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Protective Effect of Diallyl Disulfide on the Bromobenzene-Induced Hepatotoxicity in Mice

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ABSTRACT

The present work was undertaken to investigate the protective effect of diallyl disulfide on the bromobenzene toxicity in mice.

It was observed that the aniline hydroxylase and epoxide hydrolase activities were not changed by the treatment of diallyl disulfide for 5 days. But glutathione S-transferase activity was significantly increased. A striking enhancement of serum alanine aminotransferase activity and hepatic lipid peroxide content after bromobenzene administration was markedly decreased by diallyl disulfide pretreatment.

These results indicate that the inducing effects of diallyl disulfide on the bromobenzene intermediate detoxifying enzyme such as glutathione S-transferase are believed to be a possible protective mechanism for the bromobenzene toxicity in mice.

Key Words: Diallyl disulfide, Bromobenzene, Aniline hydroxylase, Epoxide hydrolase, Glutathione S-transfrease

INTRODUCTION

We previously reported that the treatment with diallyl disulfide, which is major breakdown product of allicin (Brondnitz *et al.*, 1971) (Scheme I), cause a significant increase of hepatic glutathione S-transferase activity (Huh *et al.*, 1986; Huh *et al.*, 1987).

Such a marked increment of glutathione S-transferase activity may prevent the cell damage due to electrophilic compounds and several organic hydroperoxides (Boyland and Chasseud, 1969; Prohaska and Ganther, 1977; Jakoby, 1978).

It is well known that bromobenzene is metabolized into chemically reactive metabolite by phase I enzyme and the toxic metabolite induces liver damage (Brodie *et al.*, 1971; Reid *et al.*, 1971). Meanwhile, bromobenzene-3,4-oxide, which is toxic intermediate of bromobenzene, is converted to inactivated form by glutathione S-transferase (Fijellstedt *et al.*, 1973) and epoxide hydrolase (Zampaglion *et al.*, 1973) (Scheme II).

Therefore, the present study was designed to investigate the protective effect of diallyl disulfide on the bromobenzene-induced hepatotoxicity.

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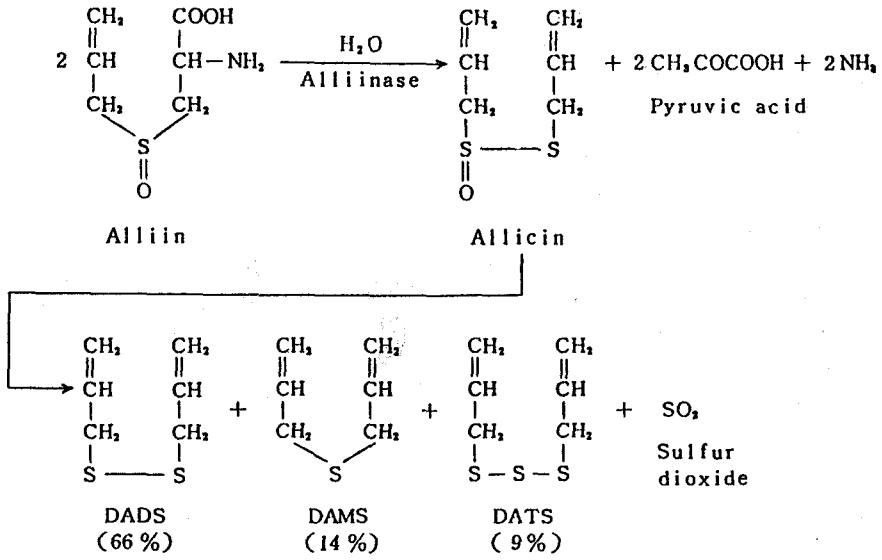
EXPERIMENTAL METHODS

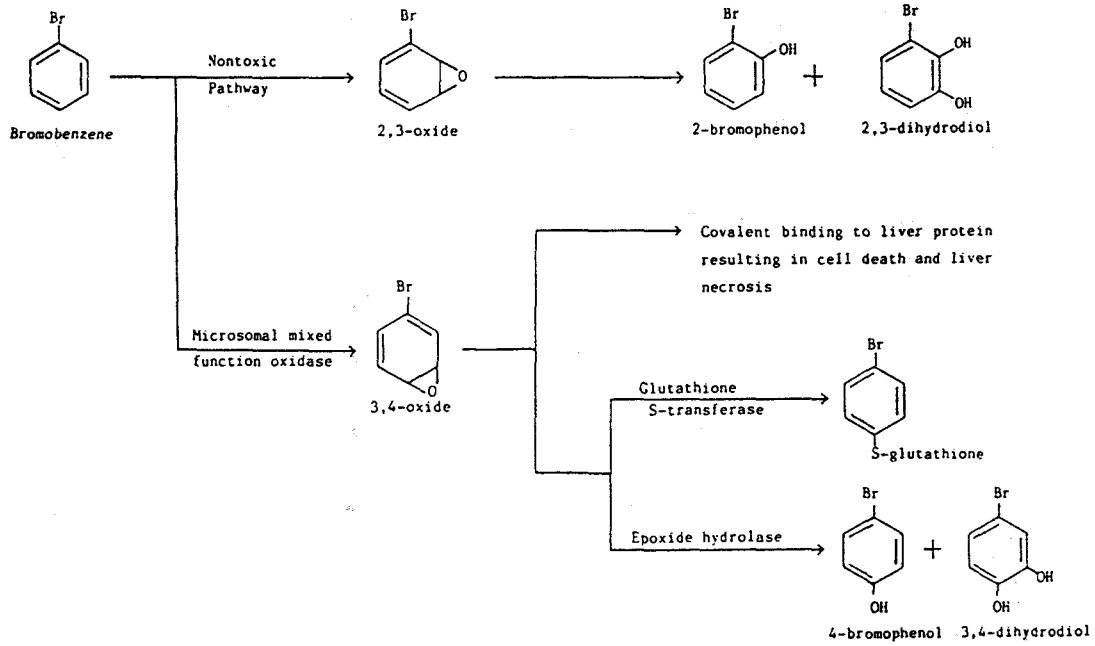
Animal treatment

ICR-male mice weighing 25g were used for all studies. Mice were given diallyl disulfide (20 mg/kg) intraperitoneally once daily for 5 days (Huh *et al.*, 1987). Control mice were injected olive oil intraperitoneally. Bromobenzene (460 mg/kg) was



DADS ; Diallyl disulfide
DAMS ; Diallyl monosulfide
DATS ; Diallyl trisulfide

Scheme I. Breakdown products of alliin.



Scheme II. Pathways of biotransformation of bromobenzene.

treated 8 hr after the last injection of diallyl disulfide for 2 days. Mice were killed 4 hr after the last dose of bromobenzene. All animals have free access to food and water but deprived of food for 20 hr prior to sacrifice.

Preparation of cytosolic and microsomal fractions

The animals were killed by exsanguination from inferior vena cava. The liver was exhaustively perfused with cold 0.15M sodium chloride solution through the portal vein and quickly removed. The liver was homogenized in cold 0.25M sucrose solution until it was uniformly homogenized. The homogenate (20% w/v) was sequentially centrifuged at 600×g, 10,000×g and 105,000×g. Then the 105,000×g supernatant was used as the cytosolic fraction and pellet as the microsomal fraction.

Partial purification of glutathione S-transferase

Purification of mouse liver cytosolic glutathione S-transferase was a modification of those described for the purification of rat liver transferases by Habig and Jakoby (1981).

Mouse liver cytosolic protein (40mg) was passed over a DEAE-cellulose column (1.6×5.5cm; Whatman DE52) equilibrated with 10mM Tris-HCl (pH 7.0) in 5mM 2-mercaptoethanol. Fractions containing transferase activity were pooled and made 150μM with respect to glutathine, and crystalline ammonium sulfate was added to 95% saturation. The precipitate was collected, dissolved and dialyzed against 10mM potassium phosphate (pH 6.1) containing 1mM EDTA, 150μM glutathione, 7mM 2-mercaptoethanol. The dialyzate was subjected to chromatography on a carboxymethylcellulose column (1.6×5.5cm; Whatman CM52) equilibrated with same buffer. After the column had been washed with the pH 6.1 buffer, different forms of mouse transferase was eluted with linear salt gradient from 0 to 200mM potassium chloride in pH 6.1 buffer.

Assay methods

Aniline hydroxylase activity was assayed by monitoring the formation of p-aminophenol according to the procedure of Bidlack *et al.* (1982). Epoxide hydrolase activity was estimated by the method of Hasegawa *et al.* (1982) with trans-stilben oxide as substrate. Glutathione S-transferase activity was measured by the amount of thioether formed according to Habig *et al.* (1974) with glutathione and 1-chloride-2,4-dinitrobenzene as substrates. Serum alanine aminotransferase (ALT) activity was measured by the method of Reitman and Frankel (1957). Content of reduced glutathione in liver tissue was estimated by the method of Ellman (1959). Lipid peroxide level of liver tissue was followed by measuring malondialdehyde with thiobarbituric acid method of Ohkawa *et al.* (1979). Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Student's t-test was used to establish significant differences in mean values between the control and treated groups.

Table 1. Effect of diallyl disulfide on the hepatic microsomal aniline hydroxylase, the cytosolic epoxide hydrolase and glutathione S-transferase activities in mice

Enzymes \ Treatment	Control	Diallyl disulfide
Microsomal AH[1]	0.78±0.06	0.88±0.10
Cytosolic EH[2]	8.06±0.56	7.19±0.08
Cytosolic GST[3]	1127.3±52.4	1419.6 ±76.1*

Mice were injected diallyl disulfide (20mg/kg) i.p. daily for 5 days and killed 24 hr after the last dose.
The assay procedure was described in the experimental methods.
Values are mean±SE for 6 animals.
1): p-aminophenol formed n moles/mg protein/min
2) decreased trans-stilben oxide n moles/mg protein/min
3) conjugated 2,4-dinitrobenzene-glutathione n moles/mg protein/min

RESULTS

Effect of diallyl disulfide on the hepatic microsomal aniline hydroxylase, cytosolic epoxide hydrolase and glutathione S-transferase activities

The influence of diallyl disulfide on the bromobenzene metabolizing enzymes activities are shown in Table 1. By the treatment of diallyl disulfide for 5 days, phase I reaction enzyme of bromobenzene metabolism, which is microsomal aniline hydroxylase, was not significantly changed. But, the detoxifying enzyme for reactive metabolite of bromobenzene, glutathione S-transferase acitvity was powerfully enhanced. Whereas, epoxide hydrolase activity was not altered by the treatment of diallyl

disulfide.

Effect of diallyl disulfide on the hepatic glutathione S-transferase activity in vitro

Diallyl disulfide effect on the cytosolic glutathione S-transferase was demonstrated in vitro and the results are shown in Fig. 1. The activities of mice liver glutathione S-transferase in the presence of diallyl disulfide were not changed.

Partial purification of liver cytosolic glutathione S-transferase in diallyl disulfide-treated mice

The elution profile is represented in Fig. 2. Two main activity fractions were recovered. In the unretained material, first peak, enzyme activity of diallyl disulfide-treated group was increased about 1.3 fold and second peak, the retained material, was increased about 1.9 fold as compared with control group.

Effect of diallyl disulfide on the kinetics of glutathione S-transferase

Double reciprocal plots on partial purified hepatic glutathione S-transferase of second peak was described in Fig. 2 for 1-chloro-2,4-dinitrobenzene are represented in Fig. 3. The Vmax value in diallyl disulfide-treated group was increased about 1.74 fold as compared with control. However, Km value was not changed.

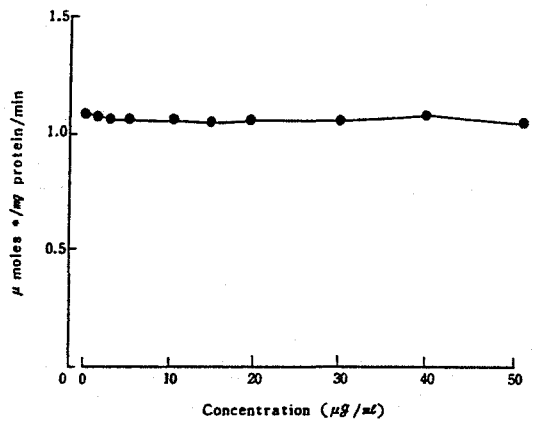


Fig. 1. Effect of diallyl disulfide on the liver cytosolic glutathione S-transferase activity in vitro. The assay procedure was described in the experimental methods. Values are mean for 3 separate experiments.
*; conjugate 2,4-dinitrobenzene-glutathione

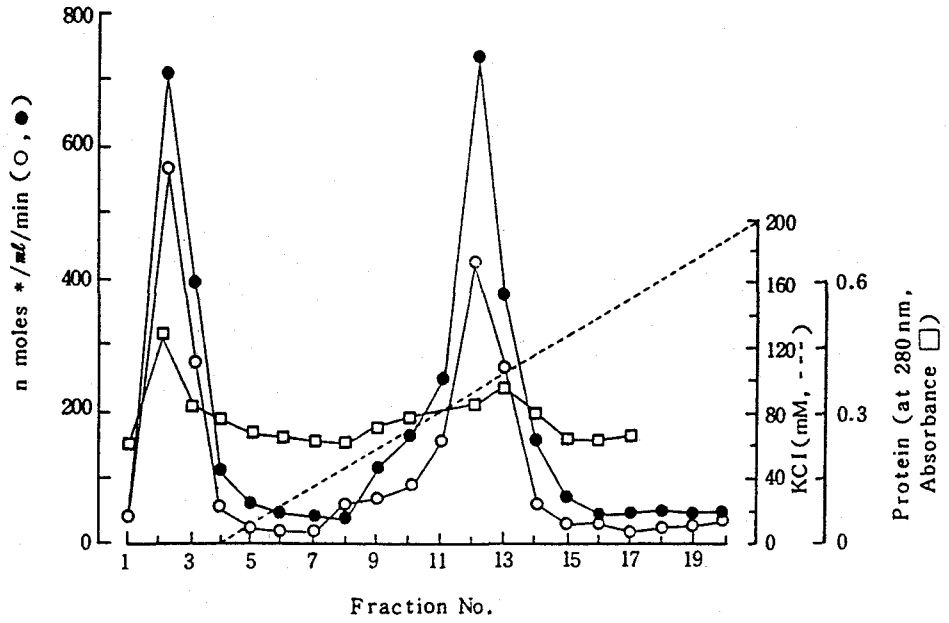


Fig. 2. CM-52 column chromatography of the liver cytosolic glutathione S-transferase. Liver cytosolic glutathione S-transferase from control and diallyl disulfide-treated mice were isolated as described in the experimental methods. Fractions of 5ml volume were collected and assayed for glutathione S-transferase activity. ——○——; Control, ——●——; Diallyl disulfide, *; conjugated 2,4-dinitrobenzene-glutathione.

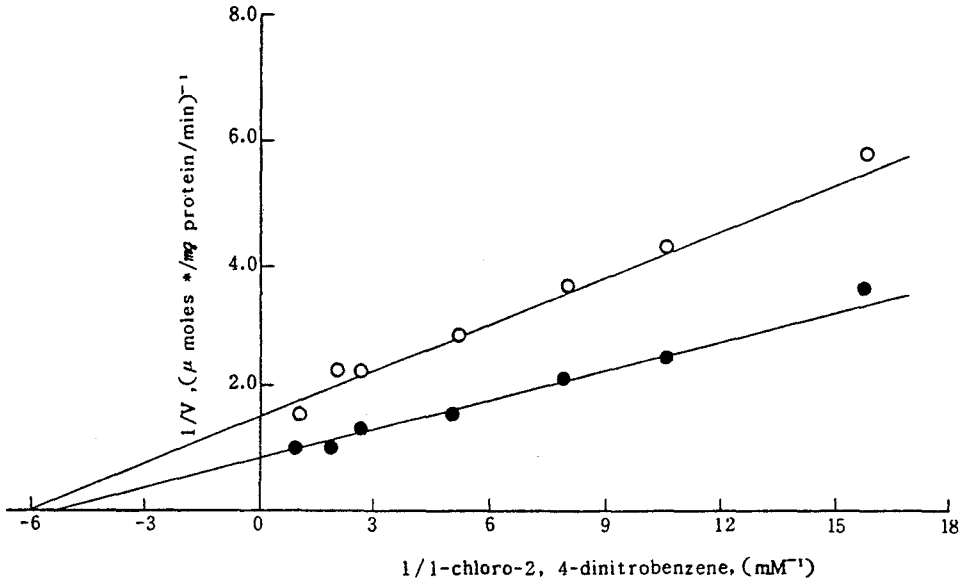


Fig. 3. Double reciprocal plots of the partial purified liver cytosolic glutathione S-transferase activity as a function of 1-chloro-2,4-dinitrobenzene at fixed level of reduced glutathione (1mM). Liver cytosolic glutathione S-transferase was partial purified by DE-52 and CM-52 column chromatography in Fig. 3. ——O——; Control, ——●——; Diallyl disulfide, *; conjugated 2,4-dinitrobenzene-glutathione.

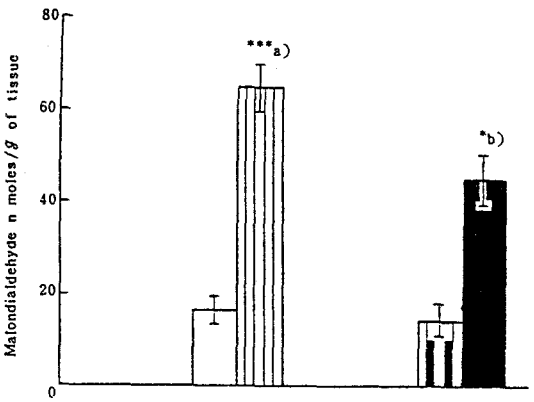


Fig. 4. Effect of diallyl disulfide on the formation of lipid peroxide in bromobenzene-treated mice livers. Mice were injected diallyl disulfide (20 mg/kg) i.p. daily for 5 days and bromobenzene (460 mg/kg) i.p. for 2 days. Mice were sacrificed 4 hr after the last dose of bromobenzene. The assay procedure was described in the experimental methods. Values are mean ± SE for 6 animals. □; Control, ▥; Bromobenzene, ▨; Diallyl disulfide, ■; Diallyl disulfide + Bromobenzene.
a); Significantly different from control, b); Significantly different from bromobenzene (*; $p<0.05$, ***; $p<0.001$).

Effect of diallyl disulfide on the hepatic lipid peroxide in bromobenzene-treated mice

Fig. 4 shows the effect of diallyl disulfide pretreatment on the formation of hepatic lipid peroxide in bromobenzene-treated mice. Bromobenzene administration produced a significant increase in the lipid peroxide. Whereas, the increment of lipid peroxide was remarkably decreased by the pretreatment of diallyl disulfide for 5 days.

Effect of diallyl disulfide on the hepatic glutathione level in bromobenzene-treated mice

The influence of diallyl disulfide on the hepatic reduced glutathione level is shown in Fig. 5. When bromobenzene was injected to the control mice, glutathione content was significantly decreased. But, decrease of glutathione level was lower by the pretreatment of diallyl disulfide than that of control group given bromobenzene alone.

Effect of diallyl disulfide on the hepatic cytosolic glutathione S-trasnferase activity in bromobenzene-treated mice

As shown in Fig. 6, cytosolic glutathione S-

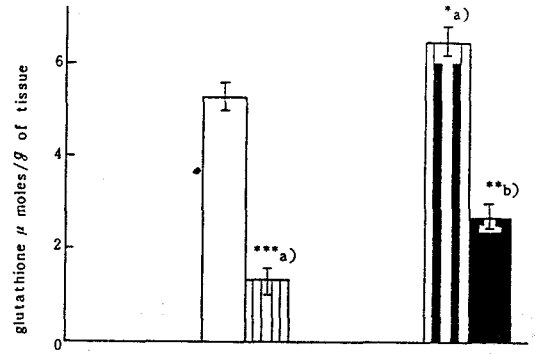


Fig. 5. Effect of diallyl disulfide on the hepatic glutathione level bromobenzene-treated mice. The assay procedure was described in the experimental methods. The other conditions are the same as described in Fig. 4. *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$.

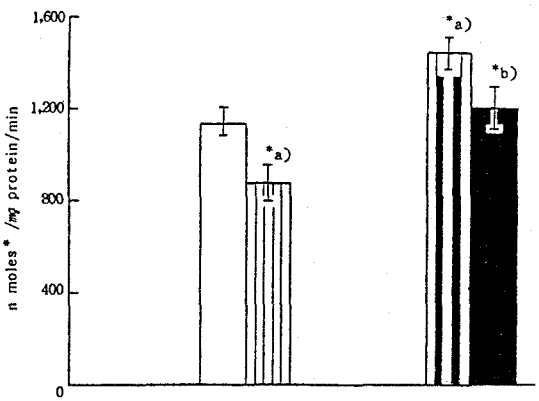


Fig. 6. Effect of diallyl disulfide on the liver cytosolic glutathione S-transferase activity in bromobenzene-treated mice. The other conditions are the same as described in Fig. 4. *; conjugated 2,4-dinitrobenzene-glutathione

transferase activity in the liver was significantly enhanced by the treatment of diallyl disulfide for 5 days. Meanwhile, when bromobenzene was injected to control mice, the enzyme activity strikingly decreased. But, in diallyl disulfide pretreated group, the decreasing effect on the enzyme activity was remarkably inhibited.

Effect of diallyl disulfide on ther serum ALT activity in bromobenzene-treated mice

The influence of diallyl disulfide on the hepatotoxicity in acute bromobenzene-treated mice is shown in Table 2. The average increment of serum ALT activity after bromobenzene administration was 20 fold compared to the control group. This increment of ALT activity was significantly reduced by the pretreatment of diallyl disulfide.

Table 2. Effect of diallyl disulfide on the serum ALT activity in bromobenzene-treated mice

Treatment	ALT (Karmen unit/ml of serum)
Control	29.3 + 5.4
Bromobenzene	580.1 + 36.3***a)
Diallyl disulfide	26.9 + 4.7
Diallyl disulfide + Bromobenzene	170.4 + 25.8***b)

Mice were given diallyl disulfide (20 mg/kg) i.p. daily for 5 days, and bromobenzene (460 mg/kg) i.p. for 2 days. Mice were sacrificed 4 hr after the last dose of bromobenzene.

a): significantly different from control, b): significantly different from bromobenzene-treated group. ***; $p<0.001$

DISCUSSION

It is well known that garlic has various pharmacological effect (Cavallito *et al.*, 1944; Dipaolo and Carruthers, 1960). Recent studies have shown that garlic components inhibited many metabolic diseases, such as atherosclerosis (Jain, 1975; Bordia *et al.*, 1977), diabetes (Mathew and Augusti, 1973; Jain and Vyas, 1975) and gout (Huh *et al.* 1985).

In this report, we have examined the protective mechanism of diallyl disulfide on the bromobenzene-induced hepatotoxicity in experimental model.

It was observed that treatment with diallyl disulfide did significantly increase glutathione S-transferase activity and glutathione level. Enzyme activity, however, in the presence of diallyl disulfide were not affected in vitro. These results imply that the increment of glutathione S-transferase activity was unlikely to have arisen directly by diallyl disulfide. Meanwhile, the elevation of glutathione S-transferase in elution profile of ion-exchange chromatography was found and Vmax of partial purified glutathione s-transferase for 1-chloro-2, 4-dinitrobenzene was significantly increased by the treatment of diallyl disulfide for 5 days. Therefore, the characteristics of the increase in the glutatione S-transferase activity by diallyl disulfide may include

induction of enzyme proteins. It was also observed that bromobenzene activating enzyme such as aniline hydroxylase and detoxifying enzyme such as epoxide hydrolase were not changed by the treatment with diallyl disulfide. In this conditions, serum alanine aminotransferase activity and hepatic lipid peroxide were significantly increased in control group by the injection of bromobenzene. But this increasing effects were markedly lowered in diallyl disulfide-pretreated group.

These results indicate that diallyl disulfide may improve the bromobenzene-induced hepatic cell membrane damage (Takeda *et al.*, 1964; Plaa and Witschin, 1976). Furthermore, the injection of bromobenzene results in the significant decrease in the level of hepatic glutathione and the activity of glutathione S-transferase, this decreasing effects were powerfully inhibited in diallyl disulfide-pretreated group.

In the above mentioned, glutathione S-transferase is regarded as the detoxifying enzyme which catalyzed the first step in mercapturic acid formation through the glutathione conjugation. Therefore, these results suggest that diallyl disulfide may prevent the tissue damage due to electrophilic compounds such as reactive bromobenzene metabolite by the increment of hepatic glutathione level and glutathione S-transferase activity. But further research in this field is needed.

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= 국문초록 =

Bromobenzene 간손상에 대한 Diallyl Disulfide의 예방효과

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Diallyl disulfide의 간손상 예방효과를 검토할 목적으로 mouse에 bromobenzene을 투여해 간 손상의 model을 만든 다음 실험을 행하였을 때, diallyl disulfide를 전처치한 실험군이 대조군에 비하여 간손상의 정도가 경미하게 나타났다. 한편 diallyl disulfide의 간손상예방 효과 기전을 구명할 목적으로 bromobenzene의 활성화 및 해독 관련 효소들의 활성을 검토하였을때 활성화 효소인 aniline hydroxylase와 해독 효소의 일종인 epoxide hydrolase의 활성은 diallyl disulfide 전처치에 의해 변동이 없었으나, 나머지 해독 효소인 glutathione S-transferase의 활성은 유의 하게 증가되었고 이의 포합인자인 glutathione의 함량도 증가하였다. 또한 diallyl disulfide에 의한 glutathione S-transferase 활성 증가현상이 어떠한 기전으로 나타나는지 검토 하였을때, diallyl disulfide를 전처치 함으로서 Km치는 별다른 차이가 없었으나 Vmax치는 대단히 증가하였다. 그러나 시험관내에서 diallyl disulfide의 첨가농도를 증가시켰을 때에는 효소활성은 변하지 않았다. 이러한 실험결과들을 종합해 볼 때, diallyl disulfide 전처치에 의한 bromobenzene이 유도한 간손상의 예방효과는 diallyl disulfide가 bromobenzene의 독성 중간 대사산물인 bromobenzene 3,4-oxide를 해독하는 glutathione S-transferase 단백의 합성을 유도함으로서 나타난 결과로 사료되어지나 이점에 대해서는 추후 계속적인 연구 검토가 행해져야 할것이다.