Free Rad. Res., Vol. 34, pp. 363-378
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Trypanosoma Cruzi Trypanothione Reductase is Inactivated by Peroxidase-Generated Phenothiazine Cationic Radicals

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Accepted for publication by Prof. V. Darley-Usmar

(Received 28 June 2000; In revised form 2 August 2000)

Inactivation of Trypanosoma cruzi trypanothione reductase by peroxidase/H_2O_2/PTZ systems

Trypanosoma cruzi trypanothione reductase (TR) was irreversibly inhibited by peroxidase/H_2O_2/phenothiazine (PTZ) systems. TR inactivation depended on (a) time of incubation with the phenothiazine system; (b) the peroxidase nature and (c) the PTZ structure and concentration. With the most effective systems, TR inactivation kinetics were biphasic, with a relatively fast initial phase during which about 75% of the enzyme activity was lost, followed by a slower phase leading to total enzyme inactivation. GSH prevented TR inactivation by the peroxidase/H_2O_2/$PTZ^{+\bullet}$ systems. Production of $PTZ^{+\bullet}$ cation radicals by PTZ peroxidation was essential for TR inactivation. Horseradish peroxidase, leukocyte myeloperoxidase (MPO) and the pseudo-peroxidase myoglobin (Mb) were effective catalysts of $PTZ^{+\bullet}$ production. Promazine, thioridazine, chlorpromazine, propionylpromazine prochlorperazine, perphenazine and trimeprazine were effective constituents of the HRP/H_2O_2/PTZ system. The presence of substituents at the PTZ nucleus position 2 exerted significant influence on PTZ activity, as shown by the different effects of 2-trifluoromethyl and 2-H or 2-chlorophenothiazines. The $PTZ^{+\bullet}$ cation radicals disproportionation regenerated the non-radical PTZ molecule and produced the PTZ sulfoxide that was inactive on TR. Thiol compounds including GSH interacted with $PTZ^{+\bullet}$ cation radicals transferring an electron from the sulfide anion to the $PTZ^{+\bullet}$, thus nullifying the $PTZ^{+\bullet}$ biological and chemical activities.

Keywords: Trypanothione reductase, phenothiazine, cationic radicals, horseradish peroxidase, myeloperoxidase, myoglobin

Abbreviations: TR, trypanothione reductase; HRP, horseradish peroxidase; MPO, myeloperoxidase; Mb, myoglobin; PTZ, phenothiazine; PZ, promazine; CPZ, chlorpromazine; TFPZ, triflupromazine; PPZ, propionylpromazine; TFP, trifluoperazine; TMPZ, trimeprazine; PMTZ, promethazine; PCP, prochlorperazine; TRDZ, thioridazine; PFZ, perphenazine; FFZ, fluphenazine; PCYZ, propericyazine; PTZ-SO, phenothiazine sulfoxide; $T(S_2)$, diglutathionylspermidine disulfide

INTRODUCTION

Trypanosoma cruzi, the agent of American trypanosomiasis (Chagas' disease) possesses trypanothione reductase (TR) as a constitutive enzyme. TR catalyses the NADPH-dependent

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reduction of glutathionyl spermidine derivatives and plays an essential role in the regulation of oxidative stress in the parasitic cell.[1] TR is a suitable model for structure-based drug-design because of its well-known molecular structure and its biological specificity.[2–5] Among the many compounds assayed as potential trypanocidal agents, especially against *T. cruzi*, stand phenothiazines.[2,6–16] These drugs exert biological activity against *T. cruzi* and some phenothiazines (chlorpromazine, trifluprom azine and trifluoperazine) inhibit reversibly TR, at relatively low concentration.[2,4,17] The $MPO/H_2O_2/CPZ$ system inhibits *T. cruzi* dihydrolipoamide dehydrogenase,[18] an effect that suggests the intervention of $PTZ^{+\bullet}$ cation radicals.[19- 22] Therefore, it seemed of interest to examine the action of peroxidase-activated phenothiazines on *T. cruzi* TR. Three different peroxidases were used, namely, (a) horseradish peroxidase (HRP) extensively used as catalyst of phenothiazine peroxidation; (b) myeloperoxidase (MPO) an effective drug-peroxidizing enzyme, including phenothiazines;[23] and (c) Mb, a heme protein which, in addition to its function as dioxygen stabilizer in striated muscle,[24] has a relatively low peroxidase activity, especially on drugs.[25–28] Phenothiazine oxidation by HRP can be represented by Reactions 1–3.

The results reported here support the hypothesis that cation radicals produced by

$$\mathrm{HRP} + \mathrm{H_2O_2} \longrightarrow \text{Compound I} + \mathrm{H_2O} \quad (1)$$

$$\text{Compound I} + \mathrm{PTZ} \longrightarrow \text{Compound II} + \mathrm{PTZ^{+\bullet}} \quad (2)$$

$$\text{Compound II} + \mathrm{PTZ} + 2\mathrm{H^+} \longrightarrow \mathrm{HRP} + \mathrm{PTZ^{+\bullet}} + \mathrm{H_2O} \quad (3)$$

phenothiazine peroxidation irreversibly inhibit TR at concentrations lower than those directly inhibiting TR.[17] Table I summarizes the structural features of the assayed phenothiazines.

TABLE I Phenothiazine structure

Phenothiazine (PTZ)	*Substituent*	
	Position 2	*Position 10*
Promazine (PZ)	H	3-Dimethyl-amino-propyl
Chlorpromazine (CPZ)	Cl	3-Dimethyl-amino-propyl
Propionylpromazine (PPZ)	$COCH_2CH_3$	3-Dimethyl-amino-propyl
Triflupromazine (TFPZ)	$-CF_3$	3-Dimethyl-amino-propyl
Trimeprazine (TMPZ)	H	2 Methyl-3-dimethyl-amino-propyl
Promethazine (PMTZ)	H	2-Dimethyl-amino-propyl
Prochlorperazine (PCP)	Cl	3-(1-methyl-4-piperazinyl)propyl
Trifluoperazine (TFP)	$-CF_3$	3-(1-methyl-4-piperazinyl)propyl
Perphenazine (PFZ)	Cl	3-[1-(2-hydroxyethyl)-4-piperazinyl]propyl
Fluphenazine (FFZ)	$-CF_3$	3-[1-(2-hydroxyethyl)-4-piperazinyl]propyl
Thioridazine (TRDZ)	$-SCH_3$	2-(1-methyl-2-piperidinyl)-ethyl
Propericyazine (PCYZ)	CN	CN 3-(4-hidroxypiperidinyl)propyl

MATERIALS AND METHODS

Enzyme preparations

Recombinant TR from *T. cruzi* was obtained by expression in *Escherichia coli* of the corresponding gene from *T. cruzi*, as described.[2] The enzyme was stored as a suspension in 70% saturated $(NH_4)_2SO_4$ solution at 4°C and preserved stable for at least one year. The specific activity of the TR preparation was 128 units/mg. Human leukocyte MPO as well as HRP and skeletal muscle Mb were obtained from Sigma Chemical Co, St. Louis, MO, USA, MPO specific activity ranged from 50–100 units/mg as determined by the standard guaiacol/H_2O_2 assay.[29] Original Mb samples were diluted with 0.15 M NaCl, 50 mM K-phosphate, pH 7.4, and oxi-Mb, deoxi-Mb and meta-Mb content was determined spectrophotometrically,[30]resulting in 1% oxi-Mb, 37% deoxi-Mb and 62% meta-Mb. Other experimental conditions were as described previously.[31]

Reagents

Phenothiazines were obtained from Sigma Chemical Company. $T(S_2)$ was obtained from Bachem Bioscience, Inc. King of Prussia, PA, USA. $T(S_2)$ stock solutions in 50 mM K-phosphate, pH 7.4 were stored at –20°C and $T(S_2)$ concentration was checked spectrophotometrically using the TR/NADPH system. $T(S_2)$ solutions were diluted with the TR assay mixture in order to obtain the 0.12 mM concentration. $PTZ^{+\bullet}$cation radicals were prepared by oxidation with peroxidase/H_2O_2systems in 50 mM K-phosphate, as indicated in Results. $PTZ^{+\bullet}$ concentration was determined spectrophotometrically with the Aminco DW UV/VIS (SLM) spectrophotometer, in 50 mM K-phosphate. The following wavelengths values (in nm) and ε values (in $M^{-1}.cm^{-1}$) were used: PZ, 518 (ε, 7, 714); CPZ, 530 (ε, 11,864) TRDZ, 633 (ε, 7,682); TFPZ 500 (ε, 7, 450). FFZ 500 (ε, 6,796); TFP 500 (ε, 6,538).[32–33] PZ-S0 was prepared by PZ oxidation with H_2O_2, as described.[34] The purity of the crystalized product was checked by thin-layer chromatography and spectroscopy at 341 nm; (ε, 6,300) ($M^{-1}.cm^{-1}$).[21]

TR inactivation

TR inactivation mixture contained TR, peroxidase, H_2O_2, K-phosphate and additions as stated in Results; final volume, 0.1 ml. Samples were incubated at 30°C for the time stated in each case and 10 µl aliquots of the inactivation mixture were added to the assay mixture, residual TR activity being measured as described below. Control samples without peroxidase or other components of the inactivation mixture were incubated simultaneously.

TR assays

TR activity was measured at 30°C by the rate of NADPH oxidation using $T(S_2)$ as electron acceptor. The standard reaction mixture contained 0.15 mM NADPH, 0.12 mM $T(S_2)$ 50 mM K-phosphate, pH 7.4, and 10 µl of "inactivation mixture" containing 0.33 µg/ml of TR; total volume, 0.3 ml. TR was preincubated in the presence of NADPH for 5 min before addition of $T(S_2)$ to initiate the reaction. Spectrophotometric measurements were performed using a Perkin-Elmer 550 UV/VIS spectrophotometer, at 30°C.

ESR

Measurements were performed in a Bruker (Bruker Analystische Messtechnik, Karlsruke, Germany) ER106 ESR spectrometer, at room temperature. General instrumental conditions were: microwave power, 20 mW; modulation frequency, 50.00 kHz; microwave frequency, 9.75 GHz; modulation amplitude, 0.948 G; time constant, 1,310; ms; scan rate, 167.7 s; gain, 1×10^5. The reaction mixture composition was as described in Results.

Expression of Results

TR activity values are presented as a percentage of control sample activity (100%). TR inactivation (*I*%) and protection (*P*%) were calculated as described previously.[18,31] Unless otherwise stated, values represent means of duplicate measurements, experimental values differing by less than 5% from mean value. When more than two measurements were performed, the values presented are means ± SD.

RESULTS

TR inactivation by peroxidase/H_2O_2/PTZ systems

A general survey of HRP/H_2O_2/PTZ systems action on TR (Table II) showed 95–100% inactivation of TR with (a) PZ, TMPZ, PPZ and CPZ all having an alkyl-amino-alkyl substituent at Position 10 and H, propionyl or Cl at Position 2 (Table I); (b) TRDZ, having a piperidinyl substituent at Position 10 and a thiomethyl group at Position 2 (Table I); (c) PCP and PFZ, having a piperazinyl substituent at Position 10 and Cl, at Position 2 (Table I). Other phenothiazines, especially those having a trifluoromethyl substituent at Position 2 (TFP, TFPZ and FFZ), were less active, or inactive, irrespective of the substituent at Position 10. PCYZ with a CN-group at Position 2, was inactive, in contrast to TRDZ, its homologous piperidinyl derivative (Table II). Figure 1 shows the time-course of TR inactivation by several MPO/H_2O_2/PTZ systems utilizing PZ, TRDZ, TMPZ, CPZ or PFZ. The /PZ and /TRDZ systems were the more effective ones and with these systems, TR inactivation kinetics showed an initial 10 min phase during which the enzyme was 65–70% inactivated. The initial phase was followed by a slow decay of activity that after 30 min of incubation with the /PZ /TRDZ and TMPZ systems ended in a 95%, 85% or 75% loss of TR activity, respectively. The /CPZ and /PFZ systems, used at the 100 μM concentration, were obviously less active than the /PZ, /TRDZ and /TMPZ systems. The time required for TR half-maximal inactivation was about 2.5 min with the /PZ and /TRDZ systems and 5.0 min with the TMPZ system. No significant variations in TR activity were observed in samples lacking MPO or phenothiazine as described in Figure 1.

Figure 2 allows to compare the activity of HRP/ and MPO/H_2O_2/PZ systems as a function of the peroxidase nature, PZ concentration and incubation time. Thus, after 5 and 15 min incubation, the HRP system was more active than its MPO counterpart but after 30 min no significant differences between their effects were observed. Moreover, TR inactivation was proportional to PZ concentration since 5.0 μM PZ was about half as active as 10 μM PZ. Observed differences between HRP/ and MPO/systems were similar to the /TRDZ and /TMPZ systems but not to the PMTZ system (Figure 3).

TABLE II Inactivation of *T. cruzi* TR by the HRP/H_2O_2/PTZ systems

Phenothiazine (μM)	*TR inactivation (%)*
Promazine (10)	100 ± 0
Chlorpromazine (10)	97 ± 1.4
Thioridazine (10)	100 ± 0.2
Propionylpromazine (10)	99 ± 0.6
Trimeprazine (10)	94 ± 0.2
Prochlorperazine (10)	100 ± 0.4
Perphenazine (10)	96 ± 1.5
Trifluoperazine (10)	37 ± 0.4
Promethazine (25)	26 ± 1.0
Fluphenazine (50)	None
Triflupromazine (50)	42 ± 1.0
Propericyazine (100)	4.0 ± 0

TR "inactivation mixture contained 10 μg/ml TR, 0.5 U/ml HRP, 0.1 mM H_2O_2, 50 mM K-phosphate, pH 7.4 and phenothiazine as stated above. Time of incubation 30 min. Other conditions in *Materials and Methods*. TR inactivation (%) by the H_2O_2/PTZ systems (HRP omitted): none with PZ, PPZ, PCP, FFZ); 8 (PMTZ); 9 (CPZ); -3 (TRDZ), 4 (PFZ) and 2 (TFP).

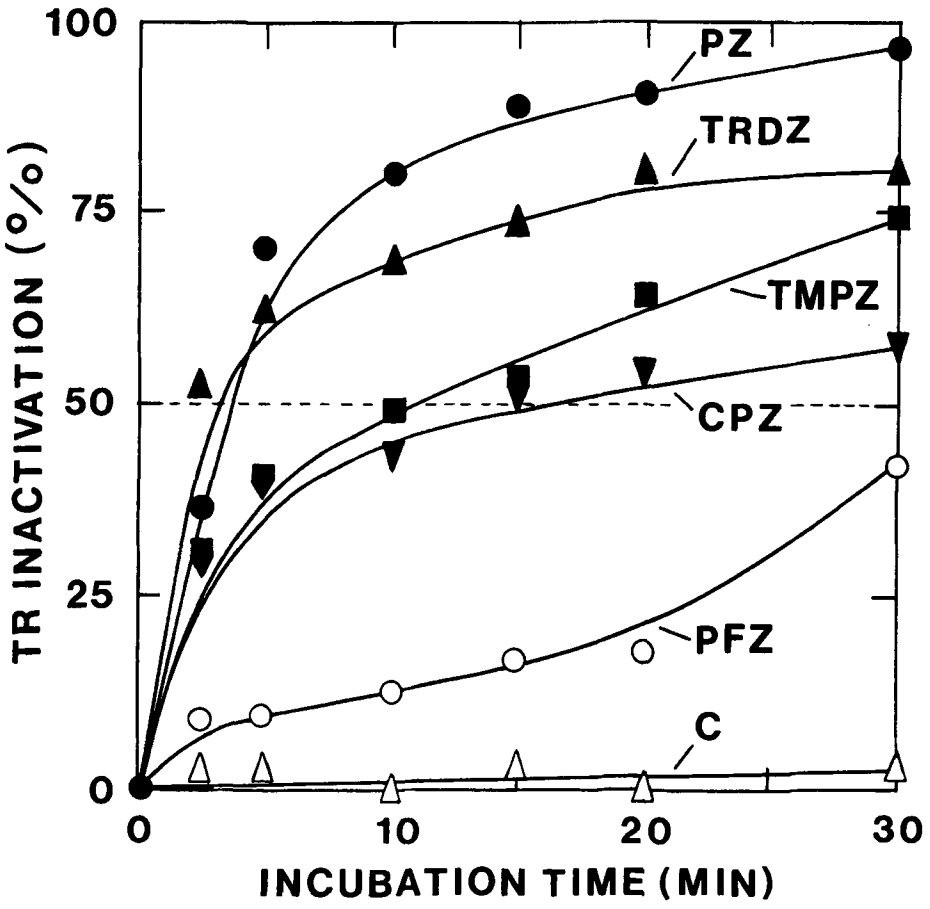

FIGURE 1 Time-course of TR inactivation by MPO/H_2O_2/PTZ systems. The inactivation mixture contained TR 10 μg/ml 0.5 U/ml MPO, 0.1 mM H_2O_2 50 mM K-phosphate, pH 7.4 and phenothiazine (10 μM, PZ, TRDZ and TMPZ; 100 μM CPZ or PFZ); total volume, 0.1 ml. Other conditions in Materials and Methods. C, control sample (MPO or Phenothiazine omitted). Value represent average of duplicate samples

Figure 4 shows the time-course of TR inactivation by the Mb/H_2O_2/PTZ systems. Mb was used at a greater concentration than MPO or HRP because Mb-seff is not a true peroxidase and with peroxidase substrates can only perform a limited number of catalytic cycles, thus being less active than HRP.[35] In close agreement with MPO/ and HRP/systems, the Mb/H_2O_2/PZ, /TMPZ, /TRDZ and /CPZ systems were the more effective as compared with other phenothiazine systems assayed (data not shown). It should be noted, however, that with the Mb system (Figure 4) TMPZ (trimeprazine) was more active than TRDZ (thioridazine), at variance with the effects observed with the MPO-systems (Figure 1). Omission of Mb or phenothiazine prevented TR inactivation. Allowance made for PZ and TMPZ, phenothiazines were used at relatively high concentrations, as compared with those used with the HRP/ and MPO/ systems (Table II and Figures 1 and 3) presumably because of different substrates specificities between HRP and MPO on the one hand, and Mb on the other.

TABLE III Effect of thiol compounds on TR inactivation by the MPO/H_2O_2/PZ system

Thiol compound (μM)	*TR inactivation (%)*	
	Incubation: 10 min	*Incubation: 30 min*
None	75.6 ± 3.4	91.6 ± 1.9
GSH (200)	2.0 ± 1.0 (97)	15.3 ± 1.8 (83)
L-Cysteine (200)	1.8 ± 1.4 (98)	30.0 ± 1.4 (67)
NAC (200)	0 ± 1.0 (100)	5.9 ± 1.6 (94)

The "inactivation mixture" contained 10 μg/ml TR, 0 5 U/ml, MPO, 0.1 mM H_2O_2, 50 mM K-phosphate, pH 7.4 and 10 μM PZ. Thiol compound as indicated above. The figures in parenthesis indicate protection (P) of TR by the thiol compound. Other experimental coriditions in Materials and Methods.

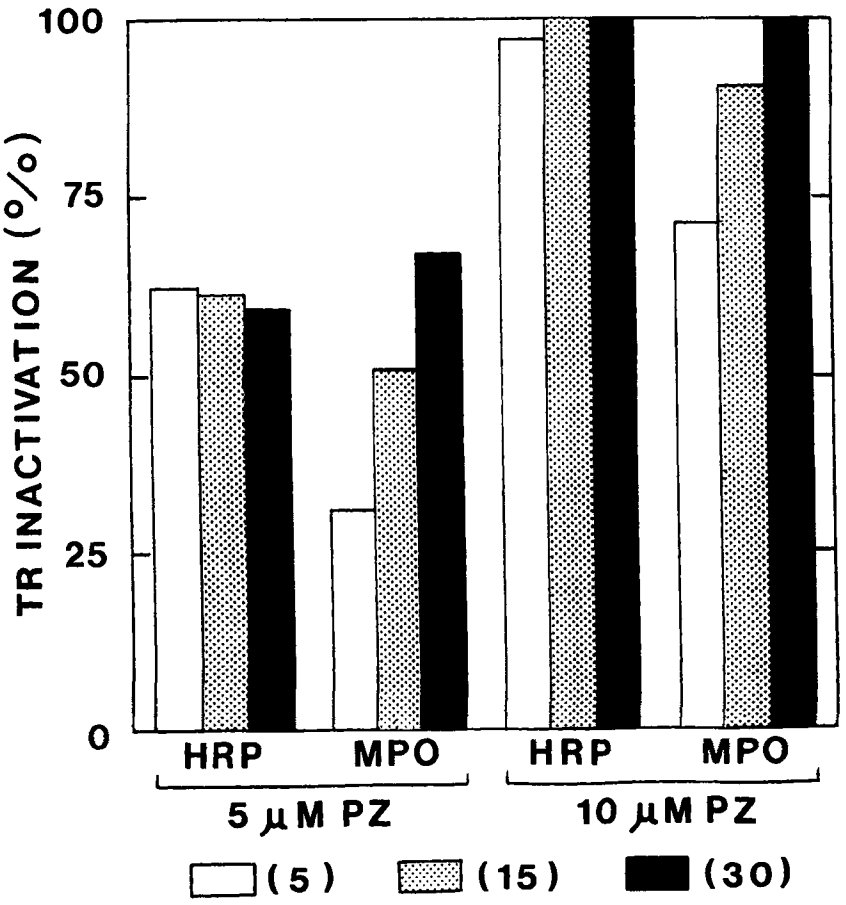

FIGURE 2 Comparative effects of HRP/ and MPO/H_2O_2/PZ systems on TR. The inactivation mixture contained 10 μg/ml TR, 0.5 U/ml HRP or MPO, 0.1 mM H_2O_2, and 50 mM K-phosphate, pH 7.4. PZ and time of incubation as indicated on the Figure. Other conditions in Figure 1 legends and Materials and Methods

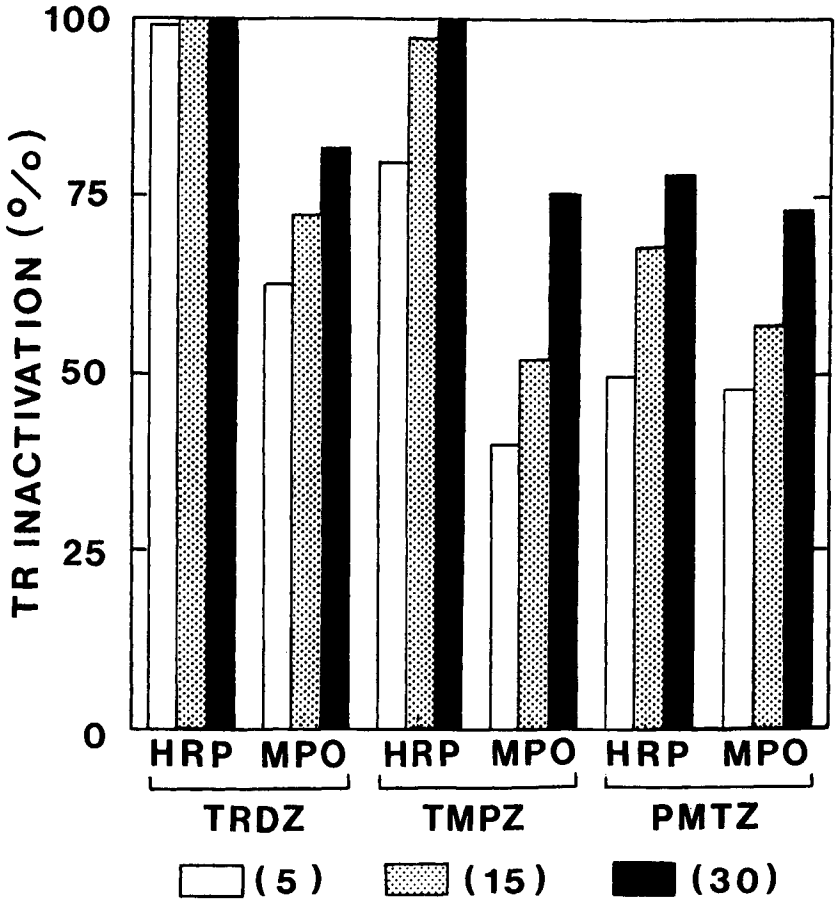

FIGURE 3 Comparative effects of HRP/ and MPO/H_2O_2/PTZ systems on TR. The inactivation mixture contained 0.5 U/ml HRP or MPO, 0.1 mM H_2O_2, K-phosphate, pH 7.4, 10 μM TRDZ and TMPZ or 100 μM PMTZ. Time of incubation (min) as indicated in the Figure. Other conditions in Figure 1 legend and Materials and Methods

TR protection by thiol compounds

In close agreement with previously described reactions of thiol compounds with phenothiazines,[37,38] GSH prevented TR inactivation by the MPO/ or HRP/H_2O_2/PTZ systems. Figure 5 shows the results obtained with MPO/H_2O_2/PZ. It is to be seen that addition of GSH to the TR inactivating mixture produced 100% protection of TR during the first 5 min of incubation but less, though significant protection (85%), after 30 min incubation. Similar results were obtained with HRP/ systems (Figure 5 inset). L-cysteine and N-acetylcysteine were also effective protectors of TR (Table III). On the other hand, 10 μM T(S_2) (TR substrate) was completely ineffective as TR protector (experimental data omitted) thus confirming the role of SH groups in TR protection.

Production of $PTZ^{+\bullet}$ cationic radicals

Figure 6 shows $CPZ^{+\bullet}$ cation radical ESR signal produced by the MPO/H_2O_2/CPZ at pH 6.5. The signal resembled the one previously

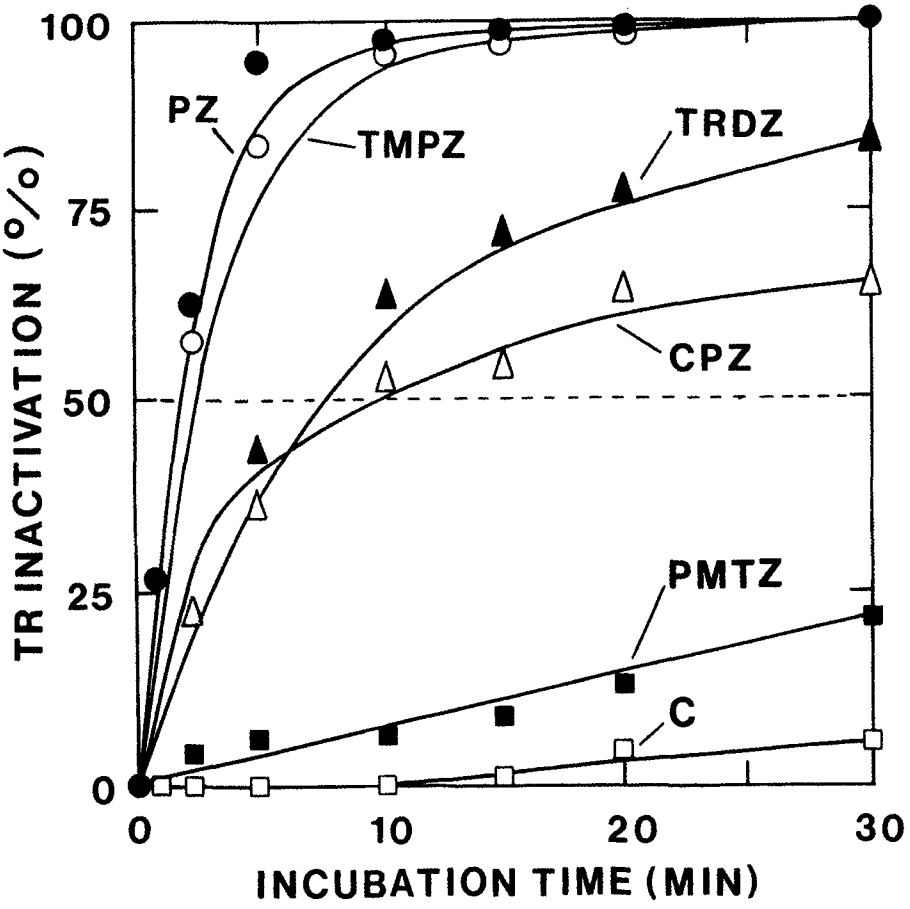

FIGURE 4 Time-course of TR inactivation by the Mb/H_2O_2/PTZ systems. The inactivation mixture contained 10 μg/ml TR, 5.0 μM Mb, 0.1 mM H_2O_2 50 mM K-phosphate, pH 7.4 and 25 μM PZ or TMPZ or 100 μM TRDZ, CPZ or PMTZ. Other conditions in Materials and Methods. C, control sample (Mb or Phenothiazine omitted)

observed with the HRP/H_2O_2/CPZ system at pH 4.5.[19] Addition of the spin-trapping agent DMPO[36] suppressed the $CPZ^{+\bullet}$ signal and prompted the appearance of DMPO-$CPZ^{+\bullet}$ adduct signal. Similar effects were observed with PZ (not shown). Production of cation radicals was confirmed by optical spectroscopy since absorption spectra are well known.[21,32,38,39] Figure 7 shows results obtained with the MPO/H_2O_2/PZ system. The radical concentration reached its maximal value shortly after completion of the reaction mixture and thenceforth it decayed slowly, thus showing typical radical instability.[39] This kinetics is explained by Reaction 4 according to which 2 moles of $PTZ^{+\bullet}$ yield one mol of parent phenothiazine and one mol of PTZ-SO.

$$2PTZ^{+\bullet} + H_2O \longrightarrow PTZ + PTZ\text{-}SO + 2H^+ \quad (4)$$

Figure 8 summarizes cation radical production by MPO/ or HRP/, H_2O_2/PZ, /TRDZ and /CPZ systems. In each condition, cation radical concentration expressed the balance between the rates of radical production and radical decay,

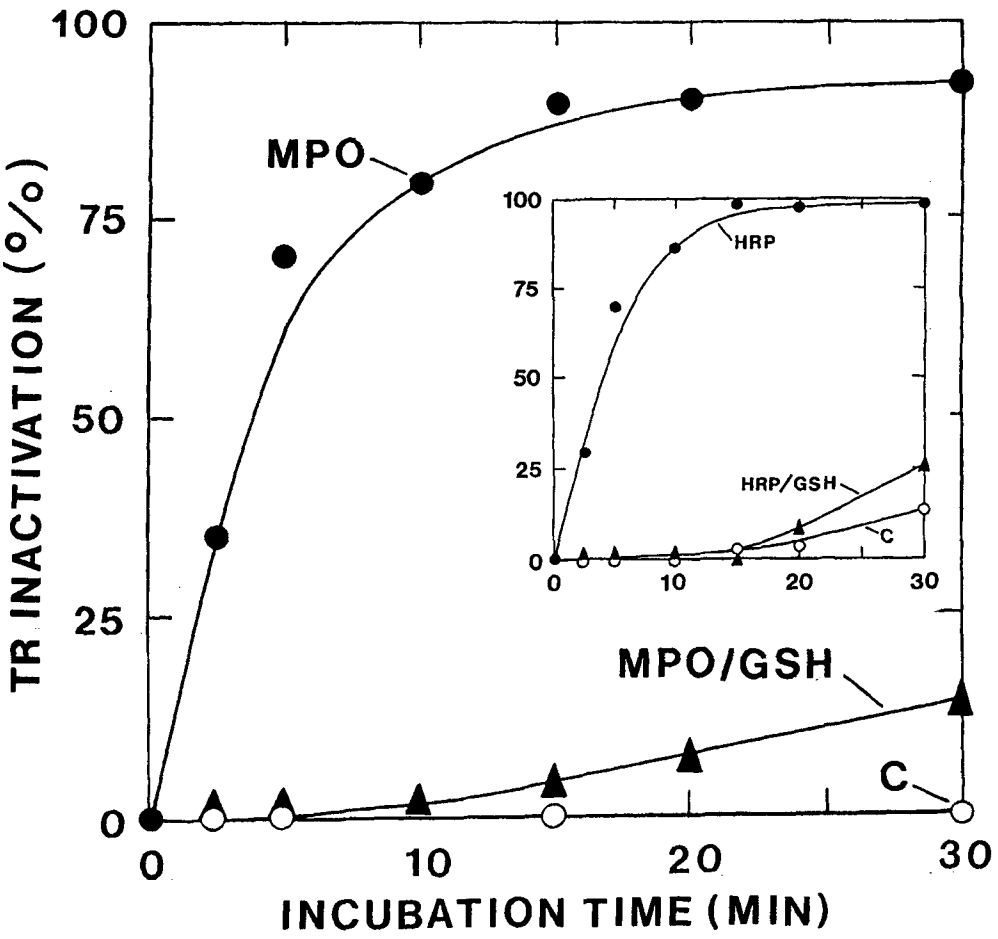

FIGURE 5 Effect of GSH on TR inactivation by the peroxidase/H_2O_2/PZ systems. The inactivation mixture contained 10 μg/ml TR 0.5 U/ml MPO, 0.1 mM H_2O_2, 10 μM PZ and 50 mM K-phosphate, pH 7.4, 0.2 mM GSH was added as indicated in the Figure. Inset. Same experimental conditions except peroxidase (HRP). Other conditions in Figure 1 legend, and Materials and Methods

respectively.[39] Since cation radical stability increases at low pH, our experiments were performed at pH 6.5, not too different from the pH used for TR inactivation. The differences in cation radical levels in Figure 8 (MPO experiment) correlated qualitatively with the MPO/H_2O_2/PTZ systems effect in Figure 1.

Production of cation radicals by the Mb/H_2O_2/PZ system is illustrated in Figure 9. The action of H_2O_2 and PZ on Mb spectrum fits in well with the peroxidase activity of perferryl and ferryl-Mb. Production of $PZ^{+\bullet}$ cation radical was demonstrated by the appearance of the characteristic absorption peak, near 520 nm.

Production and decay of $PTZ^{+\bullet}$ cationic radicals was effectively modified by GSH. Figure 10 shows the effect of GSH on the kinetics of $PZ^{+\bullet}$ production by the MPO/H_2O_2/PZ system. GSH effect depended on the GSH/PZ concentration ratio. Thus, with a relatively low GSH /PTZ concentration ratio (GSH/PZ = 25/100), $PZ^{+\bullet}$ level dropped to a minimum value at about 15 min of

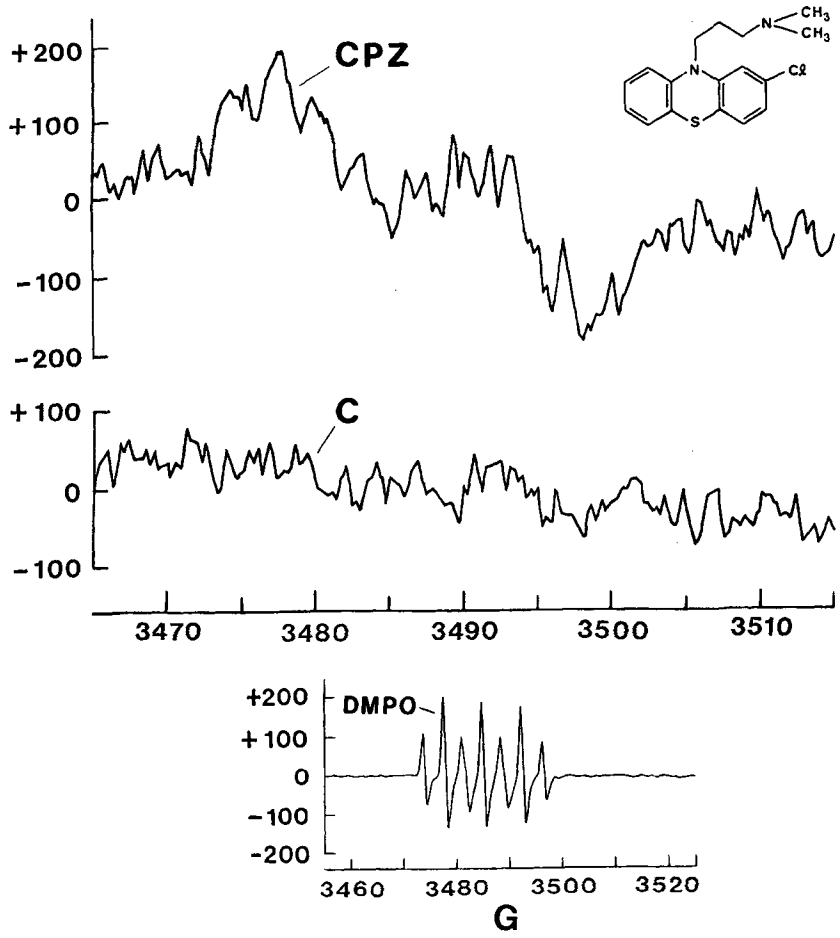

FIGURE 6 ESR signal of the Phenothiazine cationic radical. CPZ: the reaction mixture contained 2.5 U/ml MPO, 0.5 mM H_2O_2 50 mM K-phosphate pH 7.4 and 0.5 mM CPZ. Other conditions in Materials and Methods. C, control sample (MPO omitted). DMPO, reaction mixture containing MPO, H_2O_2 and CPZ as above, plus 50 mM DMPO

incubation. Then, it increased continuously to the point that at the end of the incubation, $PZ^{+\bullet}$ level was the same as the control sample level. On the other hand, with high GSH concentrations (e.g.) (GSH/PZ = 200/100) $PZ^{+\bullet}$ level decreased and remained low throughout the incubation period.

In addition to these effects, thiol compounds prevented the production of PTZ-SO by $PTZ^{+\bullet}$ disproportionation (Table IV) as expected from GSH reaction with the cation radical. PZ-SO effect on TR was investigated using the HRP/H_2O_2/PZ-SO system containing 100 μM PZ-SO. After 15 and 30 min incubation, TR inactivation values were 6.0 and 15%, respectively (average of duplicate measurements). However, addition of 10 μM PZ to the same inactivation mixture produced 100% inactivation of TR, as expected from $PZ^{+\bullet}$ formation (experimental data omitted). From these experiments it should be concluded that PZ-SO was ineffective on TR.

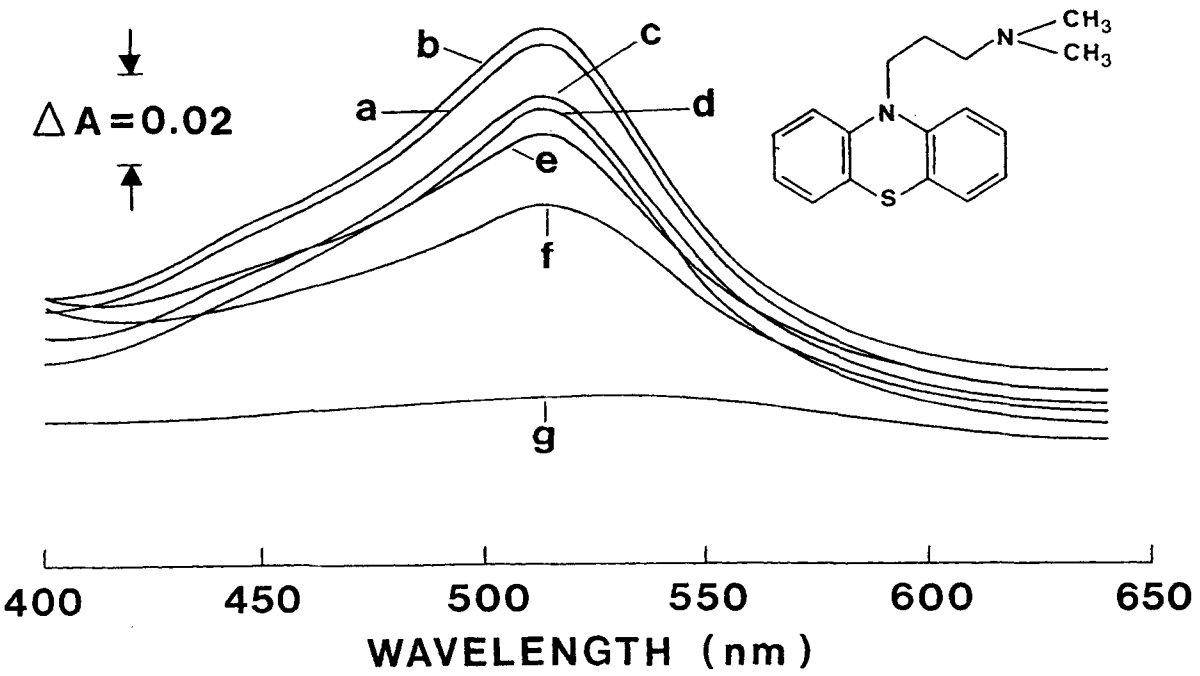

FIGURE 7 Production of $PZ^{+\bullet}$ by the MPO/H_2O_2/PZ system. The reaction mixture contained 0.5 U/ml MPO, 0.2 mM H_2O_2, 50 mM K-phosphate, pH 6.5 and 100 μM PZ, total volume, 3.0 ml. Other conditions in Materials and Methods. Lines a, b, c, d, e and f, spectra recorded 0.75, 1.5, 3.0, 5.0, 10 and 15 min after completing the reaction mixture with H_2O_2. *g*, base line

DISCUSSION

The observations described in Figures 1–4 strongly support the notion that $PTZ^{+\bullet}$ cationic radicals, produce irreversible inhibition of TR, the peroxidase/H_2O_2/PTZ systems proving the immediate generators of the cationic radical. HRP, MPO and Mb were effective pro-oxidants thus supporting the postulated mechanism for TR inactivation. Phenothiazines are direct inhibitors of TR[2,17] and therefore, the non-radical molecules could contribute to the effect here described. This assumption may be, however, minnimized since (a) dilution of TR "inactivation mixtures" with the TR "assay mixture" decreased phenothiazines concentrations to levels that would not inhibit TR[2,17]; (b) according to TR inactivation kinetics, initial samples fully conserved TR activity, at variance with a direct effect of phenothiazines; (c) TR inactivation by the peroxidase/H_2O_2/PTZ systems was always dependent on the presence of peroxidase and H_2O_2 in the "inactivation mixture".

TR inactivation by Mb/H_2O_2/PTZ systems (Figure 4) deserves a special comment. The Mb samples used in the present study contained a significant proportion of met-Mb and deoxi-Mb. In the reaction between ox-Mb or met-Mb with H_2O_2,[41,42] the heme iron is oxidized to its ferryl-oxo form and the globin to protein radicals[43] at least one of which reacts with dioxygen to produce a peroxyl radical. This radical production involves globin amino acid residues such as Trp and Tyr.[44–47]Production of ferryl-Mb from met-Mb and the latter subsequent reaction with H_2O_2 originates a catalytic cycle that determines Mb peroxidase activity. A complementary reaction to this cycle should be

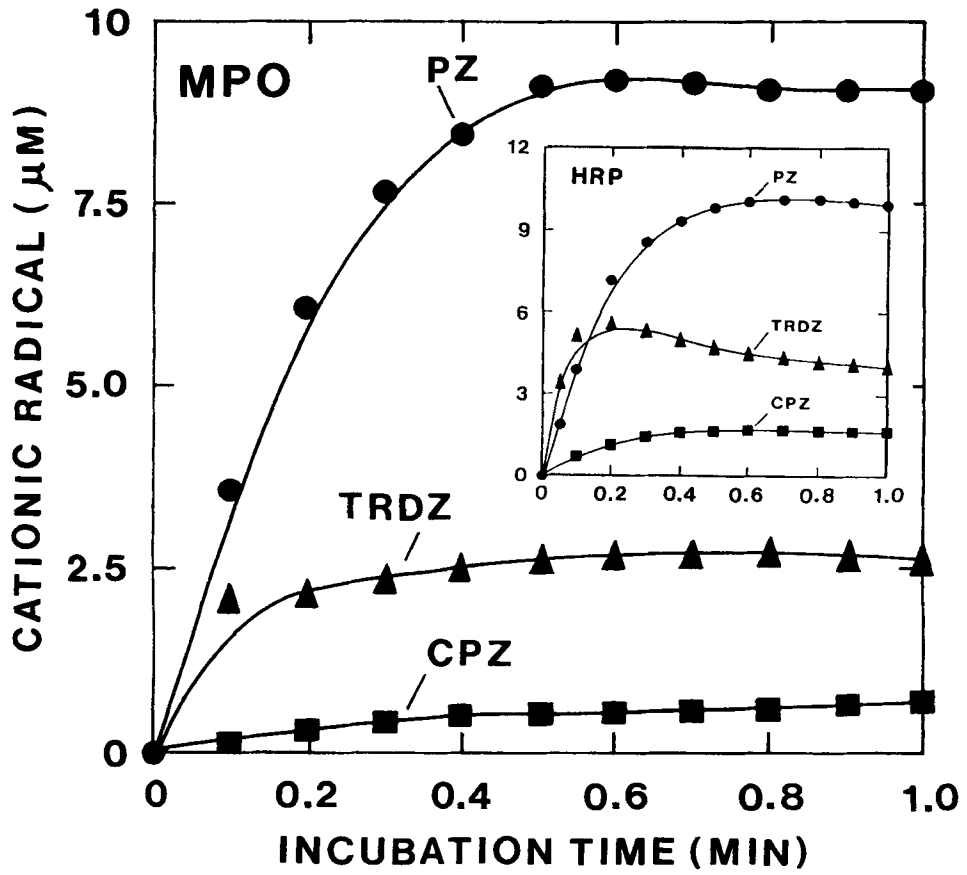

FIGURE 8 Production of $PTZ^{+\bullet}$ cation radicals by MPO/H_2O_2/PTZ systems. The reaction mixture contained 0.5 U/ml MPO, 0.5 mM H_2O_2, 50 mM K-phosphate, pH 6.5 and 100 μM phenothiazine (PZ, TRDZ and CPZ) as indicated in the Figure. Other conditions in Figure 7 legend and Materials and Methods. Inset: same experimental conditions except the peroxidase (HRP)

the H_2O_2-dependent oxidation of Mb to met-Mb.[41] Oxi-Mb autoxidation also results in the formation of superoxide anion radicals, hydrogen peroxide and other "reactive species".[30]These species production explain myosin and serum albumin oxidation as well as enzyme inactivation (myosin-ATPase) and other enzymes by ferryl-Mb.[27,35,36] Similar effects might explain TR inactivation by the Mb/H_2O_2/PTZ systems (Figure 4) but the strict requirement of phenothiazine for TR inactivation rules out a direct action of the Mb/H_2O_2 system on TR.

Phenothiazine structure-activity relationships deserve consideration in order to compare those here described with similar systems effects. Substituents at Position 2 of the phenothiazine nucleus significantly affected molecular activity since phenothiazine bearing the trifluoromethyl substituent (TFP, FFZ and TFPZ) were less active than those bearing H or the Cl atom (Table II). Examination of phenothiazine immediate actions on TR[17] indicates that (a) TFPZ and PZ were equally effective, but less than CPZ; (b) PZ and TFPZ were about three-fold more inhibitory than PPZ; (c) TFP was more inhibitor than PZ

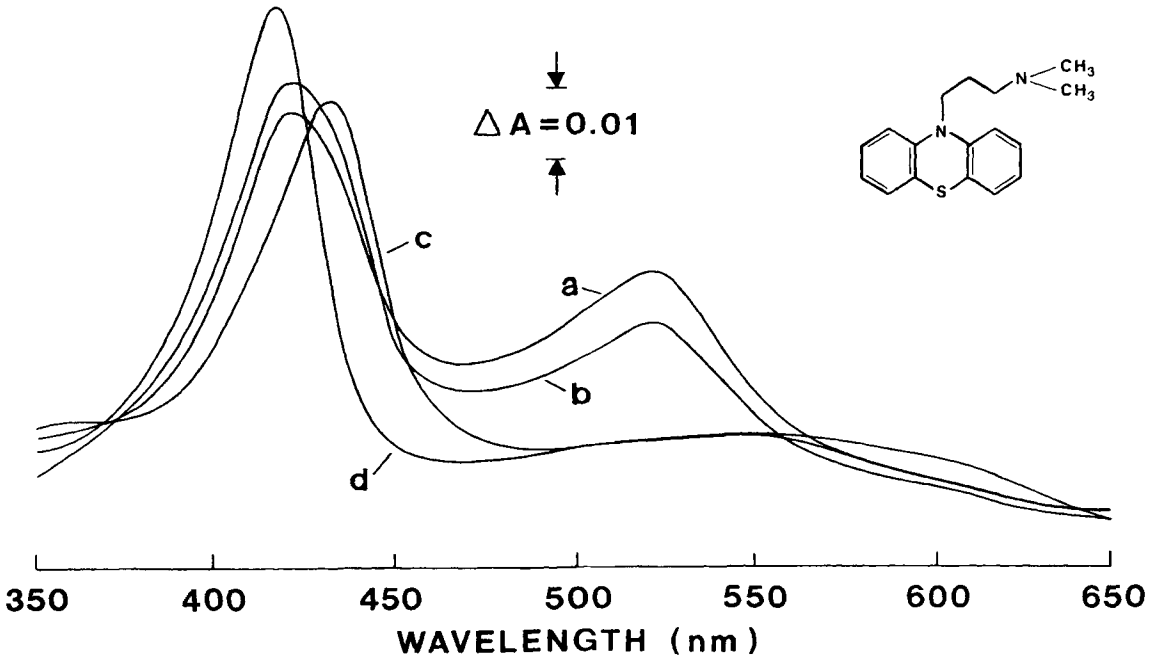

FIGURE 9 Production of $PZ^{+\bullet}$ cationic radical by the $Mb/H_2O_2/PZ$ system. The reaction mixture contained 5 μM Mb 0.2 mM H_2O_2, 50 mM K-phosphate, pH 6.5 and 100 μM PZ. Other conditions in Figure 7 legend and Materials and Methods. Lines *a* and *b* $Mb/H_2O_2/PZ$ system spectra, 1.5 and 10 min after completing the reaction mixture; *c*, Mb/H_2O_2 and *d*, Mb control samples spectra

and (d) I_{50} (μM) value for CPZ, the most active phenothiazine was 35.4 ± 7.7 μM and greater values were asigned to the other molecules assayed. Comparison of these correlations with the results reported here, especially phenothiazine concentrations effective with the $HRP/H_2O_2/PTZ$ systems (Table II) support the assumption that factors other than PTZ-TR interactions would be involved in TR inactivation by the PTZ dependent systems. Thus (a) cation radical production by peroxidase/H_2O_2/PTZ systems imply the influence of both the peroxidase and the phenothiazine structure; (b) the resulting radical concentration should depend on the balance between $PTZ^{+\bullet}$ production and decay rate, this latter being also conditioned by the phenothiazine structure; (c) the TR-$PTZ^{+\bullet}$ interaction, might determine rate and specificity of TR molecular modification. Very limited information is available on structure-related effects in these processes. Concerning PZ-SO derivatives, no evidence for any inhibitory action on TR was obtained and therefore their role may be ruled out.

TR protection by thiol compounds provides further evidence for the role of $PTZ^{+\bullet}$ cation in TR inactivation, since such reaction with thiol compounds is well known.[37,38] Moreover, the effects of GSH (Figure 10) and other thiols (Table III) suggests $PTZ^{+\bullet}$ reaction with TR thiol groups, which are essential for TR activity. Electron transfer between thiols and $PTZ^{+\bullet}$ cation radicals takes place according to Reaction 5, in which the thiol anion transfers one electron to the cation radical, thus the thiyl radical and

$$RS^- + PTZ^{+\bullet} \longrightarrow RS^\bullet + PTZ \qquad (5)$$

$$RS^\bullet + RS^- \longrightarrow RSSR^{\bullet -} \qquad (6)$$

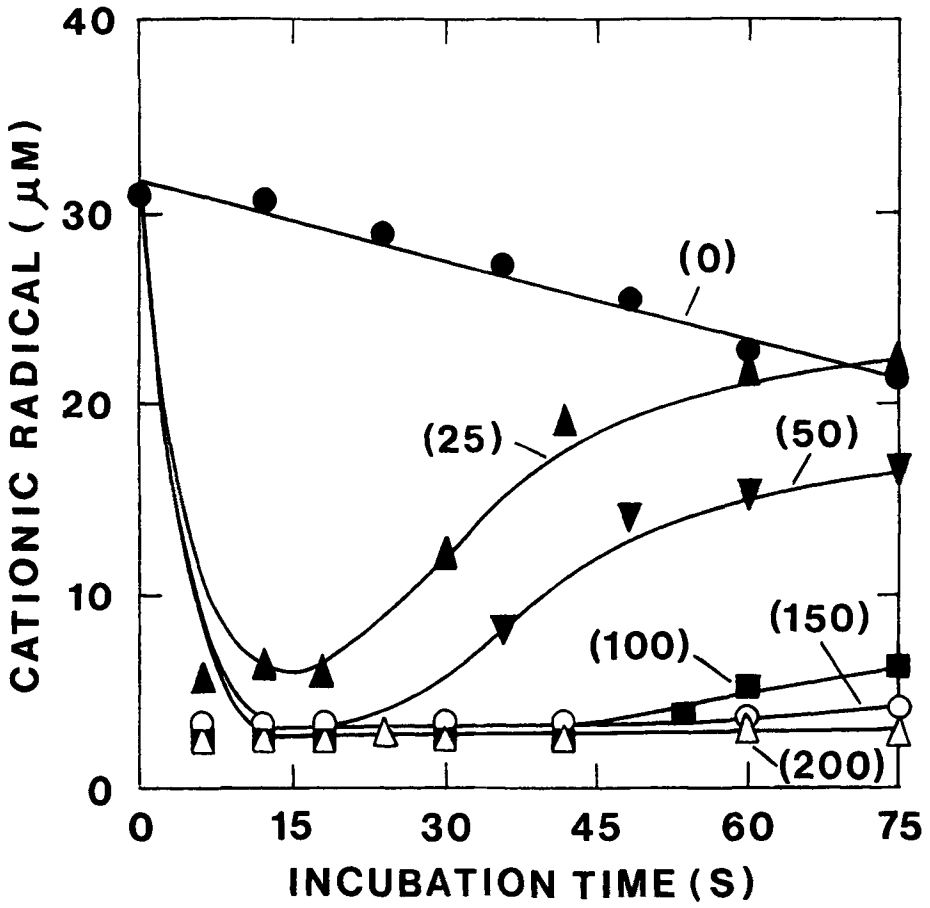

FIGURE 10 Effect of GSH on $PZ^{+\bullet}$ production kinetics. The reaction mixture contained 0.5 U/ml HRP, 0.2 mM H_2O_2, 50 mM K-phosphate, pH 6.5 and 100 μM PZ. GSH was added when $PZ^{+\bullet}$ concentration reached its maximum value. GSH concentration is indicated in parenthesis. Other conditions in Figure 7 legend and Materials and Methods

the parent phenothiazine being formed (Reaction 5). In turn, the thiyl radical forms disulfide compounds, as illustrated by Reaction 6, a reaction that contributed to the anti-oxidant function of GSH. Moreover, $PTZ^{+\bullet}$ cation radical reactions with thiol compounds in biological media might produce to phenothiazine cytotoxicity as a result of depletion of thiol dependent antioxidant mechanism. The here described results support Piette *et.al.*[19] hypothesis that $PTZ^{+\bullet}$ cation radical are essential for phenothiazine cytotoxicity.

Phorbol esters activated polymorphonuclear leukocytes produce CPZ cation radicals and CPZ-SO, in all probability as a result of CPZ peroxidation by the MPO/H_2O_2 system.[20] Moreover, in the same leukocytes, CPZ enhances H_2O_2 production and MPO release from the activated leukocytes, two effects that suggest the possibility of extraleukocyte phenothiazine activation. Taken together with TR inactivation.[2,17] $PTZ^{+\bullet}$ production by leukocyte MPO may contribute to phenothiazines trypanocidal action. Similar processes might occur with Mb in myocardial muscle; considering the high Mb concentration in striated muscle.

TABLE IV Production of FTZ-SO by peroxidase/H_2O_2/PTZ systems, inhibition by GSH

Phenothiazine (100 μM)	*GSH (μM)*	*PTZ-SO μM*		
		MPO/H2O2/system	*HRP/H2O2/system*	*Mb/H2O2*
PZ	None	56.1 ± 3.4	60.4 ± 1.0	26.9 ± 0.7
	200	15.2 ± 1.1 (73)	17.9 ± 0.3 (70)	2.9 ± 0.2 (89)
CPZ	None	17.6 ± 1.2	39.3 ± 1.2	8.8 ± 1.6
	200	1.9 ± 0.6 (89)	6.4 ± 1.4 (84)	0.0 ± 0 (100)

The reaction mixture contained 0.5 U/ml MPO or HRP, or 5.0 μM Mb, 0.1 mM H_2O_2 50 mM K-phosphate pH 7.4. Phenothiazine and GSH as indicated above. Total volume, 0.3 ml. Time of incubation, 10 min. Other experimental conditions in Materials and Methods In parenthesis, inhibition (%) of FTZ-SO production.

Acknowledgements

This work was aided by grants from the University of Buenos Aires and the Roemmers Foundation. We are grateful to Mr A. Boveris (School of Pharmacy and Biochemistry, University of Buenos Aires) for the ESR measurements, and to M.G. Gutiérrez and M.A. Verón for useful technical assistance.

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