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EFFECT OF L-PENICILLAMINE HYDANTOIN, AN ANALOGUE OF GLUTATHIONE, ON RAT LIVER GLUTATHIONE PEROXIDASE, REDUCTASE AND TRANSFERASE REACTIONS

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Abstract—In soluble fractions prepared from rat liver homogenates, L-penicillamine hydantoin appeared to be, on the basis of SH consumption measurements, a substrate for glutathione peroxidase but not transferase reactions. When glutathione is incubated with rat liver soluble proteins in the presence of penicillamine hydantoin, formation of oxidized glutathione is inhibited. Calculations from Lineweaver–Burk plots point out that inhibition by L-penicillamine hydantoin of the peroxide-dependent oxidations of glutathione is mixed, since both apparent K_m and V_{max} values are modified. Preincubation of rat liver soluble proteins with L-penicillamine hydantoin led to a progressive inactivation of glutathione peroxidase. The kinetics of this inactivation process with respect to time and inactivator concentration were studied. Inclusion in the preincubation mixture of SH-containing molecules such as dithiothreitol, L-cysteine or glutathione protected the enzyme against inactivation. However, none of these molecules and neither hydantoin, Triton X-100, phenol, nor dialysis could reverse the enzyme from inactivated to activated form. Mitochondrial glutathione peroxidase was inhibited and inactivated by L-penicillamine hydantoin to the same extent as its cytosolic counterpart. Modifications by penicillamine hydantoin of various subcellular markers enzymes (lactate dehydrogenase, *N*-acetyl β-glucosaminidase, arylsulfatase C, butyryl-CoA dehydrogenase, lauryl-CoA and glycolate oxidases) were of weak amplitude consisting of either inhibition, inactivation or stimulation.

Glutathione is a polypeptide widely distributed in mammalian cells ([1, 2], for a recent general review [3]). Its intracellular concentration varies from one tissue to another and can amount to millimolar values (e.g. liver, kidney, nervous tissue, lens) [1]. This tripeptide contributes to the cellular detoxications of endogenous (hydrogen peroxide, peroxidated lipids) [2, 4–6] and exogenous (xenobiotics) [1, 2, 4, 5, 7–10] compounds, through the action of glutathione peroxidases and transferases, respectively. Because it breaks down hydrogen peroxide, glutathione peroxidase prevents the utilization of this peroxide for other purposes, among which is the formation of hydroxyl radicals [2]. The hydroxyl radical mediates the cellular toxicity of molecular oxygen. A minor portion of molecular oxygen can, indeed, be converted to hydroxyl radical, a pathway involving superoxide anion-generating systems, enzymic superoxide dismutase and spontaneous oxidoreductions according to the Fenton reaction [6, 11, 12]. By this way, glutathione and related enzymology modulate cellular levels of hydroxyl radical [6].

The participation of hydroxyl radical to the mode of action of some anticancer drugs such as Adriamycin® is, now, well documented [13–17]. In patients, the long-term maintenance of the curative properties of this type of drug is hampered by an adaptative response of the cancer cells [18, 19]. This response presents several facets including the induction of cell membrane proteins (whose prototype is the P170 glycoprotein) and alterations of glutathione metabolism [20–22]. The cancer cell response can abolish the benefit offered by initial therapy and, for this reason, this phenomenon is called chemoresistance [18, 19]. In the chemoresistance status, the metabolism of glutathione is stimulated and is responsible for an enhanced H_2O_2 breakdown, thus resulting in a drop of the hydroxyl radical formation rates and subsequent loss of the anticancer drug-induced cytotoxicity [14, 23, 24]. The treatment of chemoresistance should rest on the reversal of increased cell membrane protein activities and accelerated glutathione metabolism. The first inhibition can be achieved by calcium channel inhibitors (e.g. verapamil) in the case of the P170 glycoprotein [25, 26] whereas the second requires the use of molecules depressing glutathione synthesis and/or metabolism [27–30]. The aim of the present work deals with the second concern in the matter of chemoresistance reversal, i.e. the concept of inhibitors of glutathione metabolism. Penicillamine hydantoin was shown to be active in two pharmacological tests representative of an antiarthritis penicillamine-like activity in man, based on the delayed hypersensitivity in mice, namely the haemophilus pertussis and picryl chloride tests and, on the basis of molecular modelling studies, to interact adequately with the catalytic center of glutathione peroxidase (Mestdagh *et al.*, unpublished

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work). This molecule (D- and L-isomers providing essentially the same effects) is here demonstrated to impair the enzyme activity. The non-peptidic nature of this molecule is promising for future experiments with intact cells.

MATERIALS AND METHODS

Materials. Biochemicals and their sources were: glutathione and 1-chloro-2,4-dinitrobenzene (Aldrich Chimie, S.a.r.l, Strasbourg, France); yeast glutathione reductase, *tert*-butyl hydroperoxide, cumene hydroperoxide, $NADP^+$, NADPH, L-cysteine and dithiothreitol (Sigma Chemical Co., St Louis, MO, U.S.A.); γ-glutamyl-3-carboxy-4-nitroanilide (Boehringer Pharma, Mannheim, Germany); hydrogen peroxide and other common chemicals (Merck, Darmstadt, Germany).

Animals. Adult male Wistar rats (200–300 g), fed a standard laboratory chow, were killed by decapitation before use.

Enzyme assays. Glutathione reductase was assayed at 30° by the oxidized glutathione-dependent oxidation of NADPH followed at 340 nm as described in Ref. 31.

Glutathione peroxidase activities were measured at 30° by two methods. In method A, the oxidations, have been assayed by monitoring the disappearance of SH groups measured with the Ellman's reagent (DTNB, 5,5'-dithio-bis-nitrobenzoic acid*) added at various incubation times to an aliquot part removed from the incubation mixture. The resulting reaction of residual SH groups with DTNB was followed at 412 nm. Tests and blanks were run at pH 7.6 in 0.1 M sodium phosphate buffer. For the test conditions, together 0.5 mM glutathione (or L-penicillamine hydantoin), the peroxide (either 1.25 mM hydrogen peroxide, 2 mM *tert*-butyl hydroperoxide or 1 mM cumene hydroperoxide) and the rat liver protein preparation were included in the incubation media. Different blanks were incubated in parallel, corresponding to the test conditions minus (1) the peroxide; (2) glutathione (or L-penicillamine hydantoin); (3) cytosolic proteins; (4) the peroxide and the cytosolic proteins.

At various time intervals, a 100 μL part aliquot was removed from the incubation medium to be added to reagent mixtures (final volume = 1 mL) containing 100 mM sodium phosphate buffer, pH 7.6 and 0.2 mM DTNB. The resulting stable absorbance values were read at 412 nm.

In method B, the appearance of the disulfide product (i.e. oxidized glutathione) was monitored at 340 nm in a coupled assay procedure which required addition of exogenous glutathione reductase (1 U/mL) and NADPH (0.1 mM) and which was an adaptation of the method described [32, 33]. Throughout the text, when the type of procedure for the assay of glutathione peroxidase is not specified, the mentioned results have been obtained using method B.

Glutathione transferases have been assayed on 1 mM glutathione and a substrate common to the different transferases 1-chloro-2,4-dinitrobenzene (1 mM) at 340 nm according to Habig and Jakoby [34].

Established procedures have been used for the assay of other enzymes: γ-glutamyl-transpeptidase [35] and glucose-6-phosphate dehydrogenase [36]. Protein and various marker enzymes of the various subcellular liver compartments were assayed as described previously [37, 38].

Synthesis of penicillamine hydantoins. The isomers of penicillamine hydantoin were synthesized from hydantoin and the corresponding penicillamine isomers according to the procedure of Poupaert *et al.* [39]. Purity was checked by a published HPLC method [39]. A K' value of 2.25 was obtained. The material was found to be devoid of 5-isopropylidenehydantoin as well as penicillamine hydantoin disulfides. Within the conditions of the assay, purity was higher than 99%. The melting point was identical to the one given in the literature (m.p. 205–206°).

Preparation of subcellular fractions from rat liver homogenates. Rat livers were homogenized in a 0.25 M sucrose/1 mM EDTA/3 mM imidazole buffer, pH 7.2–7.4. Soluble proteins were obtained by submitting the liver homogenates to a high-speed centrifugation (100,000 g × 35 min) and collecting the resulting supernatants.

The mitochondrial fractions were obtained by submitting the postnuclear supernatants (initial liver extracts from which the nuclear pellet was removed after a 600 g × 10 min centrifugation) to centrifugation forces amounting to 20,000 g during 20 min, and taking the mitochondrial pellet (ML fraction according to de Duve [40]) as the enzyme sources for the assay of mitochondrial glutathione peroxidase.

Dialysis of biological samples. Dialysis was performed using a Nojax dialysis tube and about 5 mL of the enzyme preparations were dialysed overnight against 500 mL of 25 mM Tris buffer, pH 7.4. At the beginning and at the end of the dialysis process, the buffer was tested for its content in SH groups by reactivity with the Ellman's reagent.

RESULTS

Penicillamine hydantoin as a substrate of glutathione-utilizing enzymes

In a first series of experiments, the ability of L-penicillamine hydantoin to replace glutathione as a substrate for enzyme reactions was checked.

As evidenced by monitoring the disappearance of free thiol groups, the penicillamine hydantoin was found to be a substrate of glutathione peroxidase (Fig. 1). The activity recorded on this compound amounted to 4 and 2% of that obtained on glutathione when *tert*-butyl hydroperoxide and hydrogen peroxide were used as the peroxide source, respectively. Comparable results were found with cumene hydroperoxide (Fig. 1), a peroxide which serves as a substrate for not only glutathione peroxidase (EC 1.11.1.9) but also glutathione transferase-catalysed peroxidase activities [31]. Penicillamine hydantoin was apparently inefficient in substituting glutathione as a substrate for glutathione transferases (data not shown).

* Abbreviation: DTNB, 5,5'-dithio-bis-nitrobenzoic acid (Ellman's reagent).

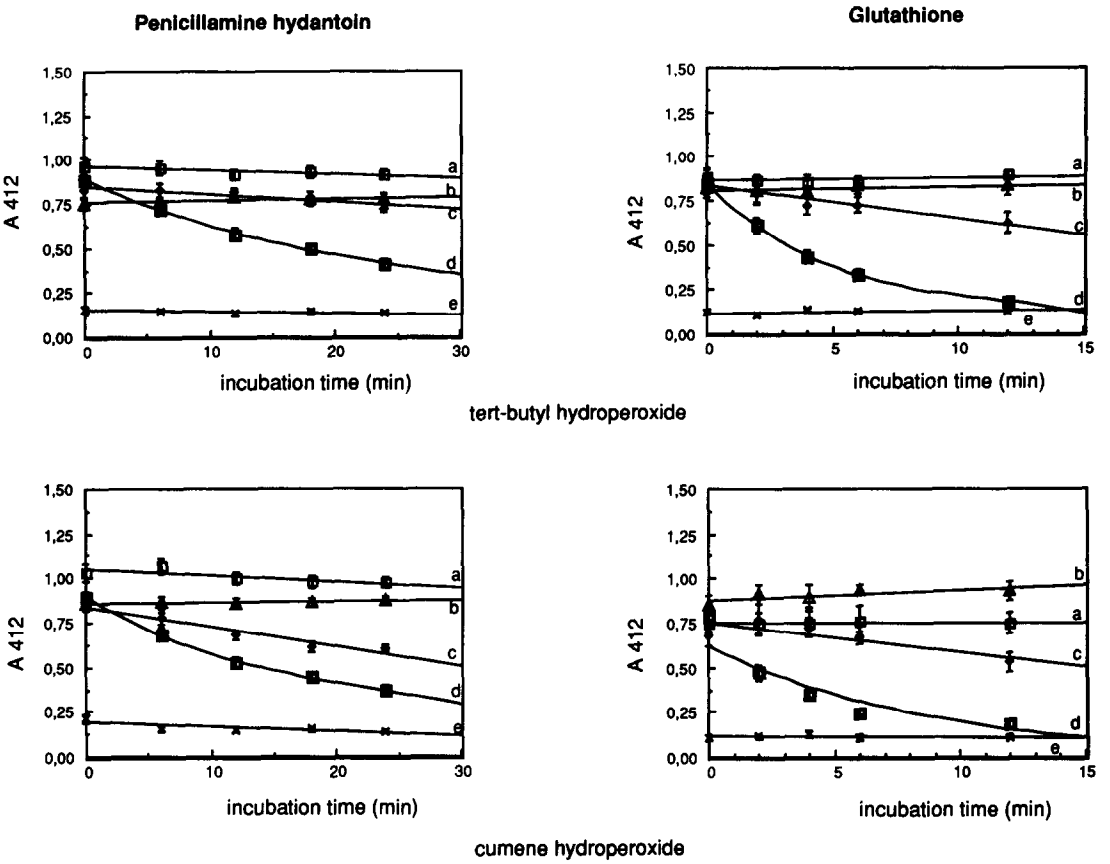

Fig. 1. Peroxide-dependent oxidations catalysed by rat liver soluble proteins of L-penicillamine hydantoin and glutathione. Stable absorbance values obtained at 30° are reported on the ordinates (A_{412}) versus incubation periods. Ten-fold more proteins than in the assay mixtures containing glutathione as a substrate (right panel) were actually used for the assays of L-penicillamine hydantoin oxidation (left panel). (d) SH consumption (from either added glutathione or L-penicillamine hydantoin) occuring in the presence of both peroxide and rat liver soluble proteins; (c) SH consumption rate induced by the peroxide alone; (a) SH consumption induced by the rat liver soluble proteins alone; (b) incubation of added SH-containing molecules in the absence of peroxide and homogenate; (e) incubation of the homogenate with the peroxide. Values are means ± SEM for at least three determinations.

Effect of penicillamine hydantoin on glutathione-utilizing enzymes

In a second series of experiments, the effect of L-penicillamine hydantoin on glutathione-related metabolism was studied.

Glutathione peroxidase activity was inhibited by L-penicillamine hydantoin whatever the nature of the peroxide (H_2O_2, *tert*-butyl hydroperoxide or cumene hydroperoxide) used as a substrate of the reaction.

The activity of glutathione peroxidase expressed as a function of various concentrations of glutathione was measured in the presence of constant amounts of peroxide, with or without added L-penicillamine hydantoin. In the absence of this pharmacomolecule, the apparent K_m and V_{max} of glutathione for the enzyme calculated from Lineweaver–Burk plots varied with the nature of the peroxide utilized. Although comparable values were obtained with H_2O_2 and *tert*-butyl hydroperoxide, different values were recorded in the presence of cumene hydroperoxide (for details, see legend to Fig. 2). In the presence of 100 μM L-penicillamine hydantoin, the enzyme reactions catalysed by glutathione peroxidase were inhibited. As illustrated by the Lineweaver–Burk plot representation, the type of inhibition was mixed since both apparent K_m and V_{max} were affected by the penicillamine hydantoin (Table 1).

By contrast with glutathione peroxidase, neither glutathione transferases, nor glutathione reductase were affected by L-penicillamine hydantoin. γ-Glutamyl-transpeptidase was insensitive to this pharmacomolecule as was the case for glucose-6-phosphate dehydrogenase (data not shown).

Penicillamine hydantoin as an inactivator of glutathione-utilizing enzymes

In the experiments described in this subsection, penicillamine hydantoin is shown to inactivate glutathione peroxidase. It may be stressed that this inactivation process does not interfere with the inhibition process described in the preceding

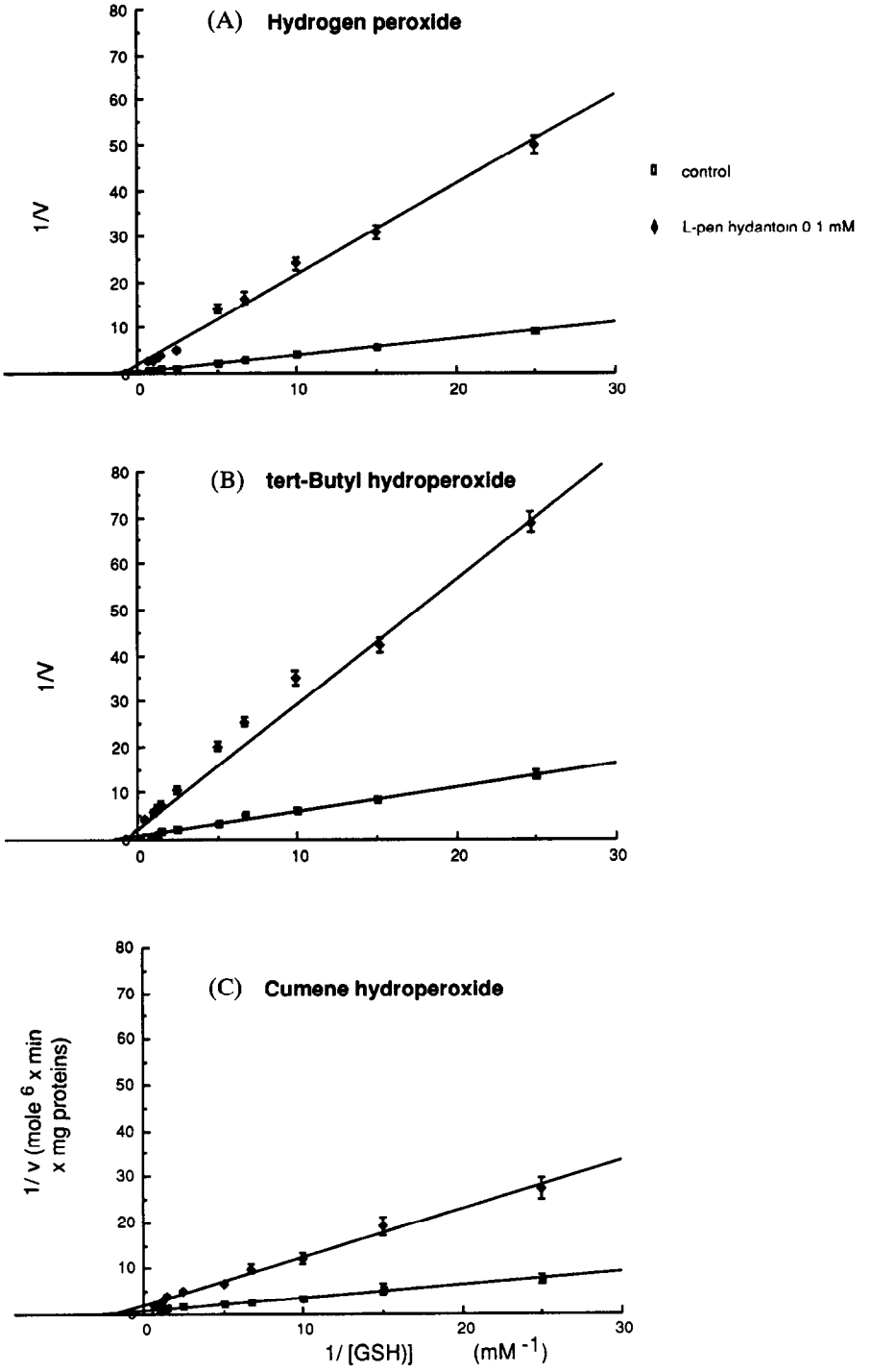

Fig. 2. Effect of 0.1 mM L-penicillamine hydantoin on the apparent V_{max} and K_m of glutathione peroxidase assayed on glutathione and (A) hydrogen peroxide, (B) *tert*-butyl hydroperoxide or (C) cumene hydroperoxide. $1/v$ is reported as a function of 1/[glutathione] (Lineweaver–Burk representation). v is expressed as μmoles glutathione oxidized per min per mg protein. Results are the mean ± SEM of at least three separate experiments.

subsection. Indeed, glutathione is, for the study of the inhibition process, included in the reaction mixture and, as evidenced in the next subsection, then prevents the inactivation process.

For the study of the inactivation of glutathione peroxidase, a 5 min preincubation of rat liver cytosolic fractions was performed at 30° in the presence of L-penicillamine hydantoin prior to

Table 1. Effect of 0.1 mM L-penicillamine hydantoin on the apparent K_m and V_{max} of glutathione peroxidase for glutathione

Peroxide	No penicillamine hydantoin		0.1 mM penicillamine hydantoin	
	K_m	V_{max}	K_m	V_{max}
H_2O_2	622	1783	1122	568
tert-Butyl hydroperoxide	633	1205	1079	353
Cumene hydroperoxide	281	1025	542	516

The enzyme was assayed in the presence or absence of penicillamine hydantoin with either hydrogen peroxide, *tert*-butyl hydroperoxide or cumene hydroperoxide as substrate. The apparent K_m and V_{max} values are expressed as micromolar concentrations and mU/mg protein, respectively.

Results are means of at least three separate experiments ± SEM.

subsequent enzyme assays. By this procedure, glutathione peroxidase was strongly inactivated (by 85%) whatever the nature of the peroxide substrate at concentrations of L-penicillamine hydantoin higher than 50 μM (Fig. 3). At lower concentrations of this pharmacomolecule, a more important residual enzyme activity could be detected after the 5 min preincubation period (see Fig. 3); the time course of this inactivation process recorded within this period is illustrated in Fig. 4.

By submitting the enzyme sources to 250 μM penicillamine hydantoin for a 5 min preincubation period, no inactivation was observed on either glutathione transferase, glutathione reductase or γ-glutamyl-transpeptidase activities.

Prevention and reversal of the inactivation by penicillamine hydantoin of glutathione peroxidase

Addition to the preincubation mixture (in which the effector was included) of free SH-containing molecules such as glutathione, cysteine or dithiothreitol, prevented the inactivation by penicillamine hydantoin of glutathione peroxidase (Fig. 5, closed columns). By contrast, the addition of these thiols after the 5 min preincubation period was unable to recover the enzyme activity (Fig. 5, open columns). Attempts to reverse, at the end of the preincubation with L-penicillamine hydantoin, the enzyme from an inactivated to an activated form were unsuccessful with molecules such as hydantoin (0.25 mM), Triton X-100 (2‰, w/w) or phenol (1 mM), the use of which was aimed at probing the hydrophobic domains of glutathione peroxidase (for further comments, see Discussion). The reversibility of the inactivation process by penicillamine hydantoin has also been studied through dialysis of the enzyme preparation inactivated by the molecule. The dialysis treatment was unable to restore the enzyme activity while it was apparently associated with the removal of penicillamine hydantoin. The dialysis treatment did not significantly affect the activity of glutathione peroxidase preincubated in the absence of penicillamine hydantoin.

Effect of penicillamine hydantoin on glutathione peroxidase assayed with low levels of hydrogen peroxide

To determine whether the inactivation and inhibition properties of penicillamine hydantoin

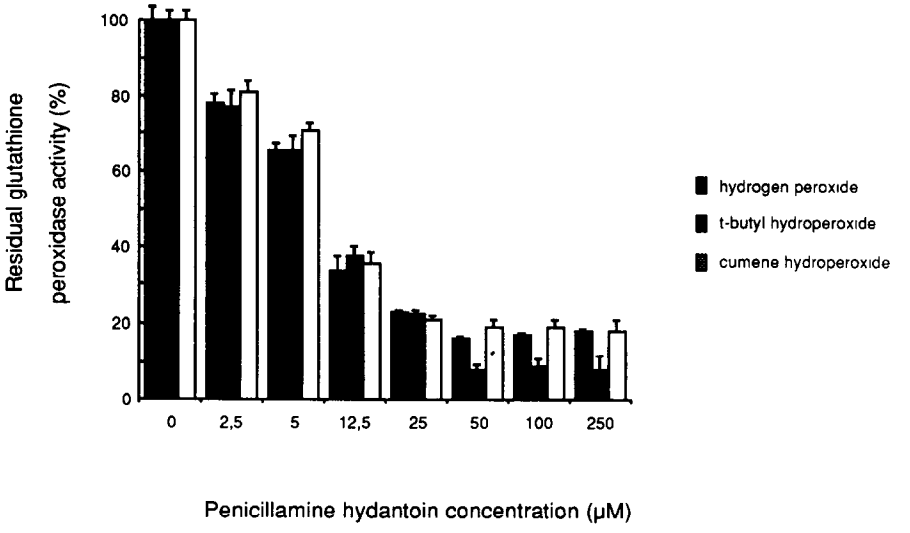

Fig. 3. Effect of a 5 min preincubation of rat liver soluble proteins with various concentrations of L-penicillamine hydantoin on the glutathione peroxidase activities assayed with glutathione and either hydrogen peroxide, *tert*-butyl hydroperoxide or cumene hydroperoxide as substrates. Glutathione peroxidase activity was measured by method B and is, here, expressed as the percentage of the activity recorded in the absence of L-penicillamine hydantoin (concentration equal to 0 μM). Each data point represents the mean ± SEM of at least three separate samples.

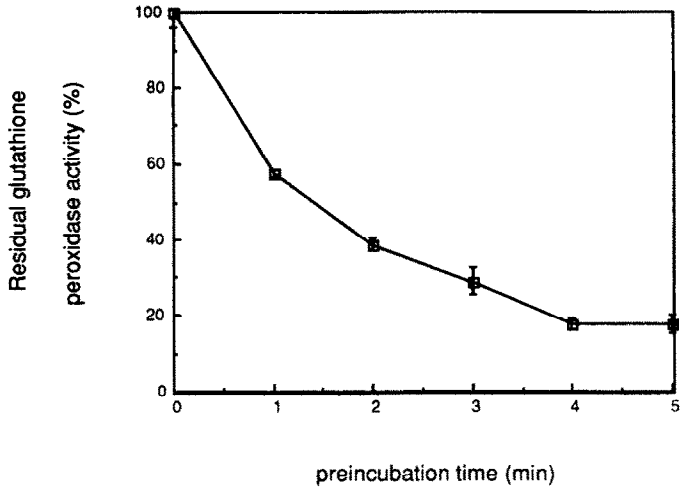

Fig. 4. Time-course of the inactivation by L-penicillamine hydantoin of glutathione peroxidase. L-Penicillamine hydantoin (25 μM) was included in the preincubation mixture. The enzyme activity was measured according to method B. Values are the means ± SEM of triplicate determinations.

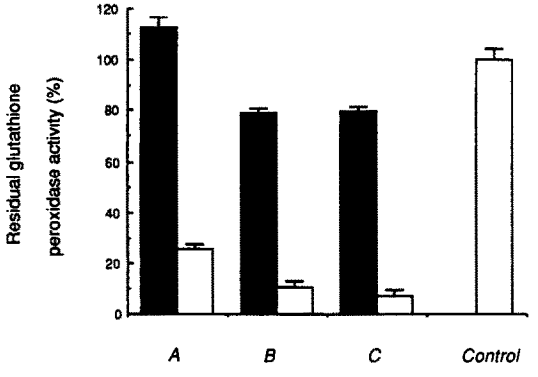

Fig. 5. Inactivation of glutathione peroxidase by L-penicillamine hydantoin in various experimental conditions. (A–C) Rat liver cytosolic proteins were preincubated at 30° in the presence of 50 μM L-penicillamine hydantoin for a 5 min period prior to the glutathione peroxidase assay. (A) No addition of molecules other than penicillamine hydantoin and classical reagents of the assay mixture. Glutathione (as 1 mM substrate) has been included in either the preincubation (closed columns) or the incubation (open columns) mixtures. (B) Addition of 1 mM cysteine in the preincubation (closed columns) or the incubation (open columns) mixtures. (C) Addition of 1 mM dithiothreitol in the preincubation (closed columns) or the incubation (open columns) mixtures. Control: the rat liver cytosolic proteins were preincubated for 5 min at 30° in the absence of pharmacomolecule or substrate before measuring glutathione oxidation rates (referred to as 100%). Data points represent the means ± SEM of at least three measurements.

could be relevant in the physiological conditions occurring in the intact cells, the molecule was tested on hydrogen peroxide levels lower than 50 μM. Figure 6 illustrates the results obtained when glutathione peroxidase is either incubated or preincubated with penicillamine hydantoin and assayed with H_2O_2 concentrations ranging from 10 to 250 μM. At low concentrations in H_2O_2 (10–50 μM), penicillamine hydantoin is still efficient at inactivating and inhibiting glutathione peroxidase.

Others

The original results presented above have been here illustrated for the use of the L-isomer of penicillamine hydantoin, only. Roughly similar inhibition and inactivation properties were observed with the D-isomer and the racemic mixture. No significant effect of the hydantoin molecule could be detected whereas DL-penicillamine exhibited inhibition and inactivation properties like penicillamine hydantoin. Interestingly enough, DL-penicillamine, although it displayed on glutathione peroxidase activities an inactivation whose amplitude (23–26% residual activities upon 50 μM DL-penicillamine) was of the same order of magnitude as that observed with the penicillamine hydantoin (8–20% of residual activities upon 50 μM DL-penicillamine hydantoin), was unable to reproduce thoroughly the inhibition achieved by its hydantoin derivatives. At a 250 μM concentration, the penicillamine was capable of inhibiting glutathione peroxidase by less than 5%, only, while the activities of this enzyme were at least halved by a similar concentration of the penicillamine hydantoin (data not shown). On the other hand, the mitochondrial glutathione peroxidase was also found to be, to a similar extent, sensitive to the inhibition and inactivation properties of the penicillamine hydantoin isomers (data not shown).

In order to know whether the impairment of glutathione peroxidase activity was a very specific feature or not, the sensitivity to penicillamine hydantoin has been also studied in whole liver homogenates on marker enzyme activities from various subcellular compartments including lactate

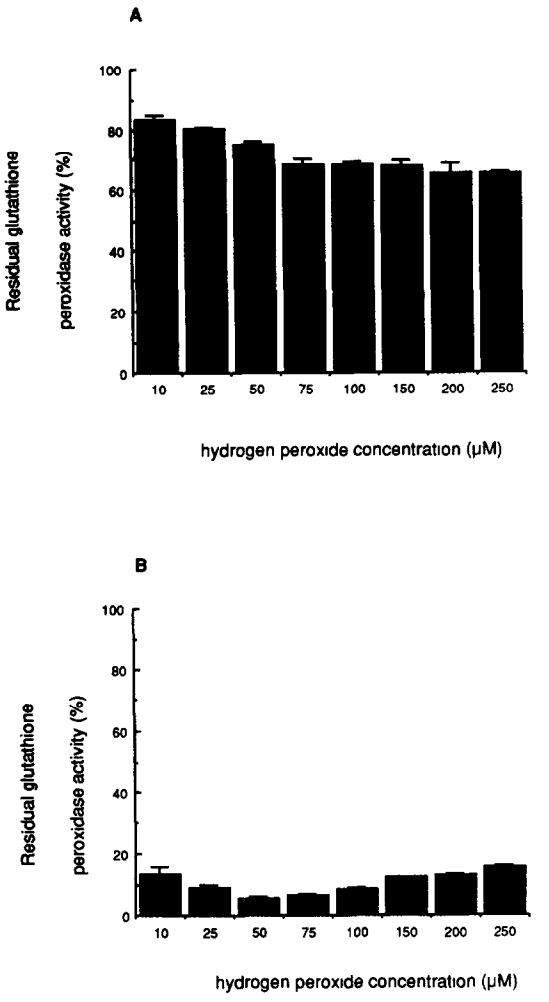

Fig. 6. Effect of L-penicillamine hydantoin on glutathione peroxidase activity assayed on low levels of hydrogen peroxide. (A) Inhibition process; (B) Inactivation process. Results are means of at least three separate experiments ± SEM.

dehydrogenase (cytosol), *N*-acetyl-β-glucosaminidase (lysosomes), arylsulfate C (microsomes), butyryl-CoA dehydrogenase (mitochondria), lauryl-CoA and glycolate oxidases (peroxisomes). With 0.1 mM L-penicillamine hydantoin neither lactate dehydrogenase, nor arylsulfatase C activities were affected. *N*-Acetyl-β-glucosaminidase was not inhibited but was inactivated by 10%. Lauryl-CoA and glycolate oxidases were inhibited by 10% but could not be inactivated. Butyryl-CoA dehydrogenase activity was increased 1.4- and 1.9-fold upon incubation and preincubation with the effector, respectively. Increasing the concentration of penicillamine hydantoin to a 0.25 mM value was without any significant additional effect on these marker enzyme activities, except for lactate dehydrogenase inhibited by about 10–20% and the two peroxisomal oxidases more severely inhibited (by 20%) but not inactivated. The exact nature of the last effect recorded on the peroxisomal oxidases could be questioned since these enzymes were assayed by substrate-dependent hydrogen peroxide production and the indirect measurement of lauryl-CoA oxidase i.e. cyanide-insensitive lauryl-CoA oxidation (third step of peroxisomal β-oxidation) was insensitive to penicillamine hydantoin.

DISCUSSION

This work provides the first observation that L-penicillamine hydantoin affects selectively one of the glutathione-utilizing enzymes namely glutathione peroxidase, but neither glutathione reductase nor glutathione transferases. L-Penicillamine hydantoin displays a mixed inhibition profile. The competitive feature can be explained by a competition between glutathione and L-penicillamine hydantoin for the binding to the catalytic center of glutathione peroxidase. The non-competitive part of the inhibition is less clear and could correspond to the inactivation process which involves penicillamine hydantoin.

The inactivation process severely impairs the oxidation of glutathione (see Figs 3 and 4) but apparently not that of penicillamine hydantoin (see Fig. 1). This is likely to result from structural modifications of glutathione peroxidase inducing steric constraints which impair access to the catalytic center of glutathione but not necessarily L-penicillamine hydantoin. This inactivation process apparently requires, as an obligatory step, the interaction of the enzyme with the thiol function of L-penicillamine hydantoin. Indeed, the putative structural modifications appear to be either prevented by other SH-containing molecules or induced by penicillamine but not hydantoin. At this stage, it is unknown whether the pharmacomolecule–enzyme interaction implies the formation of a disulfide link or a selenium–thiol bond and whether this interaction primarily involves the catalytic center or another site located elsewhere in the protein. Nevertheless, the part of the protein which is the target for the inactivation effect of L-penicillamine hydantoin appears to be distinct from that which is concerned by the inhibition. The latter is apparently correlated with the conformational analogy which exists between L-penicillamine hydantoin and the natural substrate (i.e. glutathione) (Mestdagh *et al.*, unpublished work) while the former could be well accounted for by the penicillamine moiety of the pharmacomolecule. Furthermore, penicillamine is capable of replicating the inactivation but not the inhibition properties of L-penicillamine hydantoin on glutathione peroxidase. When in the inactivated form, the enzyme appears to exhibit residual glutathione peroxidase activity (see Figs 3 and 4), a feature which could be accounted for by either the existence of another cytosolic glutathione peroxidase or by the non-absolute restriction of glutathione access to the catalytic center of the protein. Confirming the last hypothesis is the fact that the enzyme, in the presence of L-penicillamine hydantoin, remains active up to 5 min when this pharmacomolecule is used as a substrate (see Fig. 1) indicating that the enzyme is not fully inactivated.

Hydrophobic interactions are commonly used to purify glutathione peroxidase [41–44], attesting to

the existence of essential hydrophobic domains in the protein. Since L-penicillamine hydantoin has an amphiphilic character, the effects of hydrophilic (hydantoin), lipophilic (phenol) and amphiphilic (detergents) compounds were studied. As none of these molecules could reverse the enzyme inactivation, it appears that neither hydrophobic forces nor hydrogen bonds play a critical role. This is in agreement with the establishment of a covalent bond between L-penicillamine hydantoin and the protein.

In view of its peptidomimetic nature and therefore its increased metabolic resistance, L-penicillamine hydantoin appears to be of value in *in vivo* studies. Work is currently being done to define its potential in the reversal of chemoresistance, via depression of glutathione peroxidase activity and subsequent decrease in hydrogen peroxide detoxifying capacity.

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REFERENCES

1. Kosower N and Kosower E, The glutathione status of cells. *Int Rev Cytol* **54**: 109–160, 1978.
2. Meister A, Selective modification of glutathione metabolism. *Science* **220**: 472–477, 1983.
3. Deleve L and Kaplowitz N, Importance and regulation of hepatic glutathione. *Seminars Liver Dis* **10**: 4, 251–266, 1990.
4. Mannervik B, Roles and functions of glutathione. *Biochem Soc Trans* **15**: 717–719, 1987.
5. Orrenius S and Moldéus P, The multiple roles of glutathione in drug metabolism. *TIPS* **5**: 432–435, 1984.
6. Halliwell B, Oxidants and human disease: some new concepts. *FASEB J* **1**: 358–364, 1987.
7. Coles B and Ketterer B, The role of glutathione and glutathione transferases in chemical carcinogenesis. *Crit Rev Biochem Mol Biol* **25**: 47–70, 1990.
8. Mantel T, McCusker F, Phillips M and Boyce S, Glutathione *S*-transferases. *Biochem Soc Trans* **18**: 175–177, 1990.
9. van Bladeren P, Formation of toxic metabolites from drugs and other xenobiotics by glutathione conjugation. *TIPS* **9**: 295–299, 1988.
10. Vos R and van Bladeren P, Glutathione *S*-transferases in relation to their role in the biotransformation of xenobiotics. *Chem Biol Interact* **75**: 241–265, 1990.
11. Halliwell B and Gutteridge J, Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch Biochem Biophys* **246**: 501–514, 1986.
12. Halliwell B and Gutteridge J, Iron and free radical reactions: two aspects of antioxidant protection. *TIBS* **11**: 372–375, 1986.
13. Cervantes A, Pinedo H, Lankelma J and Schuurhuis G, The role of oxygen-derived free radicals in the cytotoxicity of doxorubicin in multidrug-resistant and sensitive human ovarian cancer cells. *Cancer Lett* **41**: 169–177, 1988.
14. Sinha B, Katki A, Batist G, Cowan K and Myers C, Differential formation of hydroxyl radicals by adriamycin in sensitive and resistant MCF-7 human breast tumor cells: implications for the mechanism of action. *Biochemistry* **26**: 3776–3781, 1987.
15. Turner M, Everman D, Ellington S and Fields C, Detection of free radicals during the cellular metabolism of adriamycin. *Free Rad Biol Med* **9**: 415–421, 1990.
16. Fisher G, Brown J and Patterson L, Involvement of hydroxyl radical formation and DNA strand breakage in the cytotoxicity of anthraquinone antitumor agents. *Free Rad Res Commun* **11**: 117–125, 1990.
17. Chacon E and Acosta D, Mitochondrial regulation of superoxide by Ca^{2+}: an alternate mechanism for the cardiotoxicity of doxorubicin. *Toxicol Appl Pharmacol* **107**: 117–128, 1991.
18. Hayes J and Wolf C, Molecular mechanism of drug resistance. *Biochem J* **272**: 281–295, 1990.
19. Tsuruo T, Mechanisms of multidrug resistant and implications for therapy. *Jpn J Cancer Res* **79**: 285–296, 1988.
20. Lee FYF, Sciandra J and Siemann D, A study of the mechanism of resistance to Adriamycin® *in vivo*. Glutathione metabolism, P-glycoprotein expression, and drug transport. *Biochem Pharmacol* **38**: 3697–3705, 1989.
21. Wheeler C, Rader R and Kessel D, Membrane alterations associated with progressive adriamycin resistance. *Biochem Pharmacol* **31**: 2691–2693, 1982.
22. Deffie A, Alam T, Seneviratne C, Beenken S, Batra J, Shea T, Henner W and Goldenberg G, Multifactorial resistance to adriamycin: relationship of DNA repair, glutathione transferase activity, drug efflux, and P-glycoprotein in cloned cell lines of adriamycin-sensitive and resistant P388 leukemia. *Cancer Res* **48**: 3595–3602, 1988.
23. Mimnaugh E, Dusre L, Atwell J and Myers C, Differential oxygen radical susceptibility of adriamycin-sensitive and -resistant MCF-7 human breat tumor cells. *Cancer Res* **49**: 8–15, 1989.
24. Singh SV, Iqbal J and Krishan A, Cytochrome P450 reductase, antioxidant enzymes and cellular resistance to doxorubicin. *Biochem Pharmacol* **40**: 385–387, 1990.
25. Goodman G, Yen Y, Cox T and Crowley J, Effect of verapamil on *in vitro* cytotoxicity of adriamycin and vinblastine in human tumor cells. *Cancer Res* **47**: 2295–2304, 1987.
26. Yusa K and Tsuruo T, Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Res* **49**: 5002–5006, 1989.
27. Kramer R, Zakher G and Kim J, Role of the glutathione redox cycle in acquired and *de novo* multidrug resistance. *Science* **241**: 694–697, 1988.
28. Somfai-Relle S, Suzukake K, Vistica BP and Vistica DT, Reduction in cellular glutathione by buthionine sulfoximine and sensitization of murine tumor cells resistant to L-phenylalanine mustard. *Biochem Pharmacol* **33**: 485–490, 1984.
29. Hamilton TC, Winker MA, Louie KG, Batist G, Behrens BC, Tsuruo T, Grotzinger KR, McKoy WM, Young RC and Ozols RF, Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem Pharmacol* **34**: 2583–2586, 1985.
30. Hoskins LK, Whelan RDH, Shellard SA, Bedford P and Hill BT, An evaluation of the role of glutathione and its associated enzymes in the expression of differential sensitivities to antitumor agents shown by a range of human tumour cell lines. *Biochem Pharmacol* **40**: 1833–1842, 1990.
31. Carlberg I and Mannervik B, Purification and charac-

terization of the flavoenzyme glutathione reductase from rat liver. *J Biol Chem* **250**: 5475–5480, 1975.
32. Carmagnol F, Sinet P and Jerome H, Selenium-dependent and non-selenium dependent glutathione peroxidases in human tissue extracts. *Biochim Biophys Acta* **759**: 49–57, 1983.
33. Lawrence R and Burk R, Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun* **71**: 952–958, 1976.
34. Habig W and Jakoby W, Assays for differentiation of glutathione *S*-transferases. *Methods Enzymol* **77**: 398–400, 1981.
35. Szasz G, A kinetic photometric method for serum γ-glutamyl transpeptidase. *Clin Chem* **15**: 124–136, 1969.
36. Levy H and Christoff M, A critical appraisal of the effect of oxidized glutathione on hepatic glucose 6-phosphate dehydrogenase activity. *Biochem J* **214**: 959–965, 1983.
37. Vamecq J and Van Hoof F, Implication of a peroxisomal enzyme in the catabolism of glutaryl-CoA. *Biochem J* **221**: 203–211, 1984.
38. Vamecq J, Draye J-P and Brison J, Rat liver metabolism of dicarboxylic acids. *Am J Physiol* **256**: 6680–6688, 1989.
39. Poupaert J, Mukarugambwa S and Dumont P, Search for pro-drug forms of D-penicillamine: synthesis and metabolism of D-penhydantoin. *Eur J Med Chem* **15**: 511–514, 1980.
40. de Duve C, Exploring cells with a centrifuge. *Science* **189**: 186–194, 1975.
41. Forward R and Almoy R, Separation of two forms of glutathione peroxidase from human erythrocytes by hydrophobic chromatography. *J Chromatogr* **330**: 383–387, 1985.
42. Maddipati K and Marnett L, Characterization of the major hydroperoxide-reducing activity of human plasma. Purification and properties of a selenium-dependent glutathione peroxidase. *J Biol Chem* **262**: 17398–17403, 1987.
43. Li N, Reddy P, Thyagaraju K, Reddy A, Hsa B, Scholz R, Tu C and Reddy C, Elevation of rat liver mRNA for selenium-dependent glutathione peroxidase by selenium deficiency. *J Biol Chem* **265**: 108–113, 1990.
44. Stepanik T and Ewing D, Coisolation of glutathione peroxidase, catalase and superoxide dismutase from human erythrocytes. *J Biochem Biophys Methods* **20**: 157–169, 1990.