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INCREASED EFFLUX RATHER THAN OXIDATION IS THE MECHANISM OF GLUTATHIONE DEPLETION BY 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP)

Donato Di Monte, Martha S. Sandy, and Martyn T. Smith

Department of Biomedical and Environmental Health Sciences,
School of Public Health, University of California, Berkeley, CA 94720

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SUMMARY. Incubation of isolated hepatocytes in the presence of either the parkinsonian-inducing compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or its putative toxic metabolite 1-methyl-4-phenylpyridinium ion (MPP^+) led to a depletion of intracellular reduced glutathione (GSH), which was mostly recovered as glutathione disulfide (GSSG). However, both MPTP- and MPP^+-induced glutathione perturbances were relatively unaffected by the prior inhibition of glutathione reductase with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), suggesting that intracellular oxidation was not the major mechanism involved in the GSH loss. Inclusion of cystine in the incubation mixtures revealed a time-dependent formation of cysteinyl glutathione (CySSG), indicating that an increased efflux was mostly responsible for the MPTP- and MPP^+-induced GSH depletion. Therefore, the measurement of GSSG, which is apparently formed extracellularly, was not associated with oxidative stress.

The possible generation of oxygen radicals and their role in cell damage caused by the parkinsonian-inducing compound MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (1) has been the subject of much controversy. Reduced glutathione (GSH) is involved in a variety of detoxication reactions against oxidizing species produced during the metabolism of xenobiotics (2). Therefore, a selective depletion of GSH in the substantia nigra of mice injected with MPTP has been interpreted as an index of regional vulnerability to oxidative stress (3). A lower concentration of GSH has also been found selectively in the substantia nigra of patients with idiopathic Parkinson's disease (4), leading to the possibility of GSH consumption being a more general event in the pathological processes occurring in this region of the brain.

Several lines of evidence point to the fully oxidized metabolite 1-methyl-4-phenylpyridinium ion (MPP^+) as the ultimate mediator of MPTP-induced cell damage (6,7). Johannessen *et al.* (8) have measured an increase in the plasma concentrations of oxidized glutathione (GSSG) after systemic administration of

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MPP^+ to rats, suggesting that this compound induces oxidative stress in vivo. Recent work in our laboratory using isolated rat hepatocytes as an in vitro model has shown, however, that both MPTP and MPP^+ are unlikely to produce general cytotoxicity as a consequence of oxygen radical generation, even if both compounds cause a depletion of intracellular GSH (9,10). Here, we report that the GSH depletion induced by both MPTP and MPP^+ is not due to intracellular oxidation, but rather to an increased efflux of the reduced tripeptide from the cell and its subsequent oxidation in the extracellular space.

MATERIALS AND METHODS

MPTP (hydrochloride salt) and MPP^+ (iodide salt) were purchased from Research Biochemicals (Wayland, MA). Collagenase (grade II) was from Boehringer (Mannheim, West Germany). BCNU was kindly supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment of the National Cancer Institute, Bethesda, MD. Hepatocytes were isolated from male, Sprague-Dawley rats (220-280 g body weight) and incubated (10^6 cells/ml) in Krebs Henseleit buffer (11). Cell viability was assessed as exclusion of Trypan blue (11) and was always greater than 90% at the beginning of the experiments. When inhibition of glutathione reductase (EC 1.6.4.2) was desired, the cells were pretreated for 20 min with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and then incubated for 75 min in fresh media supplemented with 1 mM methionine as in (12). Glutathione reductase activity was measured spectrophotometrically by monitoring the oxidation of NADPH (at 340 nm) in the presence of 50 mM GSSG, 0.1 mM NADPH and 1% Triton X-100. Only hepatocyte preparations with > 90% inhibition were used in the experiments shown here. To measure GSH efflux from hepatocytes, 0.2 mM cystine was included in the incubation mixtures as in (13). GSH, GSSG and the cysteinyl glutathione disulfide (CySSG) were detected by HPLC as described in (14). At the indicated times, samples (1 ml) were taken and the cells were immediately separated from the media by rapid centrifugation (3 s at 13,000 g) in a microfuge (Eppendorf 5415). When cystine was present in the incubation the cell pellets were washed once and then resuspended in buffer. GSH was extracted by addition of 70% perchloric acid (1:20, v/v).

RESULTS

Incubation of isolated hepatocytes in the presence of 1.0 mM MPTP led to a progressive decline in the intracellular level of GSH (Figure 1A). The initial GSH concentration was 38.5 ± 3.9 nmoles/10^6 cells and 29.5 ± 2.7 nmoles were lost after 80 min incubation, prior to the occurrence of detectable signs of hepatocyte death (data not shown). Approximately half of this decrease in GSH occurred by 40 min. At this time point 92% of the missing GSH was recovered as GSSG (intracellular + extracellular).

The role of H_2O_2 generation in the oxidation of GSH during MPTP exposure was investigated by inhibiting glutathione reductase activity with BCNU and,

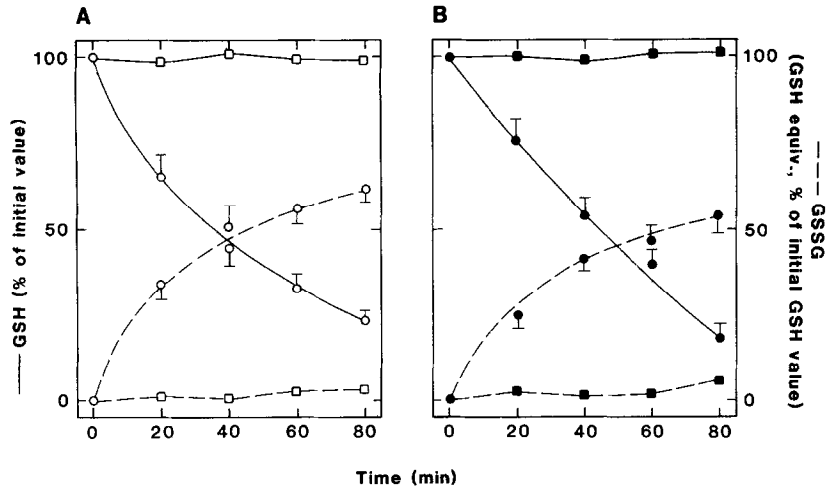

Fig. 1. GSH depletion and GSSG formation caused by 1.0 mM MPTP in untreated (A) and BCNU-treated (B) hepatocytes. Freshly isolated hepatocytes were either pretreated with BCNU (filled symbols) or not pretreated (open symbols) and then exposed to 1.0 mM MPTP (○) or no addition (□). At the time points shown, GSH and GSSG contents were analyzed as described in Methods. The data represent the mean (± S.D.) of 4 separate experiments.

thus, impairing the action of glutathione peroxidase (12). As previously reported (9), BCNU-treated hepatocytes were not significantly more susceptible to the toxic effects of MPTP than control cells. Figure 1B shows that the rate of GSH loss caused by MPTP was not significantly altered by the inhibition of glutathione reductase. Approximately 50% of the GSH was lost 40 min after MPTP addition, similar to the rate of loss in untreated hepatocytes. The initial GSH concentration was 51.1 ± 3.9 nmoles/10^6 cells and the intracellular value fell to 8.7 ± 3.1 nmoles after 80 min. The overall GSH decrease was, therefore, quantitatively greater than that measured in untreated cells, but this difference was much less than that expected for a potent H_2O_2-generating compound (12). The recovery of GSH as GSSG in Figure 1B was 88% at 40 min. From the data shown in Figure 1, one can therefore conclude that the inhibition of glutathione reductase does not affect the rate of GSH oxidation caused by MPTP, suggesting a minimal role for H_2O_2. Direct inhibition of the enzyme by MPTP could explain this phenomenon, but the maximal activity of glutathione reductase in the hepatocytes was found to be unaffected by MPTP exposure (data not shown). The possibility that GSH oxidation occurred in the extracellular

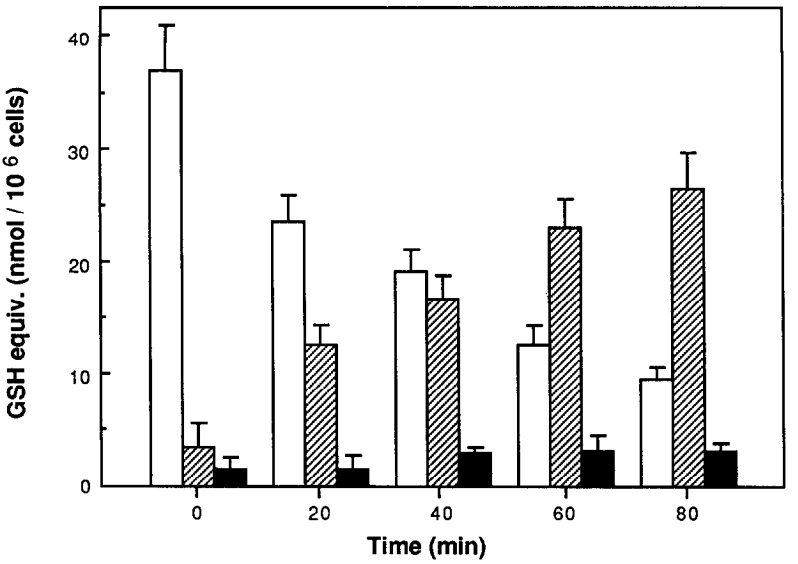

Fig. 2. Measurement of MPTP-induced GSH efflux by addition of cystine to the hepatocyte suspension. Incubations were performed in the presence of 0.2 mM cystine. At indicated times, intracellular GSH (□), extracellular CySSG (▨) and total (intracellular + extracellular) GSSG (■) were measured as described in Methods. The intracellular GSH content of the control cells was 38.8±3.3 nmol/10^6 cells and was little affected by incubation for 80 min. Bars represent the mean±S.D. for 4 separate cell preparations.

space therefore seemed likely. To test this hypothesis, hepatocytes were incubated in medium supplemented with 0.2 mM cystine and the formation of cysteinyl glutathione (CySSG) determined as an index of GSH release from the cells (13).

Figure 2 shows that a time-dependent formation of CySSG was observed after addition of MPTP to hepatocyte suspensions in the presence of cystine. Oxidation of GSSG accounted for only about 7% of the total GSH decrease in these experiments, while 84% of the GSH was recovered as CySSG at 80 min. Less than 1.5% of the CySSG was found intracellularly at all time points (data not shown). In control cells, the concentration of CySSG rose from 3.4 ± 0.9 to 6.2 ± 0.5 nmoles/ml after 1 hour incubation, in agreement with previously reported data on GSH efflux from freshly isolated hepatocytes (13). When MPTP was added to BCNU-treated hepatocytes in the presence of cystine, the loss of GSH was, indeed, due mostly to oxidation (Figure 3). Total GSSG

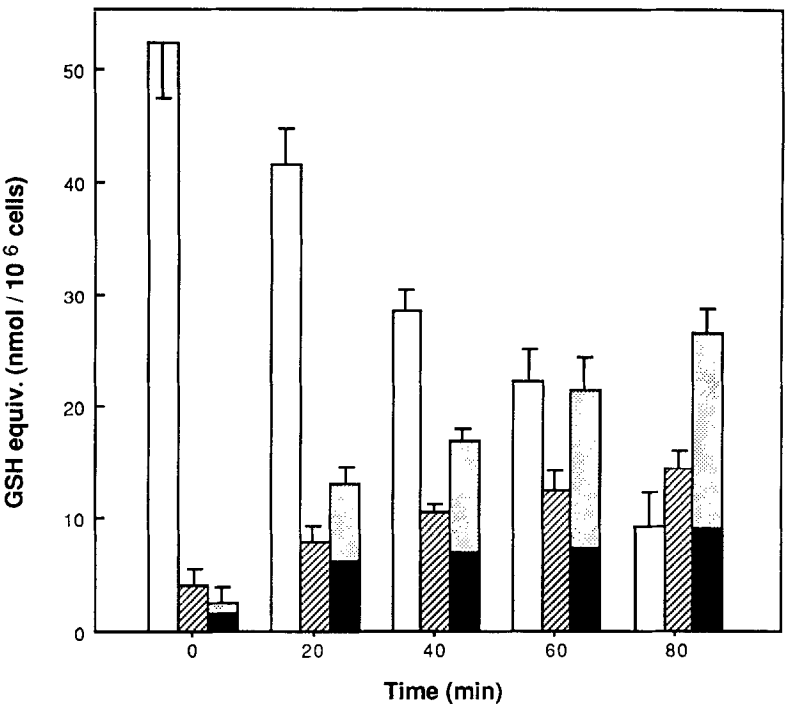

Fig. 3. Effect of cystine on glutathione measurement in BCNU-treated hepatocytes. Freshly isolated hepatocytes were preincubated as described in Methods with BCNU and then exposed to 1.0 mM MPTP in the presence of 0.2 mM cystine. At the time points indicated, intracellular GSH (□), extracellular CySSG (▨) and total GSSG (intracellular, ▩ + extracellular, ■) were measured by high performance liquid chromatography (18). In control cells, GSH values (51.2 nmol/10^6 cells at 0 min) did not change significantly during 80 min incubation and the concentration of CySSG increased by 3.2±0.7 nmoles between 0 and 80 min. Bars represent the mean±S.D. for 4 separate experiments.

accounted for 57% and CySSG for 24% of the difference between GSH concentrations at time 0 and after 80 min incubation.

The role of oxidative stress in the depletion of GSH induced by the putative toxic metabolite of MPTP, MPP^+, was then assessed in isolated hepatocytes. The results reported in Table I show that 2.0 mM MPP^+ caused a 40% decrease in the intracellular GSH level after 80 min incubation. Formation of GSSG, which accounted for approximately 80% of this loss, was not due, however, to intracellular H_2O_2 generation. When cystine was added to the incubation, the recovery of GSH as CySSG and GSSG was 76% and 5% respectively after 80 min, indicating that an increased efflux of GSH was responsible for the GSH loss induced by MPP^+, as well as for that observed with MPTP.

TABLE I

EFFECT OF 2.0 mM MPP^+ ON HEPATOCYTE GLUTATHIONE IN THE ABSENCE AND PRESENCE OF CYSTINE

Min.		Treatment			
		None	Cystine	MPP^+	Cystine + MPP^+
0	GSH	37.6±3.8	38.4±4.0	36.5±3.1	37.2±3.5
	GSSG	2.9±1.1	1.5±0.4	3.2±0.9	1.8±0.5
	CyssG	0.5±0.2	3.1±1.4	0.5±0.2	3.6±1.1
40	GSH	36.9±4.3	36.7±3.4	28.6±2.9	27.5±3.8
	GSSG	2.8±0.7	1.6±0.9	9.9±2.1	2.2±0.7
	CySSG	0.7±0.3	4.2±1.3	1.4±0.5	10.8±1.7
80	GSH	37.1±4.2	37.8±4.0	21.8±2.2	20.3±3.6
	GSSG	3.3±1.2	2.3±0.5	15.2±2.7	2.7±1.0
	CySSG	1.4±0.6	6.4±1.3	2.2±0.3	16.5±1.8

Incubations were performed in the absence or presence of cystine (0.2 mM) as indicated. Intracellular GSH, total (intracellular + extracellular) GSSG and extracellular CySSG were measured according to Reed et al. (18). Each value (expressed as GSH or GSH equiv./10^6 cells) represents the mean (± S.D.) of 3 separate preparations.

DISCUSSION

The data presented in this study allow a detailed analysis of the biochemical events following exposure of hepatocytes to MPTP, and support our recent conclusion that oxidative damage does not play an important role in the general cytotoxic effects of this compound (9). H_2O_2 does seem to be formed during MPTP metabolism in hepatocytes, probably as a product of the oxidative reaction which is catalyzed by monoamine oxidase in the outer membrane of mitochondria, generating the 2,3-dihydropyridinium derivative of MPTP (5). The rate of H_2O_2 formation does not seem to overwhelm the capacity of the cell to scavenge this oxidant, however, and did not induce apparent biochemical effects (e.g., GSH oxidation) unless glutathione reductase activity was inhibited.

The GSH loss induced by MPTP has consequently been shown to involve two different mechanisms, oxidation and efflux, in both control and BCNU-treated

hepatocytes. The balance between which of these two mechanisms predominates is dependent primarily on the activity of the glutathione peroxidase/glutathione reductase system. When this system is not compromised, no oxidative processes seem to be involved in the intracellular loss of GSH caused by either MPTP or MPP^+. This effect is due rather to an increased efflux of GSH with GSSG being formed subsequently by oxidation in the extracellular space. Therefore, no relationship links the measurement of GSSG after hepatocyte exposure to MPTP with the generation of oxygen radicals.

The loss of GSH induced by MPP^+ was much slower than that observed with MPTP (even at twice the concentration), reflecting the limited access of this charged metabolite to the cell (7), and suggesting that the GSH efflux induced by these pyridine compounds is triggered by intracellular events. The biochemical mechanism underlying these processes is the subject of ongoing studies in our laboratory. GSH might be released and oxidized extracellularly following the breakdown of an unstable glutathione conjugate excreted from the cells (15). This hypothesis, however, is unlikely, since neither MPTP nor MPP^+ have been found to react with GSH and form a glutathione conjugate. A more likely explanation is based on the fact that GSH is negatively charged within cells and perturbations of membrane potentials can influence its efflux (16,17). Both MPTP and MPP^+ toxicity in hepatocytes seem to be correlated with an earlier rapid depletion of ATP (18). The relationship between these toxic events and glutathione status might provide the key to fully interpret the phenomenon described in this study. In accordance with this hypothesis, preliminary results show that other compounds which perturb cell membrane potentials (e.g., valinomycin) and/or impair the supplies of cellular ATP (e.g., antimycin A) also deplete intracellular GSH via a mechanism of increased efflux (D. Di Monte, M.S. Sandy and M.T. Smith, manuscript in preparation).

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