Biochemical Pharmacology, Vol. 35, No. 18, pp. 3143–3146, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

INHIBITION OF GLUTATHIONE PEROXIDASE AND GLUTATHIONE TRANSFERASE IN MOUSE LIVER BY MISONIDAZOLE

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(*Received* 22 *July* 1985; *accepted* 6 *February* 1986)

Abstract—The mechanisms of toxicity and sensitization by the radiosensitizer misonidazole [1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol] are not well understood. We report here on the inhibition of total glutathione peroxidase (GSHPx), selenium-dependent glutathione peroxidase (selenium-GSHPx) and glutathione transferase (GSHTx) activities by misonidazole. Mouse liver cytosol GSHPx and selenium-GSHPx were inhibited *in vitro* with 0.5 mM misonidazole. On administration of the drug intraperitoneally (800 mg/kg) to mice, it was found that GSHPx, selenium-GSHPx, and GSHTx were inhibited in homogenate, cytosol, and microsomal fractions of mouse liver. GSHPx was depressed in all fractions up to 60–70% of control values, with maximum depression occurring in the cytosol and homogenate fractions in less than 2 hr. Recovery of activity was slower in the microsomes. In general, the pattern of depression of selenium-GSHPx was parallel to that of GSHPx except in microsomes, where GSHPx is minimal. Quantitatively, selenium-GSHPx was least affected. GSHTx was inhibited 70–80% of control values in cytosol and homogenate with recovery by 24 hr, whereas a second period of depression occurred at 24 hr in the microsomes. The inhibition of peroxide-metabolizing enzymes may lead to elevation of intracellular peroxide levels, contributing to the radiosensitizing effect and/or toxicity of misonidazole.

Misonidazole [1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol] is an imidazole derivative under investigation as a radiosensitizer of hypoxic tumor cells [1]. The use of this drug in radiation therapy of cancer is limited by its toxicity [2], manifested mainly as neuropathies [3, 4].

The mechanisms of radiosensitization and toxicity of misonidazole are relatively unknown. The metabolism of misonidazole depends on the oxygen content of tissues, and the metabolites formed result in sensitizing or toxic effects [5]. In hypoxic cells, reductive metabolites are formed, which can interact with critical biomolecules, such as DNA [6], and under aerobic conditions reactive oxygen species can be generated [5]. Misonidazole metabolites formed under anaerobic conditions (or by chemical reduction) can conjugate with glutathione (GSH) [5, 7], decreasing the effective concentration of this intracellular thiol vital for the detoxification of peroxides. We have examined the effects of misonidazole on the enzymes that generally act on GSH, namely, glutathione peroxidase (EC 1.11.1.9, total and selenium-dependent) and glutathione transferase (EC 2.5.1.18), and the results are presented in this paper.

MATERIALS AND METHODS

Misonidazole was dissolved in warm, neutral saline and administered (800 mg/kg body wt) intraperitoneally to male CD2F1 mice. At various times after drug injection, each animal was anesthetized with nembutal and its liver was perfused *in situ* with saline. Liver homogenates (10%) were prepared in 0.25 M sucrose, and the combined mitochondrial-nuclear fraction was sedimented at 10,000 *g* for 20 min. Cytosol and microsomal fractions were obtained by centrifuging the postmitochondrial supernatant fraction at 105,000 *g* for 1 hr. These fractions were diluted, and the assay system for glutathione peroxidase contained 0.0250, 0.0700 and 0.0080 mg protein of homogenate, microsomes and cytosol respectively. For the glutathione transferase assay, the respective amounts of the fractions used were 0.0042, 0.0170 and 0.0013 mg protein.

For *in vitro* studies, the 105,000 *g* liver cytosol fraction from control mice was used as the source of glutathione peroxidase. Misonidazole was added to the assay system to test any direct effect of the drug on glutathione peroxidase (GSHPx) activity in mouse liver. The direct effect of the drug on mouse liver glutathione transferase (GSHTx) activity was not tested.

GSHPx was assayed according to the method of Lawrence and Burk [8] with modifications. The assay system in 0.9 ml contained 0.5 ml of 0.2 M potassium phosphate buffer (pH 7.0), 0.5 units glutathione reductase, 1 mM GSH, 0.16 μmoles NADPH, 1.0 mM sodium azide, tissue extract, and water. The reaction was initiated by the addition of 0.1 ml of 15 mM cumene hydroperoxide, and the absorbance change was recorded every minute at 340 nm using a Cary–Varian spectrophotometer. The use of cumene hydroperoxide as a substrate provides for the determination of total glutathione peroxidase activity, which includes selenium-dependent glutathione peroxidase. The selenium-dependent glutathione peroxidase (selenium-GSHPx) activity was assayed sep-

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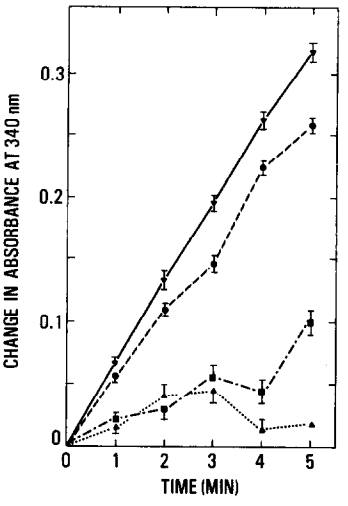

Fig. 1. Inhibition of mouse liver cytosol total glutathione peroxidase *in vitro* by misonidazole. Mouse liver cytosol fraction was incubated with misonidazole at various concentrations for 2 min and assayed with cumene hydroperoxide. Values given are means of five separate determinations ± S.E.M. Key: (▼——▼) control; misonidazole concentrations: (●——●) 0.2 mM; (■—·—■) 0.4 mM; and (▲----▲) 0.5 mM.

arately using 0.1 ml of 2.5 mM hydrogen peroxide (H_2O_2) instead of cumene hydroperoxide [8]. Glutathione transferase activity was assayed by the method of Habig *et al.* [9] by the nucleophilic attack of glutathione anion on 1-chloro-2,4-nitrobenzene measured at 340 nm. The amount of GSH added was in excess so that any direct effect of misonidazole on GSH could not be a factor in the glutathione peroxidase measurement. The amount of glutathione reductase was also in excess so that any decrease in this enzyme would not be interpreted as a depression in glutathione peroxidase. This was determined in a separate study of the effects of misonidazole on pure glutathione reductase (conversion of GSSG to GSH). Misonidazole did not have any effect on the absorption of NADPH used in the assay system.

Misonidazole was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. All other reagents were purchased from the Sigma Chemical Co., St. Louis, MO.

RESULTS

When misonidazole was tested *in vitro* for its effect on glutathione peroxidase at a concentration of 0.2 mM, a significant ($P < 0.05$) inhibition of total GSHPx was seen from 2 min onward, compared to control activity. Almost total inhibition of the activity was seen at 0.4 and 0.5 mM (Fig. 1). Higher concentrations of the drug could not be tested due to its absorbance at 340 nm. The effect of misonidazole on selenium-GSHPx was not significant at 0.2 mM (Fig. 2). At 0.5 mM it was inhibited significantly, but even at this concentration the inhibition was less than that observed for GSHPx with 0.4 mM misonidazole. Increasing the GSH concentration from 1 to 5 mM, 10-fold more than the highest concentration of misonidazole used (0.5 mM), did not relieve the inhibition.

The data for GSHPx, selenium-GSHPx, and GSHTx in the homogenate, microsomes, and cytosol fractions of liver from control mice (saline administered) and from mice killed at different periods after the administration of misonidazole are given in Tables 1, 2, and 3 respectively. In general, total GSHPx activity (Table 1) decreased significantly at 0.5 hr after the drug administration, and the decrease persisted up to 12 hr after the drug was given. Significant inhibition of selenium-dependent GSH-Px activity (Table 2) was seen in cytosol and homogenate at 1 hr and inhibition at some later periods. The inhibition, compared to control, was less than that observed with GSHPx. Selenium-GSHPx in the microsomes was insufficient to assay.

GSHTx was also inhibited, with the greatest inhibition observed in the cytosol fraction, although recovery was seen at 12 hr. In microsomes, GSHTx was inhibited at 3 hr and a second period of depression occurred at 24 hr.

Table 1. Total glutathione peroxidase activity in mouse liver fractions at various periods after administration of misonidazole (800 mg/kg body wt)

Period after treatment (hr)	Enzyme activity (units/mg protein) in: Homogenate		Microsomes		Cytosol	
	Saline	Misonidazole	Saline	Misonidazole	Saline	Misonidazole
0.5	0.58 ± 0.01	0.48 ± 0.02*	0.22 ± 0.01	0.15 ± 0.01*	1.46 ± 0.04	1.15 ± 0.01†
1.0	ND	0.36 ± 0.01†§	0.19 ± 0.01	0.10 ± 0.01‡	ND	0.75 ± 0.04†§
2.0	0.62 ± 0.02	0.39 ± 0.06‡	0.21 ± 0.02	0.15 ± 0.01‡	1.50 ± 0.09	1.08 ± 0.02*
3.0	0.55 ± 0.05	0.49 ± 0.02	ND	ND	1.45 ± 0.06	1.05 ± 0.04†
4.0	ND	0.41 ± 0.02§	0.29 ± 0.02	0.14 ± 0.01†	1.34 ± 0.12	0.98 ± 0.05‡
6.0	0.61 ± 0.01	0.49 ± 0.04‡	0.17 ± 0.01	0.08 ± 0.02*	1.39 ± 0.03	1.22 ± 0.04*
12.0	0.56 ± 0.02	0.39 ± 0.01†	0.29 ± 0.04	0.21 ± 0.03	1.28 ± 0.06	0.99 ± 0.03*
24.0	0.62 ± 0.03	0.54 ± 0.03	0.24 ± 0.02	0.15 ± 0.01*	1.27 ± 0.05	1.15 ± 0.04

Values are expressed as mean of activities from four to six animals ± S.E.M. ND = not determined.
*–‡ Significantly different compared with saline value: *$P < 0.01$, †$P < 0.001$, and ‡$P < 0.05$.
§ These values were compared with mean of all saline (control) values of respective fractions.

Table 2. Selenium-dependent glutathione peroxidase activity in mouse liver fractions at various periods after administration of misonidazole (800 mg/kg body wt)

Period after treatment (hr)	Enzyme activity (units/mg protein) in: Homogenate		Cytosol	
	Saline	Misonidazole	Saline	Misonidazole
0.5	0.36 ± 0.02	0.39 ± 0.02	0.86 ± 0.05	0.74 ± 0.07
1.0	0.42 ± 0.02	0.29 ± 0.01*	0.86 ± 0.05	0.59 ± 0.08†
2.0	0.43 ± 0.02	0.42 ± 0.05	0.89 ± 0.02	0.75 ± 0.03‡
3.0	0.44 ± 0.03	0.44 ± 0.03	1.02 ± 0.08	0.76 ± 0.06†
4.0	0.35 ± 0.02	0.38 ± 0.02	1.00 ± 0.06	1.00 ± 0.03
6.0	0.49 ± 0.01	0.35 ± 0.04†	1.12 ± 0.06	0.99 ± 0.08
12.0	0.42 ± 0.03	0.41 ± 0.02	0.93 ± 0.04	0.93 ± 0.07
24.0	0.44 ± 0.01	0.40 ± 0.03	1.25 ± 0.09	1.15 ± 0.03

Values are expressed as mean of activities from four to six animals ± S.E.M.
*–‡ Significantly different compared with saline value: *$P < 0.001$, †$P < 0.05$, and ‡$P < 0.01$.

DISCUSSION

Glutathione peroxidase is a selenoenzyme that can detoxify hydrogen peroxide or organic hydroperoxides [10]. When enzyme activity is assayed with organic hydroperoxides like cumene hydroperoxide, the activity obtained includes a non-selenium-dependent component that was later shown to be associated with glutathione transferase [11]. In the present study, GSHPx (assayed with cumene hydroperoxide) was more sensitive to misonidazole *in vitro* and *in vivo* than selenium-GSHPx (assayed with H_2O_2). GSHTx was also inhibited by misonidazole *in vivo*. The pattern of inhibition of GSHPx was somewhat similar to GSHTx in the liver fractions, but the quantitative results suggest that the GSHTx component of GSHPx is more affected than the selenium-GSHPx.

The aerobic and anaerobic metabolism of misonidazole appear to result in different end products [5], probably explaining its sensitizing and toxic effects. Hypoxic reduction of misonidazole by microsomes is known to produce a radical anion [12], which may be responsible for the formation of adducts with glutathione or DNA [6, 7]. Aerobic metabolism of heterocyclic nitrocompounds produces superoxide, which may undergo interconversions to other reactive oxygen species [13]. The *in vitro* inhibition of glutathione peroxidase and *in vivo* inhibition of glutathione peroxidase and glutathione transferase may be due to the direct reaction of protein thiols of these enzymes with misonidazole. However, this reaction would be more likely under anaerobic conditions where both protein and nonprotein thiols react with misonidazole [5]. Under the conditions of the present experiments, it is unlikely that depression of GSH concentration contributed to the decrease in enzyme activity. The inhibition (*in vivo*) may also be related to the cyclic reduction–oxidation of the nitro-radical anion [14, 15] of misonidazole. The probable generation of superoxide from this cyclic reaction may result in the inhibition of the enzyme although it is reported that misonidazole is not as good a promoter of superoxide formation [12] or of oxygen consumption as other heterocyclic nitro compounds [5]. The greater inhibition of the microsomal glutathione peroxidase and transferase may be due to the activation of misonidazole in the microsomes [12] and its

Table 3. Glutathione transferase activity in mouse liver fractions at various periods after administration of misonidazole (800 mg/kg body wt)

Period after treatment (hr)	Enzyme activity (units/mg protein) in: Homogenate		Microsomes		Cytosol	
	Saline	Misonidazole	Saline	Misonidazole	Saline	Misonidazole
0.5	1.92 ± 0.06	1.80 ± 0.05	0.25 ± 0.01	0.27 ± 0.01	5.50 ± 0.07	5.70 ± 0.14
1.0	1.92 ± 0.06	1.92 ± 0.09	0.21 ± 0.01	0.18 ± 0.04	6.41 ± 0.14	5.03 ± 0.14*
2.0	ND	1.50 ± 0.19‡	0.22 ± 0.01	0.22 ± 0.01	5.80 ± 0.13	4.97 ± 0.07*
3.0	1.90 ± 0.12	1.70 ± 0.08	ND	0.12 ± 0.01*‡	5.99 ± 0.11	4.09 ± 0.08*
4.0	ND	1.40 ± 0.04‡	0.22 ± 0.01	0.21 ± 0.01	ND	4.46 ± 0.08*‡
6.0	2.20 ± 0.04	1.80 ± 0.09†	0.21 ± 0.01	0.19 ± 0.01	6.30 ± 0.19	4.17 ± 0.06*
12.0	1.94 ± 0.03	1.82 ± 0.06	0.21 ± 0.01	0.19 ± 0.01	6.09 ± 0.15	5.90 ± 0.17
24.0	1.97 ± 0.07	1.94 ± 0.06	0.24 ± 0.01	0.13 ± 0.01*	6.15 ± 0.08	6.26 ± 0.12

Values are expressed as mean of activities from four to six animals ± S.E.M. ND = not determined.
*,† Significantly different compared with saline value: *$P < 0.001$, and †$P < 0.01$.
‡ These values were compared with mean of all saline (control) values of respective fractions.

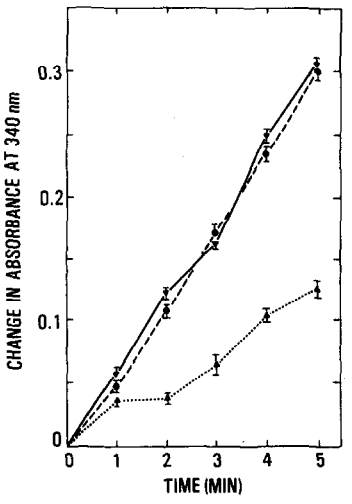

Fig. 2. Inhibition of mouse liver cytosol selenium-dependent glutathione peroxidase *in vitro* by misonidazole. Mouse liver cytosol fraction was incubated with misonidazole at various concentrations for 2 min and assayed with hydrogen peroxide. Values given are means of five separate determinations ± S.E.M. Key: (▼——▼) control; misonidazole concentrations: (●——●) 0.2 mM; and (▲----▲) 0.5 mM.

subsequent binding to these enzymes. Alternatively, the enzyme may be irreversibly bound at the active site with glutathione–misonidazole conjugate.

The inhibition of these two enzymes may be one of the causes of the elevation of hydrogen peroxide levels reported in the presence of misonidazole [5]. The present investigation may provide more insight into toxicity resulting from misonidazole treatment than on sensitization, which is limited more likely to hypoxic cells. Small increases in lysosomal enzyme activity [16] in the nerve region and the increase in lysosomal membrane permeability [17] in the presence of misonidazole may be due to an increase in lipid peroxidation resulting from elevated peroxide level. Thus, one of the probable mechanisms of action of misonidazole may be the susceptibility of tissue to lipid peroxidation due to the inhibition of glutathione peroxidase and glutathione transferase. The inhibition of these enzymes and the lipophilicity of the drug could contribute to the neuropathies observed in the use of misonidazole. Further studies on neural tissue after treatment with misonidazole are needed to obtain direct evidence for this view.

Acknowledgements—We are grateful to William E. Jackson III for performing statistical analyses. Dr. Kumar is a National Research Council-AFRRI Research Associate. This research was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under Work Unit MJ00026. The views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred. Research was conducted according to the principles enunciated in the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council.

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