Biochemical Pharmacology, Vol. 37, No. 3, pp. 473–479, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

ROLE OF NONPROTEIN THIOLS IN ENZYMATIC REDUCTION OF 2-NITROIMIDAZOLES*

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(*Received* 29 *October* 1986; *accepted* 30 *June* 1987)

Abstract—The role of nonprotein thiols (NPSH) in the enzymatic reduction of the nitro function in 2-nitroimidazoles (2-NI) has been investigated. The addition of NPSH has been shown previously to protect cells from the hypoxic cytotoxicity of 2-NI, whereas depletion of NPSH enhances the hypoxic cytotoxicity. In this report, we have investigated the effects of thiol depleting agents, *N*-ethylmaleimide (NEM) and diethyl maleate (DEM), on the enzymatic reduction of the nitro group. Cytosolic and microsomal fractions of rat hepatic tissue and xanthine oxidase were employed as sources of nitro reductases. Addition of NPSH caused an enhancement in the reduction of the nitro group of 2-NI; cysteine was significantly more effective than glutathione (GSH) in stimulating the enzymatic reduction. The reduction of the nitro function was decreased markedly in the presence of NEM or DEM. Addition of cysteine or GSH reversed the inhibition with NEM. Both NEM and DEM also attenuated the enhancement of reduction observed after the addition of NPSH. These results suggest that the addition of NPSH facilitates the reduction of the nitro function to the reduced intermediates that may be inactivated by an excess of NPSH, whereas the depletion of NPSH allows the accumulation of the toxic nitro radicals causing increased cytotoxicity.

The metabolism of xenobiotics usually leads to detoxification and subsequent elimination from the body. However, for certain drugs, the metabolites formed are more toxic than the parent compound. This property can sometimes be exploited as a means of new drug design. The 2-nitroimidazoles (2-NI)‡ were designed primarily as radiosensitizers of hypoxic tumor cells [1]. However, they have also exhibited preferential toxicity towards hypoxic cells [2]. The 2-NI, such as misonidazole (MISO), have been shown to undergo enzymatic reduction as shown in the following scheme [3]:

$$RNO_2 \rightarrow RNO_2 \rightarrow RNO \rightarrow RNOH \rightarrow RNHOH \rightarrow RNH_2$$
$$(RNO_2 \leftarrow RNO_2: O_2)$$

The cytotoxic species in this sequence could either be the radical intermediates [3] or a non-radical intermediate metabolite, the hydroxylamine [4], that may subsequently react with macromolecules under hypoxic conditions, causing injury or death. The nitro radical is oxidized back to the parent compound under aerobic conditions, and further reduction is stopped. Reactive oxygen species generated during this sequence may account for the aerobic toxicity of the drugs [5]. Because of the dose-related peripheral neuropathy associated with MISO [6], SR2508 was developed as an agent that has a lower lipophilicity and a more favorable tumor/brain ratio [7].

Glutathione (GSH) is the major endogenous detoxifying agent in combating against the cytotoxicity caused by 2-NI under hypoxic conditions [8]. Thiol depleting agents such as NEM and DEM have been reported to enhance the cytotoxicity of MISO primarily due to removal of the protection offered by GSH [3]. Besides participating in detoxification, GSH is also involved in modulating enzyme activities in glycolysis and gluconeogenesis [9]. Thiols have also been shown to be involved in the conversion of the dehydrogenase to the oxidase form of xanthine oxidase (XO) [10]. The effects of GSH on the activities of drug-metabolizing enzymes have not been investigated thoroughly. We therefore studied the role of nonprotein thiols (NPSH) in the enzymatic reduction of MISO and SR2508 in the presence or absence of the thiol depleting agents NEM and DEM. Since the liver is the major organ for drug metabolism, we studied the reduction of the nitro group in the hepatic cytosolic and microsomal fractions of the rat. In addition, purified xanthine oxidase was also employed to study the role of NPSH on the reduction of the nitro group.

MATERIALS AND METHODS

Materials. Buttermilk XO (EC 1.1.3.22), hypoxanthine, NADH, NADPH, GSH, cysteine, NEM

* Supported by PHS Grant 21050 awarded by the National Cancer Institute, DHSS.

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‡ Abbreviations: DEM, diethyl maleate; GSH, glutathione; MISO, misonidazole; NEM, *N*-ethylmaleimide; 2-NI, 2-nitroimidazoles; NPSH, non-protein thiol(s); SR2508, 2-(2-nitro-1-imidazolyl)-*N*-hydroxyethyl acetamide; and XO, xanthine oxidase.

and DEM were purchased from the Sigma Chemical Co. MISO and SR2508 were obtained from the National Cancer Institute. The solvents used were of HPLC grade.

Enzymatic preparation. Male Sprague–Dawley rats (200–300 g) were starved for 24 hr and then decapitated. Their livers were perfused with normal saline, minced, and homogenized. The microsomal and cytosolic fractions were obtained by the calcium sedimentation and centrifugation technique according to the procedure of Schenkman and Cinti [11]. The initial pellet from the 12,000 *g* spin was discarded, and solid $CaCl_2$ was added to the supernatant fraction to reach a final concentration of 8 mM. The solution was stirred and centrifuged at 25,000 *g* for 15 min. The supernatant fraction was used as the cytosolic fraction which was freeze-dried for storage and later resuspended in 10 mM Tris buffer, pH 7.4, when used. The pellet consisting of microsomes was suspended in 10 mM Tris buffer, pH 7.4, and frozen at −70° until assayed. The protein in each fraction was determined by using the Bio-Rad protein assay.

The NPSH contents of the cytosolic and microsomal fractions were determined by the procedure of Varnes and Biaglow [12]. The NPSH content was also determined after treatment of each fraction with various amounts of NEM or DEM.

Enzyme assay. The incubation mixture consisted of 6.5 mg cytosolic protein or 2 mg microsomal protein in 100 mM Tris–HCl buffer, pH 7.4. The mixture was purged with nitrogen for 30 min at 37°. To study the effect of depletion of NPSH, NEM or DEM was added before the gassing. The amount of NEM or DEM added in the cytosol was two or five times the amount of NPSH measured in each cytosolic fraction which ranged from 1.3 to 2.5 mM. This represented the low or high concentration of thiol depleting agents. Since NPSH levels in the microsomal fractions and XO were insignificant, 1.5 and 3 μmol of NEM or DEM were employed as low and high concentrations of the thiol depleting agents in the experiments with these enzyme systems. The reaction was started by adding MISO or SR2508 (0.36 μmol in the case of the microsomes and 0.18 μmol in the case of the cytosol), followed by the addition of the cofactors NADH (1.1 μmol for the cytosol) or NADPH (1.1 to 3.3 μmol for the microsomes). Equimolar amounts of either GSH or cysteine in relation to the amount of the thiol depleting agents were used in the reversal experiments, before addition of the substrates (2-NI). The total volume of the mixture was 1.5 ml. In some experiments, the protein fractions were heated in a water bath for 5 min at 100° to destroy the enzymatic activity. The reaction time was generally 30 min, and nitrogen was flushed continuously through the mixture during this time. Some experiments were performed under aerobic conditions and subjected to the same sequence of treatment as the hypoxic samples. The reaction was stopped by the addition of 1.5 ml of methanol. The mixture was filtered through a 0.22 μm Millipore filter, and the filtrate was then analyzed by employing the HPLC method.

The reduction of MISO by XO was performed by incubating a mixture consisting of 0.5 units XO, 0.5 mmol hypoxanthine and 0.36 μmol MISO in a total volume of 1.5 ml. The NEM or DEM (1.5 to 3.0 μmol) and the molar equivalent amount of either GSH or cysteine were used to study the effect of thiol depletion and repletion respectively. GSH or cysteine (1.5 to 15 μmol) was also added directly to the enzyme mixture to study the effect of NPSH on the rate of reduction of the nitro function.

HPLC analysis. The samples were analyzed by a Beckman HPLC unit model 341 equipped with a reverse phase 15.0 cm econosphere C18 5 μm column (Alltech). The absorbance of the nitro function of 2-NI was measured at 320 nm. The flow rate was 1 ml/min with a mobile phase of 20% methanol–80% water. The retention times for MISO and SR2508 were 4.3 and 2.1 min respectively. SR2508 was used as the internal standard for analyzing the reduction of MISO, and MISO was used as the internal standard for analyzing the reduction of SR2508. The internal standards were added after the enzymatic reduction. The calculation of the percentage of 2-NI reduced was based on the decrease in the absorbance of the nitro function peak at 320 nm. The reaction of NADH or NADPH with thiol depleting agents was measured by using an LKB diode array detector controlled by an IBM XT computer. The column and flow rate were the same, but the mobile phase was 15% methanol–85% water. The percent change of NADH or NADPH was quantitated by the change in peak heights at 265 and 340 nm respectively.

Statistical analysis. The values were expressed as mean ± SEM. The significance among samples was measured by an independent Student's *t*-test, and P values of less than 0.05 were considered statistically significant.

RESULTS

The reduction of 2-NI by the microsomes (Fig. 1) and cytosol (Fig. 2) was determined initially at concentrations ranging from 18 to 900 nmol/1.5 ml. Concentrations of 360 nmol/1.5 ml for the microsomes and 180 nmol/1.5 ml for the cytosol were selected for further experiments since these concentrations were in the linear phase of the curve. The amount of 2-NI reduced as a function of time is shown in Fig. 3. The reduction was linear for the first 30 min with both cytosolic and microsomal fractions. Therefore, subsequent reductions were carried out for a 30-min reaction time. In the cytosol, the amounts of MISO and SR2508 reduced after 30 min were 12.7 and 10.4 nmol/mg protein respectively. The amounts of MISO and SR2508 reduced by the microsomal enzymes were significantly higher at the level of 76.5 and 92 nmol/mg protein respectively. The 2-NI were not reduced either by the cytosolic or microsomal fraction if incubation was carried out under aerobic conditions.

The NPSH contents of the cytosolic and microsomal fractions were initially determined. Addition of NEM or DEM at a 2 molar equivalent concentration of the NPSH content in cytosolic fraction depleted the NPSH to approximately 41 or 51% of the control respectively. At a 5 molar equivalent concentration, the NPSH content in the cytosolic fraction was depleted to 4 and 23% of the control

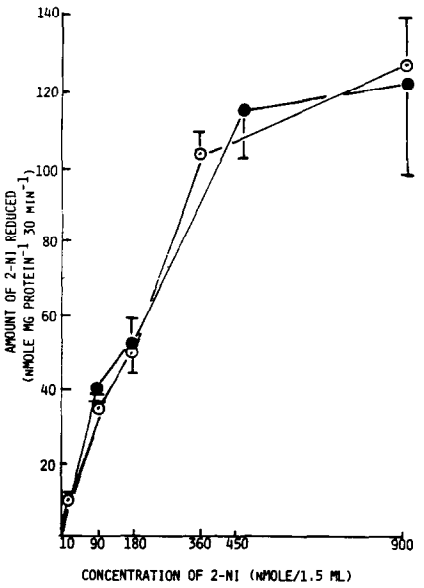

Fig. 1. Nitro reduction of MISO and SR2508 by rat hepatic microsomes at various concentrations of MISO and SR2508 after a 30-min incubation. The reaction mixture consisted of 2 mg microsomal protein, 1.1 μmol NADPH, and 18–900 nmol 2-NI in 100 mM Tris–HCl buffer, pH 7.4, in a total volume of 1.5 ml. Key: (○) MISO, and (●) SR2508.

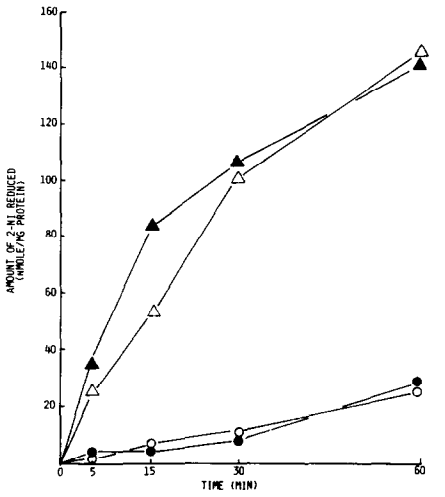

Fig. 3. Nitro reduction of MISO and SR2508 by rat hepatic microsomes or cytosol as a function of time. The reaction conditions were the same as described in Fig. 1 for the microsomes: (△) MISO or (▲) SR2508 and in Fig. 2 for the cytosol: (○) MISO or (●) SR2508. The concentrations of 2-NI used were 360 nmol in the case of microsomes and 180 nmol with cytosol in a total volume of 1.5 ml.

by NEM and DEM respectively (data not shown). Depletion of NPSH by DEM was relatively less, perhaps due to its rapid metabolism by the cytosolic enzymes. The NPSH content in the microsomal fraction was generally about 10-fold less than the cytosolic fraction.

Since NADH and NADPH were used as cofactors in the NPSH depletion assay and in the nitro reduction assay, the effects of thiol depleting agents on these nucleotides were also studied. The complete UV spectrum (190 to 370 nm) of NADH or NADPH was generated every second by using the LKB diode array detector. The absorption maxima at 265 and 340 nm were chosen for the quantitation of NADH and NADPH. Addition of DEM or NEM did not change significantly the peak height or the shape of the spectrum after a 30-min incubation at 37°. No new product was detected at the wavelength range of 190 to 370 nm.

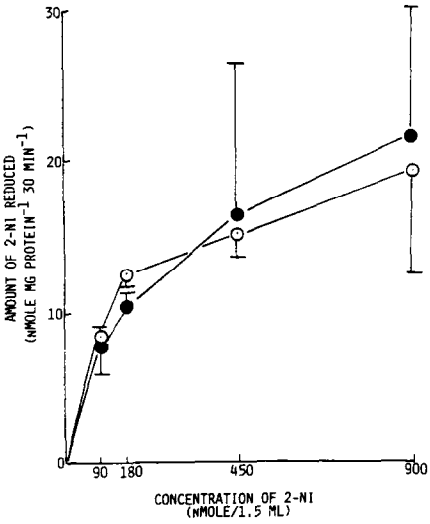

Fig. 2. Nitro reduction of MISO and SR2508 by rat hepatic cytosol at various concentrations of MISO and SR2508 after a 30-min incubation. The reaction mixture consisted of 6.5 mg cytosolic protein, 1.1 μmol NADH and 18–900 nmol 2-NI in 100 mM Tris–HCl buffer, pH 7.4. The total volume was 1.5 ml. Key: (○) MISO, and (●) SR2508.

The effects of thiol depleting agents on the reduction of MISO and SR2508 by the cytosolic fraction are shown in Fig. 4a and 4b respectively. The results are compared to the normalized base line reduction and are expressed as percent of the control. The amount of thiol depleting agents used was dependent upon the NPSH content of each fresh batch of cytosolic enzyme. Thus, NEM at two times the amount of NPSH, was able to inhibit the nitro reduction of the 2-NI by 20%, while the inhibition was increased significantly to more than 70% when five times the NEM was used. Similarly, DEM also inhibited the nitro reduction of MISO and SR2508 but the inhibition was less pronounced than that caused by NEM. Addition of a molar equivalent amount of GSH or cysteine in the presence of the thiol depleting agents not only abolished the inhibition but was found to stimulate the reduction of the nitro function in both MISO (Fig. 4a) and SR2508 (Fig. 4b). There was no reduction observed under aerobic conditions. Since similar reduction patterns were observed with either MISO or SR2508, most studies of nitro reduction by the hepatic microsomal fraction and XO were carried out with MISO only.

The effects of NEM and DEM on the nitro reduction of MISO by the microsomes are shown in Fig. 5. NEM inhibited the reduction of MISO by 70

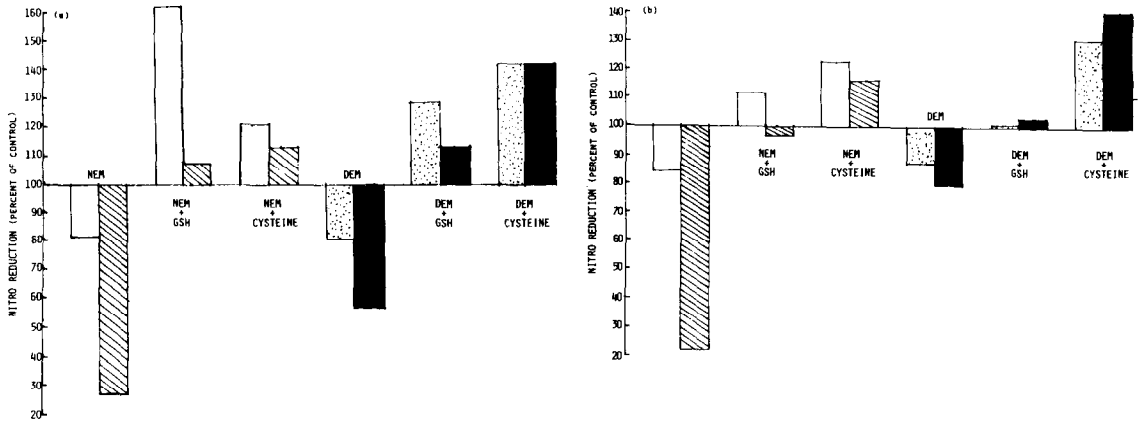

Fig. 4. Effects of thiol depleting agents and NPSH on the nitro reduction of MISO (a) and SR2508 (b) by rat hepatic cytosol. The reaction mixture consisted of 6.5 mg cytosolic protein, 1.1 μmol NADH, 180 nmol MISO in 100 mM Tris–HCl buffer, pH 7.4, and NEM (□, ▨) or DEM (▩, ■) at a 2 or 5 molar equivalent amount of NPSH respectively. In the reversal experiments, an equal amount of GSH or cysteine relative to the amount of NEM or DEM used was added.

and 90% of the control at the level of 1.5 and 3 μmol respectively. The inhibition of NEM was not reversed by addition of an equal molar amount of GSH. The effects of DEM on the nitro reduction of MISO at 1.5 and 3 μmol concentrations were comparatively insignificant. The effects of the addition of NPSH on the microsomal reduction of MISO are shown in Fig. 6. Addition of GSH at concentrations greater than 3 μmol caused a significant increase in the rate of reduction of MISO by the microsomes. However, cysteine stimulated the reduction rate at a significantly higher level and was approximately three to five times more effective than GSH.

Various factors as described in Table 1 were studied for the microsomal reduction of MISO. The results demonstrate that there was no reduction of MISO under aerobic conditions even with the addition of GSH or cysteine (at concentrations which caused stimulation under anaerobic conditions). Boiling the microsomal preparation abolished the enzymatic activity. The cofactor NADPH was also required since the reduction did not proceed in the presence of NADH.

The results of the effects of NEM on the reduction of MISO with purified XO are shown in Fig. 7. Addition of NEM at the level of 1 and 2 μmol/ml inhibited the nitro reduction of MISO by 36 and 53% of the control respectively. The inhibition was reversed by adding an equimolar amount of GSH. In contrast, DEM at these concentrations did not cause an inhibition of the nitro reduction; however, it did inhibit the effect of the GSH stimulation (Fig. 7). The effects of increasing the concentration of GSH or cysteine on the enzymatic activity of XO in reducing the nitro function of MISO are shown in Fig. 8. The results demonstrate that addition of both GSH and cysteine significantly stimulated the enzy-

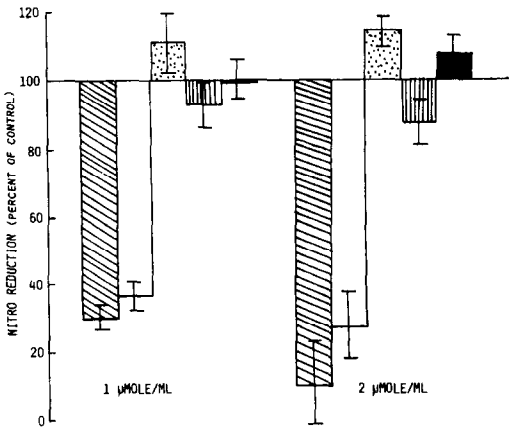

Fig. 5. Effects of NEM, DEM and GSH on the nitro reduction of MISO by rat hepatic microsomes. The reaction mixture consisted of: 2 mg microsomal protein, 1.1 μmol NADPH, 360 nmol MISO with either 1.5 or 3 μmol of NEM (▨), DEM (■), NEM + GSH (□), DEM + GSH (▥), or GSH (▩) in a total volume of 1.5 ml.

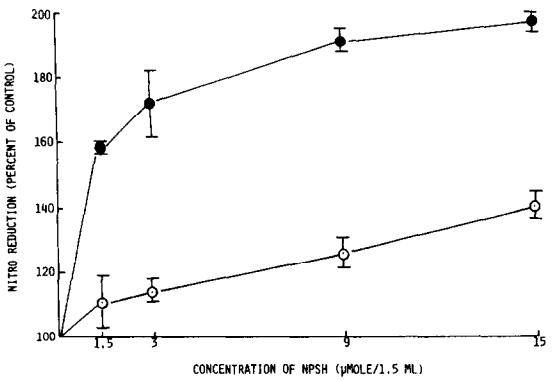

Fig. 6. Nitro reduction of MISO by rat hepatic microsomes as a function of various concentrations of GSH or cysteine. The reaction conditions were: 2 mg microsomal protein, 1.1 μmol NADPH, 360 nmol MISO with 1.5 to 15 μmol GSH (○) or cysteine (●) in Tris–HCl buffer, pH 7.4.

Table 1. Reduction of misonidazole by rat hepatic microsomes

No.	Incubation conditions	Amount of MISO reduced (nmol/mg protein/30 min) In air	In hypoxia
1	Microsomes (2 mg) + NADPH (1.1 μmol)	0	76.5 ± 1.4
2	Microsomes (2 mg) + NADH (1.1 μmol)		0
3	Boiled microsomes (2 mg) + NADPH (1.1 μmol)		4.4 ± 4.8
4	Microsomes (2 mg)		0
5	Microsomes (2 mg) + NADPH (1.1 μmol) + GSH (3 μmol)	0	99.6 ± 1.0*
6	Microsomes (2 mg) + NADPH (1.1 μmol) + cysteine (1.5 μmol)	0	138 ± 9.2*
7	Microsomes (2 mg) + NADPH (3.3 μmol)		118 ± 1.1
8	Microsomes (2 mg) + NADPH (3.3 μmol) + GSH (3 μmol)		142 ± 3.8*
9	Microsomes (2 mg) + NADPH (3.3 μmol) + cysteine (1.5 μmol)		152 ± 5.3*
10	Microsomes (2 mg) + GSH (15 μmol)		0
11	Microsomes (2 mg) + cysteine (3 μmol)		0
12	Boiled microsomes (2 mg) + NADPH (1.1 μmol) + GSH (15 μmol)		1.0 ± 1.1

Reductions were carried out in a 100 mM Tris–HCl buffer, pH 7.4, in a total volume of 1.5 ml containing 0.36 μmol misonidazole.

* Significantly different at the level of $P < 0.05$ (Student's t-test) when No. 5 or 6 is compared to 1 and No. 8 or 9 is compared to 7.

matic activity, and this stimulation was approximately similar for GSH and cysteine in contrast to the effects observed in the microsomal system (Fig. 6).

DISCUSSION

The reduction of 2-NI involves a six-electron reduction process, and the nitroso and hydroxylamine are relatively stable intermediates before they are further reduced to the amine. Radicals are also formed in between each intermediate step [3]. Any or all of these intermediates may be responsible for the cytotoxicity of nitroimidazoles, either directly because of their high reactivity towards biologically important molecules, or indirectly by the formation of toxic metabolites [13]. Fragmentation of the imidazole ring, after the formation of the hydroxylamine intermediate, has been reported to yield cytotoxic species such as glyoxal and guanidine derivatives [14]. Glyoxal has been shown to form covalent adduct products with guanosine [13]. More recently, Heimbrook and Sartorelli [15] have also reported that the anaerobic reduction of misonidazole results in the formation of glyoxal in a yield of 25%. GSH is important in protecting against the cytotoxic effects of the reduced products of 2-NI by conjugation followed by subsequent excretion [3, 13]. Apparently,

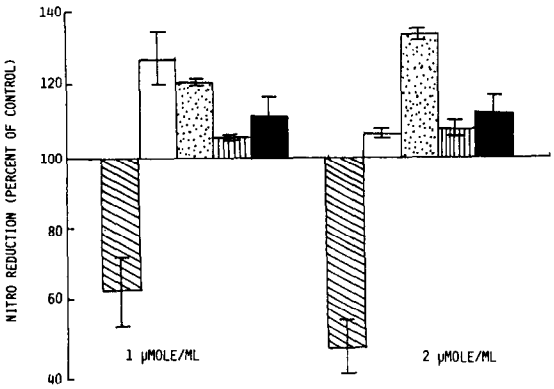

Fig. 7. Effects of NEM, DEM and GSH on the nitro reduction of MISO by xanthine oxidase. The incubation mixture included 0.5 units XO, 0.5 mol hypoxanthine, 360 nmol MISO and either 1.5 or 3 μmol NPSH depleting agent in a total volume of 1.5 ml. Key: (▨) NEM; (□) NEM + GSH; (▩) GSH; (▥) DEM; and (■) DEM + GSH.

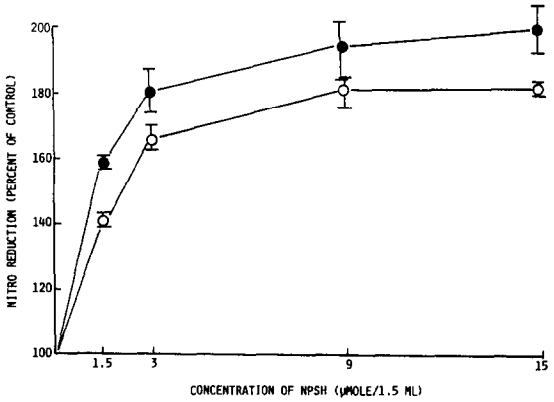

Fig. 8. Nitro reduction of MISO by xanthine oxidase as a function of increasing concentrations of GSH (○) or cysteine (●). The reaction conditions were the same as described in the legend of Fig. 7.

this is a primary rationale in using thiol depleting agents for enhancing the cytotoxicity of MISO [3, 16]. McManus *et al.* [17] have shown that GSH reduces the binding of the reduced MISO to DNA, suggesting that GSH may be binding to the reactive metabolites. However, very little is known about how the depletion of NPSH will affect the metabolism of 2-NI. There is an increasing amount of evidence suggesting that thiol levels are important in regulating enzymatic activities especially in the glycolytic and other synthetic pathways [9, 18, 19].

To isolate the effects of NPSH depletion on the microsomal enzymes, NADPH itself was employed as a cofactor instead of using an NADPH-generating system (which may be affected by the thiol depleting agents). The thiol depleting agents, NEM and DEM, did not react directly with either NADH or NADPH (data not shown). Boiled microsomes under the assay conditions employed did not reduce the 2-NI, suggesting that the reduction of the nitro group is enzymatic. Since GSH may combine with one or more of the metabolites formed [13], measurement of the metabolites or GSH-conjugates cannot distinguish the decrease in formation of the metabolites as being due to either the inhibition of nitroreductase activity or the inhibition of GSH-conjugate formation by the thiol depleting agents. Moreover, GSH may supply reducing equivalents to facilitate the rapidity of reduction and its dependency on nitroreductase activity and NADPH. Therefore, the nitro reduction was followed by measuring the disappearance of the parent compound. Nevertheless, metabolites were observed in the reduced mixture using the LKB diode array detector, and efforts are underway to identify and quantitate the metabolites formed as a result of modulation of the enzymatic steps.

The results in this report have provided evidence that the thiol depleting agents NEM and DEM inhibit the enzymatic reduction of 2-NI by the cytosolic fraction of rat hepatic tissue. However, in the experiments with microsomes and XO, only NEM and not DEM inhibited the nitro reduction. The inhibition in cytosol appears to be mediated by interaction with thiols because the addition of GSH or cysteine reversed the inhibition. Previous reports have suggested the involvement of the sulfhydryl group in the activities of certain cytosolic drug-metabolizing enzymes. For example, the electron transfer mechanism in the case of lipoamide dehydrogenase (NADH: lipoamide oxidoreductase, EC 1.6.4.3) can be modified by sulfhydryl reagents in a two-electron to a one-electron transfer type mechanism [20]. Involvement of the thiol function has also been implicated in conversion of the XO enzyme dehydrogenase (type D) to the oxidase (type O) form [10]. Our results using buttermilk XO have demonstrated that the nitro reduction of 2-NI was inhibited by NEM and that this was reversed by the addition of GSH. This also suggests that the oxidase instead of the dehydrogenase form of XO is the major nitroreductase for 2-NI in the cytosol since the reduction was sensitive to sulfhydryl agents and oxygen. DEM did not inhibit the reduction but inhibited the stimulation by GSH. Apparently, the SH group of XO may not be accessible to DEM. Stimulation of the XO activity with GSH or cysteine illustrates that the reduction of the nitro function is carried out more effectively when free sulfhydryl groups are available.

The microsomal reducing activity per mg protein was significantly higher than that of the cytosolic fraction. Therefore, the nitro reduction by the microsomes was investigated in detail. Addition of NPSH, such as GSH or cysteine, stimulated the reduction of the nitro function, although the stimulation by an equimolar concentration of cysteine was 5-fold more effective than GSH. The concentrations of GSH used in these experiments were similar to the physiological concentrations in most cell types (e.g. 1–12 mM) as reported by Kosower and Kosower [21]. Fahey *et al.* [22] have also reported that the levels of GSH and cysteine in liver are 7.8 and 0.05 μmol/g respectively. Increased intracellular cysteine levels can be achieved by exogenous addition of cysteine or indirectly by adding L-2-oxothiazolidine-4-carboxylate that has been employed to allow the cysteine to be incorporated into GSH through the γ-glutamyl cycle [23]. Since the microsomal reduction is NADPH dependent, GSH and cysteine alone are not likely to stimulate the enzymatic activity by directly providing the reducing equivalents. Taylor and Rauth [24] have also reported earlier that the addition of GSH to a homogenate of Chinese hamster ovary cells appears to increase the metabolism of MISO. However, they did not elaborate further on their findings. The results in this report have provided evidence that the reduction of the nitro group did not progress with GSH or cysteine alone, and that the reduction was stimulated by GSH or cysteine even in the presence of excess NADPH. Moreover, the stimulation of the nitro reduction by GSH or cysteine was associated with the enzymatic fraction since the nitro reduction was abolished after boiling the microsomes. In addition, GSH or cysteine did not stimulate the nitro reduction under aerobic conditions, suggesting that the NPSH may be affecting the reducing steps beyond the formation of the nitro radical anion under hypoxia.

NEM inhibited both the nitro reduction by the microsomes and also the stimulatory effect of GSH. The inhibition of the microsomes by NEM was not reversed by the addition of GSH, indicating that perhaps NEM reacts irreversibly with the SH groups in the microsomal enzymes [25]. The other thiol depleting agent, DEM, was relatively less effective than NEM in inhibiting the stimulatory effect of GSH. These data, however, do not rule out the possibility of the indirect involvement of NPSH in the nitro reduction of microsomes by influencing the redox status of the NADPH pool [26, 27].

Both the microsomal and cytosolic enzymes are capable of reducing 2-NI under hypoxic conditions. However, the relative contribution or reduction by the microsomes would be expected to be higher than by the cytosol as was demonstrated in this study. The reduction patterns of MISO or SR2508 were similar in the cases of cytosolic and microsomal fractions, suggesting that these agents are essentially similar in their abilities to be reduced.

Enhancement of nitro reduction by NPSH in this report has suggested that the NPSH involvement is probably beyond the nitro radical anion step. The depletion of NPSH would therefore inhibit the

reduction of 2-NI from the toxic nitro radical anion to the more stable intermediates, and then to the relatively nontoxic amine. This scheme would result in the accumulation of the nitro radicals under anaerobic conditions, leading to increased cytotoxicity. In addition, the depletion of NPSH would not only cause a decrease in the formation of one of the possible reactive intermediates, the hydroxylamine, but would also be expected to decrease the formation of cytotoxic fragmentation products [13]. Under aerobic conditions, the nitro radicals will be expected to be reoxidized to the parent compound. This hypothesis is consistent with the findings that have shown enhancement of the hypoxic cytotoxicity of MISO and other 2-NI used in combination with thiol depleting agents *in vitro* [12] and also explains the paradox that the thiol depletion decreases the nitro reduction but increases the cytotoxicity. The paradox may also involve the fact that the depletion of NPSH may lead to a loss of scavengers that may intercept reactive metabolites before they reach target molecules.

The role of NPSH in the enzymatic nitro reduction can be explained in several ways. The NPSH may be important in maintaining the enzymes in the appropriate reduced (−SH) form through a sulfhydryl exchange mechanism [28]. Also, the sulfhydryl group may be important at the active or allosteric site of the enzymes, and thiol reactive agents such as NEM or DEM may bind directly to this site and inhibit the enzymatic reaction. However, the inhibition of the enzyme activity was reversed upon the addition of GSH or cysteine in the case of cytosol and XO, indicating that a competitive process exists. Furthermore, GSH may also affect the kinetics of enzymes, since the interaction of GSH with the reactive intermediates may facilitate the reduction. This report has presented evidence that the attempts to modulate the NPSH levels by thiol depleting agents to increase hypoxic cytotoxicity under *in vivo* conditions should be undertaken with caution in view of the fact that overall nitro reduction was decreased significantly after NPSH depletion.

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