ORIGINAL

ENHANCEMENT OF PARAQUAT TOXICITY BY GLUTATHIONE DEPLETION IN MICE *IN VIVO* AND *IN VITRO*

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(Received August 3, 1995 ; Accepted August 28, 1995)

ABSTRACT — Effect of glutathione (GSH) depletion on paraquat (PQ) toxicity in the liver and kidneys of mice was examined. Glutamic-pyruvate transaminase (GPT) and blood urea nitrogen (BUN) levels in plasma of mice were hardly changed by treatment with 150 μ mol/kg of PQ. However, significant increases in the plasma GPT and BUN levels after PQ injection were observed in mice which were pretreated with L-buthionine-SR-sulfoximine (BSO), an inhibitor of GSH synthesis, at 4 hr prior to PQ administration. This result supports the previous observation that hepatotoxicity of PQ was enhanced in diethyl maleate-pretreated mice (Cagen and Gibson, 1977). In the present study, lipid peroxidation evaluated by thiobarbituric acid-reactive substances (TBA-RS) level in the liver of mice given PQ was elevated by pretreatment with BSO. Moreover, enhancement of PQ cytotoxicity by BSO pretreatment was also observed in cultured mouse hepatoma cell line (NCTC clone 1469). Vitamin E, an antioxidant, and Desferal, an iron chelator, significantly prevented mice from the BSO-enhanced hepato- and nephrotoxicity of PQ. These findings suggest that the tissues or cells of low GSH concentration are highly vulnerable to PQ toxicity and GSH may play a major role in diminishing the toxic action of PQ exerted through oxidative stress.

KEY WORDS: Glutathione depletion, Hepatotoxicity, Nephrotoxicity, Paraquat, Buthionine sulfoximine.

INTRODUCTION

Paraquat (1,1-dimethyl-4,4'-bipyridium dich-

loride, PQ) is a non-selective herbicide, and extremely toxic to man and most animal species (Haley, 1979). PQ gives damages in the lungs, kidneys and liver and may result in death (Haley, 1979). The biochemical mechanism of PQ toxicity has well been investigated and generally

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considered to exert its toxicity by the induction of oxidative stress. PQ undergoes a redox cycling reaction in which PQ is metabolized by NADPH-cytochrome P-450 reductase to a reduced intermediate, paraquat radical (Bus *et al.*, 1974). This intermediate produces reactive oxygens during the oxidation with molecular oxygen (Farrington *et al.*, 1973). Disruption of the balance between reactive oxygen generation and their dissipation by cellular defense systems may allow reactive oxygen species to attack biomolecules.

Glutathione (GSH), tripeptide containing cysteine, plays a role as a cellular defense factor against reactive oxygen species generated in tissues (Meister, 1991). Some studies have been conducted to elucidate the relationship between cellular GSH and PQ toxicity *in vivo* and *in vitro* (Yamamoto, 1993; Hagen *et al.*, 1986; Peter *et al.*, 1992). Bus *et al.* (1975) reported that diethyl maleate-induced GSH depletion enhanced lethal toxicity of PQ in mice. Liver damage following PQ administration was observed in diethyl maleate-pretreated mice (Cagen and Gibson, 1977). However, the treatment with diethyl maleate itself caused lipid peroxidation and liver necrosis (Maellaro *et al.*, 1990). Thus, diethyl maleate appears to be inadequate to assess the effect of GSH depletion on toxicity of PQ. On the other hand, the hepatic ultrastructural change and renal functional impairment have also been recognized as acute phase toxic effects of PQ (Matsumori *et al.*, 1984; Ecker *et al.*, 1975). However, the effect of GSH depletion on hepato- and nephrotoxicity of PQ has scarcely been well documented. In the present study, therefore, we examined PQ-toxicity in the liver and kidneys of mice treated with L-buthionine-SR-sulfoximine (BSO) to deplete GSH by inhibiting its synthesis. Protective effects of vitamin E, an inhibitor of lipid peroxidation, and Desferal, an iron chelator acting as an anti-oxidative drug *in vivo*, against toxicity of PQ in GSH depleted mice were also investigated.

MATERIALS AND METHODS

Animals and chemicals : Male CD-1 (ICR) mice (4 weeks old) were purchased from Charles River Japan, Inc., Atsugi, Japan. NCTC clone 1469 mouse hepatoma cell line was given from

the National Institute of Hygiene, Japan. PQ was purchased from Sigma Chemical Co., St. Louis, MO, USA. DL- α -tocopherol acetate (vitamin E) was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Desferrioxamine mesylate (Desferal) was purchased from Ciba-Geigy Japan, Takarazuka, Japan. PQ and Desferal were dissolved in saline prior to use. Vitamin E was dissolved in olive oil or ethanol. L-buthionine-SR-sulfoximine (BSO, Sigma Chemical Co.) was dissolved in 0.15 M sodium hydroxide solution, and the pH of the solution was adjusted to 7 with 1 M hydrochloric acid.

Treatment of animals : Mice were given a single dose of PQ (s.c.; 150 μ mol/kg) 4 hr after BSO (s.c.; 0.75, 1.5 and 3.0 mmol/kg) administration. Vitamin E (s.c.; 0.56 mmol/kg) in olive oil was administered twice 24 hr and 1 hr prior to the PQ treatment. Desferal (s.c.; 0.19 mmol/kg) was administered three times 4 hr and 1 hr before and 4 hr after the PQ treatment. Blood samples were obtained from femoral artery and heparinized. Plasma was prepared from the blood samples by centrifugation for determination of glutamic-pyruvate transaminase (GPT) activity as an indicator of hepatotoxicity and blood urea nitrogen (BUN) as that of nephrotoxicity. Liver and kidneys were excised for determination of tissue GSH and thiobarbituric acid-reactive substances (TBA-RS).

Cell culture : NCTC clone 1469 were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The cells were treated with BSO (500 μ M). After incubation for 15 hr, the medium was removed and PQ (0.01–3 mM) was added with fresh medium. These cultures were served for MTT assay after 6 hr incubation with PQ.

Analysis : Tissue GSH level was determined by the method of Toyo'oka and Imai (Toyo'oka and Imai, 1983) modified as described by Tanaka-Kagawa *et al.* (Tanaka-Kagawa *et al.*, 1993). GPT and BUN level were measured by automatic analyzer (Hitachi 705, Hitachi Ltd., Tokyo, Japan) using AutoseraGPT and AutoseraUN (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). TBA-RS level was determined by method of Ohkawa *et al.* (1989) and expressed as nmol malondialdehyde (MDA)/g tissue.

MTT assay : Cultured cells plated at 3×10^4

cells/well in 96-well microplate were treated with chemicals as described above and then 50 $\mu\text{g}/10\ \mu\text{l}$ of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution was added. After 2 hr reaction at 37°C, 100 μl of 20% sodium dodecylsulfate solution was added and absorbance at 600 nm of each well was measured.

Statistics : Statistical analyses were performed using one-way analysis of variance with pairwise comparison by the Bonferroni method.

RESULTS

In order to reduce tissue GSH levels, mice were treated s.c. with 0.75, 1.5 or 3.0 mmol/kg of BSO. At 4 hr after BSO treatment, GSH levels in the liver and kidneys were reduced depending on the dose of BSO (Table 1). Since 3.0 mmol/kg of BSO reduced hepatic and renal GSH levels to 63% and 19% of the control levels, respectively, without affecting plasma GPT and BUN levels even at 28 hr after the administration (Fig. 1), 3.0 mmol/kg of BSO was used in subsequent experiments.

In a preliminary experiment, 150 $\mu\text{mol}/\text{kg}$ or 200 $\mu\text{mol}/\text{kg}$ of PQ was given to mice with or without BSO pretreatment. Most of mice treated with BSO and 200 $\mu\text{mol}/\text{kg}$ of PQ were died

within 24 hr, whereas the plasma GPT and BUN levels were increased to 3-fold and 6-fold, respectively, by 150 $\mu\text{mol}/\text{kg}$ of PQ in combination with 3.0 mmol/kg of preadministered BSO as shown in Fig. 1. Although the data was not shown, none of the mice was died by the treatment with this combination during 24 hr after PQ dose. Therefore, we finally decided to use 150 $\mu\text{mol}/\text{kg}$ of PQ in the present experiment. Significant increases in the plasma GPT and BUN levels were also observed at 12 hr, but not at 3 hr after PQ treatment (data not shown). The TBA-RS level was also determined to evaluate the extent of lipid peroxidation. Hepatic TBA-RS level de-

Table 1. Tissue glutathione level 4 hr after pretreatment with BSO in mice.

Group	Glutathione ($\mu\text{mol}/\text{g}$ tissue) ^{a)}	
	Liver	Kidney
Control	8.530 $\pm$ 1.314	2.949 $\pm$ 0.304
BSO 0.75 mmol/kg	6.290 $\pm$ 1.103	0.679 $\pm$ 0.103 ^{b)}
1.5 mmol/kg	5.768 $\pm$ 0.710 ^{b)}	0.581 $\pm$ 0.015 ^{b)}
3.0 mmol/kg	5.571 $\pm$ 0.898 ^{b)}	0.557 $\pm$ 0.018 ^{b)}

Tissue samples were taken 4 hr after a 3 mmol/kg s.c. dose of BSO.

^{a)} : Mean $\pm$ S.D. (n=5).

^{b)} : Significantly different from control (P<0.01).

Fig. 1. Effect of pretreatment with 3 mmol/kg of BSO on plasma GPT and BUN level in mice 24 hr after the PQ (150 $\mu\text{mol}/\text{kg}$) treatment. PQ was given s.c. 4 hr after the BSO s.c. administration. Each value is the mean $\pm$ S.D. of 4–5 animals. **: Significantly different from the control (P<0.01).

terminated at 3 hr after PQ treatment was significantly increased to 170% of those of the control in mice pretreated with BSO (Fig. 2), whereas the increased TBA-RS value was then declined to the control level during subsequent 20 hr (data not shown).

Fig. 3 shows the effect of vitamin E or Desferal on the lipid peroxidation observed at 3 hr after PQ injection in BSO pretreated mice.

Fig. 2. Effect of pretreatment with 3 mmol/kg of BSO on TBA-RS level in liver of mice 3 hr after the PQ (150 μ mol/kg) treatment. PQ was injected s.c. 4 hr after the BSO administration. Each value is the mean $\pm$ S.D. of 4–5 animals. **: Significantly different from the control ($P < 0.01$).

Doses of vitamin E (0.56 mmol/kg) and Desferal (0.19 mmol/kg) were chosen according to the data reported previously (Shahar *et al.*, 1989; Hoffer *et al.*, 1992). The PQ-induced increase in the hepatic TBA-RS level in BSO pretreated mice was reduced to the control level by vitamin E or Desferal. The hepatotoxicity and nephrotoxicity of PQ evaluated by plasma GPT and BUN, respectively, were enhanced by BSO pretreatment. The increases in these indicators were significantly reduced to below 50% by vitamin E (Fig. 4) or Desferal (Fig. 5).

Next, we examined the effect of BSO on PQ toxicity in cultured cells. Concentrations of BSO and PQ in cultured medium were chosen according to the experimental conditions reported previously by Shrieve and Harris (1986) and Peter *et al.* (1992), respectively. Cellular GSH concentration in NCTC clone 1469 mouse hepatoma cell line was reduced to 10% of those of untreated cells by pretreatment with BSO (data not shown). Pretreatment of mouse hepatoma cell line with BSO for 15 hr prior to PQ addition induced a significant augmentation of the cytotoxicity of PQ (Fig. 6). The IC_{50} value of PQ in NCTC clone 1469 cells pretreated with BSO was 1.87 mM, but over 3 mM in untreated cells.

Fig. 3. Effect of pretreatment with vitamin E (VE) (a) or Desferal (DF) (b) on TBA-RS level in liver of mice 3 hr after PQ treatment. Mice were treated s.c. with 0.56 mmol/kg of vitamin E 24 hr and 1 hr prior to PQ treatment or with 0.19 mmol/kg of DF 4 hr and 1 hr prior to PQ treatment. PQ (150 μ mol/kg, s.c.) was given 4 hr after the BSO (3 mmol/kg, s.c.) administration. Each value is the mean $\pm$ S.D. of 5 animals. *: Significantly different from mice not treated with vitamin E or Desferal ($P < 0.05$).

Fig. 4. Effect of pretreatment with vitamin E (VE) on plasma GPT level and BUN level in mice treated with BSO and/or PQ. Mice were treated s.c. with 0.56 mmol/kg of vitamin E 24 hr and 1 hr prior to PQ (150 μ mol/kg) treatment. PQ was treated s.c. 4 hr after the BSO (3 mmol/kg) administration. Each value is the mean $\pm$ S.D. of 4–5 animals. **: Significantly different from mice not treated with vitamin E ($P < 0.01$).

Fig. 5. Effect of treatment with Desferal (DF) on plasma GPT level and BUN level in mice treated with BSO and/or PQ. Mice were treated s.c. with 0.19 mmol/kg of Desferal 4 hr and 1 hr before and 4 hr after PQ (150 μ mol/kg) treatment. PQ was treated s.c. 4 hr after BSO (3 mmol/kg) administration. Each value is the mean $\pm$ S.D. of 4–5 animals. **: Significantly different from mice not treated with Desferal ($P < 0.01$).

Fig. 6. Effect of pre-exposure to BSO (500 μ M) on cytotoxicity of PQ in NCTC clone 1469 mouse hepatoma cells. BSO was added into culture medium 15 hr prior to PQ treatment. The cells were treated with PQ for 6 hr, and MTT solution was added to each well. After 2 hr, crystals were dissolved by 20% SDS solution and optical density of each well at 600 nm was measured. Percent of survival cells was calculated as a ratio of absorbance of treated cells to those of control. Each value is the mean $\pm$ S.D. of 6 wells. **: Significantly different from cells not treated with BSO ($P < 0.01$).

DISCUSSION

A number of experimental results suggesting a protective role of cellular GSH against PQ toxicity have been reported. Yamamoto (1993) recently reported that PQ decreased GSH contents in the liver of mice. Administration of thiol compounds was effective to prevent the PQ-toxicity *in vivo* and *in vitro* (Szabo *et al.*, 1986; Hagen *et al.*, 1986). Bus *et al.* reported that GSH depletion induced by diethyl maleate increased lethal toxicity of PQ in mice (Bus *et al.*, 1975). PQ showed hepatotoxic effect in diethyl maleate-pretreated mice (Cagen and Gibson, 1977). These evidences indicate that GSH may play an important role in protection against PQ toxicity. However, the treatment only with diethyl maleate induced lipid peroxidation and liver necrosis (Maellaro *et al.*, 1990). Further, the relation of tissue GSH level to hepato- and nephrotoxicity of PQ has not been well documented. Therefore, we attempted to utilize BSO, a specific inhibitor of γ -glutamylcysteine synthetase (Griffith, 1982), for depletion of tissue

and cellular GSH to examine the protective role of GSH against tissue specific toxicity and cytotoxicity of PQ, because it was confirmed that the amount of BSO used did not affect values of the indicators for hepatic and renal functions. The depletion of GSH by pretreatment of mice with BSO significantly increased the levels of plasma GPT and BUN after PQ administration (Fig. 1). These results suggest that the intracellular level of GSH was involved in propagation of hepato- and nephrotoxicity of PQ. Because the enhancement of PQ toxicity by BSO was also observed in cultured mouse hepatoma cells (Fig. 6), the tissues having low concentration of GSH may be susceptible to PQ toxicity. On the other hand, Peter *et al.* (1992) described that GSH depletion by BSO could not sensitize the cultured cells to PQ toxicity. In the present study, mouse hepatoma cells were treated with PQ for a relatively short period, which might be adequate to observe toxic changes caused by PQ-induced oxidative stresses.

It has been suggested that the lipid peroxidation, initiated by reactive oxygens generated in the cyclic reduction-oxidation of PQ, is responsible for PQ toxicity (Bus *et al.*, 1976). In the present study, GSH depletion caused a significant progression of hepatic lipid peroxidation by PQ at relatively early period after the treatment (Fig. 2). GSH has been known as one of the intracellular factors to scavenge reactive oxygens generated in the cells (Meister, 1991). Therefore, at early stages of the treatment with PQ, generation of reactive oxygens in the tissues of PQ-treated mice may be enhanced by the depletion of GSH.

Vitamin E, an antioxidative factor, has been reported to inhibit PQ-induced lipid peroxidation and cytotoxicity in cultured cells (Watanabe *et al.*, 1986). On the other hand, Kohen and Chevion (1985) reported that lethal toxicity of PQ in mice was prevented by Desferal, an iron chelator. It has also been reported that iron salts are required to promote PQ-induced lipid peroxidation *in vitro* (Beloqui and Cederbaum, 1985). Sutton and Winterbourn (1984) indicated that PQ radicals reacted with hydrogen peroxide in the presence of low concentrations of iron formed hydroxyl radicals by the Fenton reaction. In the present study, both vitamin E and Desferal

significantly prevented hepatotoxicity and nephrotoxicity of PQ in GSH depleted mice (Fig. 4). PQ-induced lipid peroxidation in the liver of BSO-pretreated mice was also inhibited by vitamin E or Desferal (Fig. 3). Although some studies reported ineffectiveness of Desferal (Hoffer *et al.*, 1992) or vitamin E (Redetzki *et al.*, 1980; Eckert *et al.*, 1987; Combs and Peterson, 1983) for prevention against lethal toxicity of PQ, the results of the present study indicate that both vitamin E and Desferal are highly efficacious in protecting animals from the toxicity of PQ enhanced by GSH depletion. These results also suggest that, under GSH depletion, reactive oxygen species generated by PQ in the presence of iron ion may cause PQ toxicity.

REFERENCES

- Beloqui, O. and Cederbaum, A. I. (1985) : Microsomal interaction between iron, paraquat, and menadione : effect on hydroxyl radical production and alcohol oxidation. *Arch. Biochem. Biophys.*, **242**, 187–196.
- Bus, J. S., Aust, S. D. and Gibson, J. E. (1974) : Superoxide- and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. *Biochem. Biophys. Res. Commun.*, **58**, 749–755.
- Bus, J. S., Aust, S. D. and Gibson, J. E. (1975) : Lipid peroxidation : a possible mechanism for paraquat toxicity. *Res. Com. Chem. Pathol. Pharmacol.*, **11**, 31–38.
- Bus, J. S., Cagen, S. Z., Olgaard, M. and Gibson, J. E. (1976) : A mechanism of paraquat toxicity in mice and rats. *Toxicol. Appl. Pharmacol.*, **35**, 501–513.
- Cagen, S. Z. and Gibson, J. E. (1977) : Liver damage following paraquat in selenium-deficient and diethyl maleate-pretreated mice. *Toxicol. Appl. Pharmacol.*, **40**, 193–200.
- Combs, G. F., Jr. and Peterson, F. J. (1983) : Protection against acute paraquat toxicity by dietary selenium in the chick. *J. Nutr.*, **113**, 538–545.
- Ecker, J. L., Hook, J. B. and Givson, J. E. (1975) : Nephrotoxicity of paraquat in mice. *Toxicol. Appl. Pharmacol.*, **34**, 178–186.
- Eckert, H., Schnorr, R., Renner, H., Oddoy, A. and Lisotschkin, B. G. (1987) : Studies of lung damage by paraquat. 2. Effect of vitamin E on acute lung damage caused by paraquat. *Z. Erkr. Atmungsorgane*, **169**, 147–152.
- Farrington, J. A., Ebert, M., Land, E. J. and Fletcher, K. (1973) : Bipyridylum quaternary salts and related compounds. V. Pulse radiolysis studies of the reaction of paraquat radical with oxygen. Implications for the mode of action of bipyridyl herbicides. *Biochim. Biophys. Acta*, **314**, 372–381.
- Griffith, O. W. (1982) : Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. *J. Biol. Chem.*, **257**, 13704–13712.
- Hagen, T. M., Brown, L. A. and Jones, D. P. (1986) : Protection against paraquat-induced injury by exogenous GSH in pulmonary alveolar type II cells. *Biochem. Pharmacol.*, **35**, 4537–4542.
- Haley, T. J. (1979) : Review of the toxicology of paraquat (1,1'-dimethyl-4,4'-bipyridinium chloride). *Clin. Toxicol.*, **14**, 1–46.
- Hoffer, E., Zonis, Z., Tabak, A. and Taitelman, U. (1992) : The administration of desferrioxamine to paraquat-intoxicated rats. *Vet. Hum. Toxicol.*, **34**, 300–303.
- Kohen, R. and Chevion, M. (1985) : Paraquat toxicity is enhanced by iron and reduced by desferrioxamine in laboratory mice. *Biochem. Pharmacol.*, **34**, 1841–1843.
- Maellaro, E., Casini, A. F., Del Bello, B. and Comporti, M. (1990) : Lipid peroxidation and antioxidant systems in the liver injury produced by glutathione depleting agents. *Biochem. Pharmacol.*, **39**, 1513–1521.
- Matsumori, H., Matsumoto, T. and Ishikawa, H. (1984) : Acute toxic effects of paraquat on ultrastructure of rat liver. *Acta Pathol. Jpn.*, **34**, 507–518.
- Meister, A. (1991) : Glutathione deficiency produced by inhibition of its synthesis, and its reversal ; applications in research and therapy. *Pharmacol. Ther.*, **51**, 155–194.
- Ohkawa, H., Ohishi, N. and Yagi, K. (1989) : Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.*, **95**, 351–358.
- Peter, B., Wartena, M., Kampinga, H. H. and Konings, A. W. (1992) : Role of lipid peroxidation and DNA damage in paraquat toxicity and the interaction of paraquat with ionizing radiation. *Biochem. Pharmacol.*, **43**, 705–715.
- Redetzki, H. M., Wood, C. D. and Grafton, W. D. (1980) : Vitamin E and paraquat poisoning. *Vet.*

- Hum. Toxicol., **22**, 395–397.
- Shahar, E., Keidar, I., Hertzeg, E. and Barzilay, Z. (1989): Effectiveness of vitamin E and colchicine in amelioration of paraquat lung injuries using an experimental model. *Isr. J. Med. Sci.*, **25**, 92–94.
- Shrieve, D. C. and Harris, J. W. (1986): Effects of glutathione depletion by buthionine sulfoximine on the sensitivity of EMT6/SF cells to chemotherapy agents or X radiation. *Int. J. Radiat. Oncol. Biol. Phys.*, **12**, 1171–1174.
- Sutton, H. C. and Winterbourn, C. C. (1984): Chelated iron-catalyzed OH[•] formation from paraquat radicals and H₂O₂: mechanism of formate oxidation. *Arch. Biochem. Biophys.*, **235**, 106–115.
- Szabo, L., Matkovics, B., Barabas, K. and Oroszlan, G. (1986): Effects of various thiols on paraquat toxicity. *Comp. Biochem. Physiol.*, **83C**, 149–153.
- Tanaka-Kagawa, T., Naganuma, A. and Imura, N. (1993): Tubular secretion and reabsorption of mercury compounds in mouse kidney. *J. Pharmacol. Exp. Ther.*, **264**, 776–782.
- Toyo'oka, T. and Imai, K. (1983): High-performance liquid chromatography and fluorometric detection of biologically important thiols, derivatized with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F). *J. Chromatogr.*, **282**, 495–500.
- Watanabe, N., Shiki, Y., Morisaki, N., Saito, Y. and Yoshida, S. (1986): Cytotoxic effects of paraquat and inhibition of them by vitamin E. *Biochim. Biophys. Acta*, **883**, 420–425.
- Yamamoto, H. (1993): Protection against paraquat-induced toxicity with sulfite or thiosulfate in mice. *Toxicology*, **79**, 37–43.