of nisoldipine at pH > 7.4 enables the observation of two different reduction processes (Fig. 2): (a) a first reversible peak, which corresponds to the one-electron transfer process resulting in the generation of the nitro radical anion, and (b) a second irreversible peak, which corresponds to the formation of the hydroxylamine derivative involving a three-electron transfer process. With these experimental conditions, the reversibility of this first reduction step, as determined by the I_{pa}/I_{pc} ratio in the cyclic voltammogram of the isolated couple, increased with

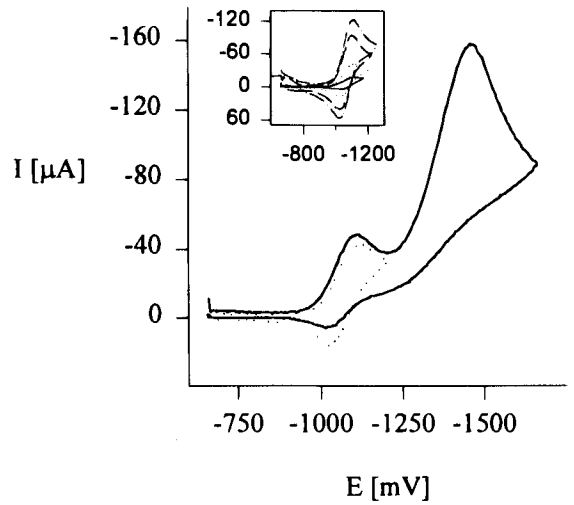

Fig. 2. Cyclic voltammograms (CV) of nisoldipine in mixed media at pH 9.0. Solid line: extended sweep. Dashed line: short sweep. Sweep Rate: 1 Vs^{-1}. Inset: Isolated Ar-NO_2/Ar-$NO_2^{\bullet -}$ couple at different sweep rates.

the addition of DMF, reaching limiting values at approximately 60% DMF.

We have studied the stability of the Ar-$NO_2^{\bullet -}$ species by changing the electrochemical conditions, i.e., the scan rate, the switching potential, while keeping the chemical conditions of the solution constant. Results show that as the scan rate increased, the I_{pa}/I_{pc} ratio increased towards unity, typical behavior for an irreversible chemical reaction following a charge-transfer step, i.e. the *E*lectron *C*hemical (EC) process (31). Furthermore, the cathodic peak potential depends on nisoldipine concentrations and sweep rates, with a $dE_{pc}/d\log c$ and $dE_{pc}/d\log v$ values varying between 20 and 22 mV. These values are in agreement with the theoretical value of 19.5 mV for an EC_i process where the chemical step follows second order kinetics (28). The fact that the current ratio does not reach a value of unity can be ascribed to competition with a small amount of heterogeneous protonation.

Also, to check the order of the following chemical reaction, I_{pa}/I_{pc} ratio dependence on concentration of the nisoldipine was studied. The theory of cyclic voltammetry for a second-order reaction initiated electrochemically has been studied exhaustively by Olmstead (31). In our experiments we have found that an increase in the nisoldipine concentration, keeping both DMF percentage and scan rate constant, resulted in a decreased I_{pa}/I_{pc} value according to the prediction of Olmstead for a second order character of the chemical reaction (31). From the above experimental data it can be concluded that the chemical reaction was of second order, i.e., a disproportionation reaction (dismutation) according to the following equation:

$$2\ \text{Ar-NO}_2^{\bullet -} + 2\text{H}^+ \rightarrow 2\ \text{Ar-NO}_2\text{H}^{\bullet}$$

$$2\ \text{Ar-NO}_2\text{H}^{\bullet} \rightarrow \text{Ar-NO}_2 + \text{Ar-NO} + \text{H}_2\text{O}$$

The influence of pH on the anion radical reaction rates was examined. These results show an increase in the stability of nitroanions formed from nisoldipine with an increase in pH, as illustrated by an increase in the I_{pa}/I_{pc} ratio (Table I). Consequently, the lifetime of the Ar-$NO_2^{\bullet -}$ species was

Table I. The Effect of Glutathione on the I_{pa}/I_{pc} Ratio for the Nisoldipine Ar-NO_2/Ar-$NO_2^{\bullet -}$ Couple as a Function of pH

	pH				
	7.4	9.0	10	11	12
$[Ipa/Ipc]_0$[a]	0.66	0.85	0.89	0.93	0.96
+ GSH[b]	0.60	0.65	0.74	0.88	0.93
% $\Delta I_{pa}/I_{pc}$[c]	−9.1	−23.5	−16.9	−5.4	−3.1
$-E_{pc}$[d],mV	1029	1031	1028	1033	1029

[a] Current ratio in the absence of GSH.
[b] Nisoldipine:glutathione/ 5:1. Sweep rate: 1 Vs^{-1}.
[c] Current ratio changes in the presence of GSH.
[d] Cathodic peak potential corresponding to the reversible couple in the absence of GSH.

increased at more alkaline pH. Studies at pH 7.4 to test the appearance of the signal corresponding to the one-electron reduction process were succesful. However, we selected pH 9.0 for kinetic characterization due to a best resolution of the signals. The natural decay of the Ar-$NO_2^{\bullet -}$ species is by a disproportionation reaction *via* the protonated radical, Ar-$NO_2H^{\bullet}$, therefore it is concluded that in alkali the unprotonated radical is more stable.

On the other hand, we have found pH-independent values of cathodic peak potentials of the reversible couple, proving that no proton transfer precedes the electrode process (Table I). It should be noted that our studies involving pH measurements were properly corrected, according to a previously published methodology (29,30).

Second-order constant for the decay of the Ar-$NO_2^{\bullet -}$ species was assessed from single cyclic voltammograms of nisoldipine, according to Olmstead's procedure (31) from the following relationship:

$$\log \omega = \log (k_2 c_0 \tau)$$

Confirming the second order character of the following chemical reaction, plots of the kinetic parameter, ω, vs. time constant, τ, were linear, with average correlation coefficients not lower than 0.97 for the drug. The absence of protons in the media favored the stability of the radical anion from the nisoldipine. As expected, the second order rate constant decreased and half-life increased as DMF percentages increased. Experimental k_2 value in optimal mixed media conditions was: $k_2 = 283 \pm 16.6\ l\ mol^{-1}\ s^{-1}$.

The addition of glutathione to nisoldipine for obtaining a 5:1 nisoldipine:glutathione ratio decreased the I_{pa}/I_{pc} value as compared with the control (without GSH) which became progressively less as the pH was increased. At higher pH, glutathione addition appeared to have little effect on I_{pa}/I_{pc}. (Table I). No change in potential was observed by CV on glutathione addition. From these results, the influence of pH is therefore apparent in modifying both radical anion stability and the interaction of the reduced drug with glutathione. This same type of behavior was observed with the other tested thiols.

To carry out a systematic study about the reactivity of the radical anion with the different thiols, we have selected a mixed medium at pH 9, taking into account the stabilization of the radical and the above described results.

The effect of additions of thiols on the I_{pa}/I_{pc} ratios demonstrated that parallel with an increase of the concentration of

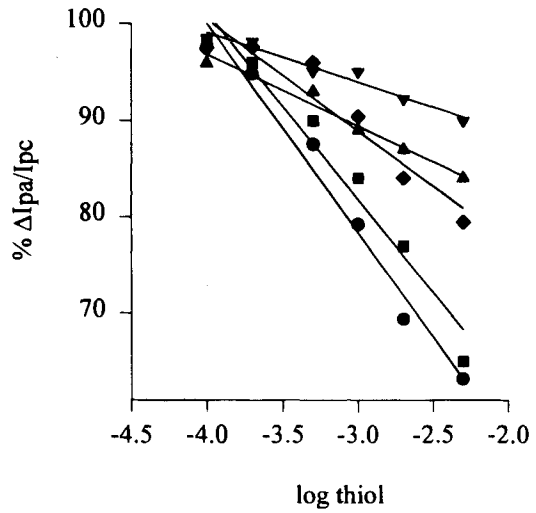

Fig. 3. Dependence of $\%\Delta I_{pa}/I_{pc}$ ratio with the logarithm of concentration of thiols. (●) GSH (■) cysteamine (▲) penicillamine (▼) captopril (◆) N-acetylcysteine. Sweep Rate: 1 Vs^{-1}

such derivatives, a decrease in the current ratio at the different sweep rates was observed for all the tested thiols (Table I). To allow a comparison between the effect produced by the thiol compounds, the I_{pa}/I_{pc} ratios were best expressed as the percentage change, %Δ, from the control value. A plot of %Δ vs. log [thiol] (Fig. 3) gave an approximately straight line relationship for the different thiols. The slopes of these curves indicate the relative sensitivity of the nitro radical anion towards the interaction with the tested thiols. Cysteamine produced a slightly higher effect than glutathione, with a decrease in the current ratio of 28%, in contrast the most weak effect corresponded to penicillamine (7.6%), at nisoldipine: thiol ratio of 5:1.

The interaction rate constants (calculated according to the procedure described in (24)) and their relationships for the reaction between the nitro radical anion from nisoldipine and the thiols at pH 9 are shown in Table II. From this table it can be concluded that in all the cases; the k_i values were higher than that corresponding to the natural decay of the radical (k_2). Consistent with the above-discussed results, cysteamine shows the higher interaction constant as compared with all the other tested thiols, except GSH . Based on the results obtained at pH 9, which were summarized in Table II, the following relative order of reactivity can be established: cysteamine ~ glutathione > N-acetylcysteine > captopril > penicillamine.

Table II. Interaction Rate Constants and Their Relationships for the Reactivity Between the Nitro Radical Anion from Nisoldipine Electrochemically Generated with Thiol of Biological Relevance at pH 9.0

Thiol	$k_i[Ms]^{-1}$	k_i/k_2[a]	k_{iGSH}/k_{ithiol}[b]
Cysteamine	16,900	59.7	0.97
Glutathione	16,454	58.1	1.0
N-acetylcysteine	4,857	17.2	3.4
Captopril	1,759	6.2	9.4
Penicillamine	753	2.7	21.9

[a] Second order decay constant (k_2) = 283 $[M\ s]^{-1}$.
[b] Constant ratio between the interaction rate constant for GSH and the interaction rate constants for the other tested thiol.

With the purpose of studying the effect of the pH on the reactivity of the radical with the different thiols, GSH was chosen. The results show an inverse linear dependence, i.e., at increasing pH values, a decrease in the k_i value is observed (i.e., at pH 9.0 k_i = 16,454 $(Ms)^{-1}$ vs. k_i = 398 $(Ms)^{-1}$ at pH 12). This type of behavior could be explained by relating the pKa value of the thiol group of GSH (pKa = 9.12, (32)) and its unionized fraction at the different studied pH. If the well-known Henderson-Hasselbach equation for a weak acid is applied (33):

$$[GS^-]/[GSH] = 10^{(pH-pKa)}$$

a significant decrease in the unionized fraction (R-SH) with pH can be calculated, e.g., 56.9% at pH 9.0 vs. 0.19% at pH 12, indicating that this fraction seems to be involved in the interaction. Probably, the unionized fraction may act as the proton donor, forming $Ar\text{-}NO_2H^{\bullet}$, therefore producing a deactivation of the radical anion. The inhibition of cytotoxicity by the thiols is traditionally viewed as:

$$Ar\text{-}NO_2^{\bullet -} + R\text{-}SH \rightarrow R\text{-}NO + R\text{-}S^{\bullet} + OH^-$$

EPR STUDIES

The *in situ* generation leading to a radical species were carried out in DMF/0.1 N aqueous NaOH pH 13 by applying the potential corresponding to the first one-electron reversible reduction process, just found in the cyclic voltammetric experiments (−1250 mV).

As can be seen in Fig. 4, the hyperfine pattern corresponding to the nitroanion of nisoldipine appears completely resolved into 35 lines. An experimental *g*-value for ascorbate of 2.0052 was determined and was used as a reference to calibrate the

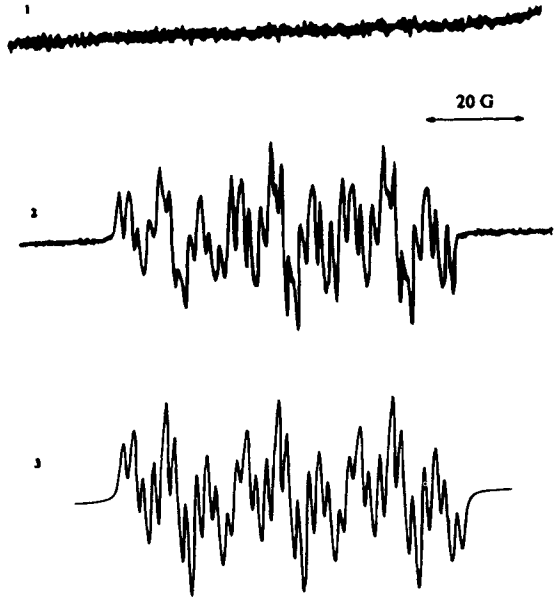

Fig. 4. EPR spectrum of nisoldipine anion radical electrochemically generated. Line 1 is the control (DMF/0.1 N NaOH, 0.1M tetrabutylammonium perchlorate). Line 2 is an EPR spectrum of nisoldipine anion radical produced in a system of 1mM nisoldipine and 0.1N NaOH, final pH 13. Line 3 is the simulated EPR spectrum of nisoldipine anion radical.

Table III. Experimental Hyperfine Splitting Constants for the Nitro Radical Anion from Nisoldipine

a_{NO2}[a]	a_{H5}	a_{H3}	a_{H4}	a_{H6}	$a_{H\text{-}DHP}$
10.87	3.59	2.83	1.09	1.09	0.65

[a] A values are expressed in Gauss.

spectra. The nisoldipine radical g-value obtained using this calibration was g(nisoldipine) = 2.0083.

The spectra were simulated in terms of one triplet due to the nitrogen nucleus of the nitro group, two doublets due to the non-equivalent hydrogens H_3 and H_5, one triplet due two equivalent hydrogens, H_4 and H_6 and one doublet due to hydrogen of the dihydropyridine ring (Fig. 1). The hyperfine constant values were obtained by an EPR simulation program and are listed in Table III. Also, from Fig. 4, it can be seen that both the experimental and the simulated spectra are in agreement.

As can be seen from Fig. 5, the addition of 20 mM glutathione to nisoldipine nitro radical anion generated by electrochemical reduction resulted in a decrease of the peak intensity until a complete inhibition occurred. Similar results were found for all the tested thiols. Thus, a complete inhibition of the nitro radical anion from a 20 mM concentration was observed. However, partial scavenging effects become evident from a 10 mM thiol concentration. Taking into account these results, it can be possible to offer direct evidence for an inhibition of the formation of nisoldipine anion radical by thiols under our experimental conditions. At present, a structure-effect relationship for the different studied thiols could not be ruled out.

In order to clear the mechanism of the reaction between thiols and the nitro radical, the feasibility of thyil radical formation by using the spin trap DMPO were conducted. However, under our experimental conditions such radical species were not detected. Probably, the dimerization of thyil radical species to give the RS-SR compound is favored on the thyil-DMPO adduct formation. However, the formation on thyil radical species can not be completely discarded.

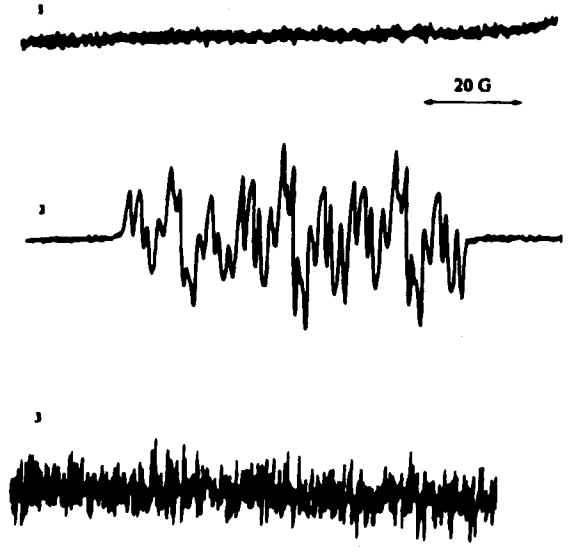

Fig. 5. Scavenging of the nisoldipine radical anion by glutathione. Line 1 is the control (DMF/0.1 N NaOH, 0.1M tetrabutylammonium perchlorate). Line 2 is the EPR spectrum of nisoldipine anion radical electrochemically generated. Line 3 illustrates the complete inhibition of free radical generation by 20 mM glutathione, final pH 13.

CONCLUDING REMARKS

The results of this study document the generation of nitro radical anion from nisoldipine directly by cyclic voltammetry and EPR spectroscopy. To our knowledge this is the first EPR study of the nisoldipine anion radical and its interaction with thiol compounds. Our results show that the generation of a stable radical intermediate occurred at alkaline range of pH (> pH 7.4 < pH13.5). On the other hand, a significant reactivity of the nitro radical anion from nisoldipine has been quantitatively demonstrated by cyclic voltammetry. In this same line of evidence, EPR results have confirmed the scanvenging of the radical by the different tested thiols (GSH, cysteamine, captopril, N-acetylcysteine and penicillamine).

The one-electron reduction product from nisoldipine significantly reacted with different relevant thiols, with the following relative order of reactivity: cysteamine ~ glutathione > N-acetylcysteine > captopril > penicillamine.

Rate interaction constants for all the thiols were higher than the second order decay constant for the nitro radical anion in all the cases. Thus, potential cytotoxicity of this redox intermediate ($Ar\text{-}NO_2^{\cdot-}$) could be efficiently reversed with any one of the tested thiol compounds. From the studies about the pH influence on the reactivity of the nitroanion, we selected glutathione. The results demonstrated that apparently, the unionized fraction (R-SH) of this endobiotic seem to be involved in the reaction.

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