J. Toxicol. Pub. Health
Vol. 14, No. 2, pp. 157-161, 1998

Aliphatic and Allyl Alcohol-Induced Liver Cell Toxicity and its Detoxification

Su-Kyung Park, Wan-Koo Lee[1], Young-Hoon Park[1] and Jeon-Ok Moon*

College of Pharmacy, Pusan National University, Keumjeung-ku, Pusan 609-735, Korea, and
[1]National Institute of Scientific Investigation Southern District Office,
Dongsam-dong, Youngdo-ku, Pusan 606-081, Korea
(Received March 12, 1998)
(Accepted June 8, 1998)

***ABSTRACT** : The mechanism of active aldehyde-induced liver disease and the enzymatic basis of detoxification were investigated using normal rat liver cell, Ac2F. Aliphatic alcohols including 1-decyl alcohol, 1-nonanol, 1-heptanol, 1-hexanol, 1-propanol and allyl alcohol exerted a dose- and time-dependent toxicity to Ac2F cells. The extent of their toxicities in buthionine sulfoximine (inhibitor of glutathione synthesis) pretreated cells was greater than in pargyline (inhibitor of aldehyde dehydrogenase, ALDH). On the other hand, the toxicity of these alcohols were not affected by 4-methylpyrazole (inhibitor of alcohol dehydrogenase, ADH). These results suggest that the contents of glutathione (GSH) seems to be very important in protecting the cells from toxicants such as aliphatic alcohols.*

***Key Words** : Aldehyde, Cytotoxicity, Glutathione*

I. INTRODUCTION

Many of the disturbances in hepatocyte structure and function associated with ethanol ingestion may result from either a direct or indirect effect of acetaldehyde (Tuma *et al.*, 1991, Lieber *et al.*,1988). There is direct evidence that increased production of acetaldehyde associated with chronic ethanol ingestion may alter mitochondrial function and specifically inhibit aldehyde dehydrogenase (ALDH) activity (Wang T. T., 1989). Furthermore, acetaldehyde-induce mitochondrial dysfunction could in turn induced still higher hepatic acetaldehyde levels. This vicious circle may cause further damage to the mitochondria and ultimately result in hepatocyte necrosis. Besides the effects of acetaldehyde on mitochondria, a number of other adverse effects of acetaldehyde on hepatocyte function have been described (Lieber *et al.*, 1988). These include alterations in DNA repair and microtubule assembly, the latter resulting in altered protein transport and secretion (Sorell *et al.*, 1987). Acetaldehyde has also been shown to enhance lipid peroxidation and collagen synthesis (Lieber *et al.*, 1988). Besides the formation of acetaldehyde from alcohol ingestion, a number other aldehydes form during normal biological process, which include aldehyde metabolites of biologic amines and the reactive aldehyde produced by peroxidation of microsomal membranes. However, only limited studies are found on the intoxification and detoxification of these aldehydes. In this paper, mechanism of the active aldehyde-induced liver disease and the enzymatic basis of detoxification was investigated using rat normal cell, Ac2F.

*To whom correspondence should be addressed

II. MATERIALS AND METHODS

1. Chemicals and materials

Dimethylsulfoxide (DMSO), glutamine, 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glutathione, rotenone, DL-buthionine-[S,R]-sulfoximine (DL-BSO), 4-methylpyrazole (4-MP), 1-hexanol, 1-heptanol, 1-nonanol and N-methyl-N-2-propynyl benzylamine (pargyl-ine, PG) were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Fetal bovine serum, Dulbecco's Modified Eagle Media (DMEM), trypsin-EDTA and antibiotic-antimycotic were purchased from Gibco Chemical

Co. (Grand Island, U.S.A.). Allyl alcohol was from Wako Chemical Co. (Tokyo, Japan).

2. Cell culture

Ac2F, the normal liver cell of Donryu rat, which was obtained from Japan Cancer Research Resources Bank, Tokyo, Japan. The cells were routinely cultured in the complete medium composed of DMEM supplemented with 10% fetal bovine serum, 2% glutamine, penicillin (100 μg/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) in a humidified atmosphere 5% CO_2, 95% air.

3. MTT Cytotoxicity assay of alcohols

The cells were placed in 24-well plates at a cell density of 5.0×10^4 cells per well. After 18-20 hrs cultivation, the complete medium of the plates was replaced by serum free medium (SFM) and the cells were exposed to varying concentration of alcohols for 2, 4 and 24 hrs. After incubation, 100 μl of MTT solution (5 mg/mL in phosphate buffered saline, PBS) was added to each well and incubated for another 4 hrs. After the culture medium had been removed, 1mL of the mixture of the same volume with DMSO and ethanol was added and mixed to dissolve the MTT formazan. The plates were measured on a microplate reader (Packard Instrument Co. U.S.A.) using wavelength of 570 nm (Tim, 1983). The survival values, used to examine the survival on cell damage by alcohols, were expressed as a percentage of the absorbance of the normal cells.

4. Exposure of alcohols after pretreatment with 4-MP, PG and DL-BSO

The cells were placed in 24-well plates at a cell density of 5.0×10^4 cells per well. After 18~20 hrs cultivation, the complete medium of the plates was replaced by SFM and the cells were preincubated with 0.1 mM 4-MP, 0.1 mM PG and 0.5 mM DL-BSO for 30 minutes before subsequent alcohols exposure and were then incubated for 24 hrs after adding alcohols. Then, MTT assay was performed.

5. Cell preparations

The cells seeded on 100 mm cell culture dish (Corning) and cultured to 80~90% confluency. The complete medium of the plates was replaced by SFM and the cells were exposed to 0.1 mM 4-MP, 0.1 mM PG , 0.5 mM DL-BSO for 2, 4, 8, 16 and 32 hrs. After the culture medium was removed from the dish, the cells were washed twice with PBS. The cells in the dish were collected by a scrapper with 1 mL PBS. From each culture dish, 1 mL cell suspension was removed and placed in a 1.5 mL tube and subsequently centrifuged at 700×g for 6 minutes. After the supernatant was removed, 200 μl phosphate buffer (pH 7.4) was added to the pellets and then were sonicated twice in the interval of 10 seconds. The mixtures were centrifuged at 14,240×g for 15 minutes (S9 fraction). For the assay of GSH content, the mixtures were centrifuged at 700×g for 10 minutes (homogenate). The treated supernatant were stored at -78°C until assay.

6. GSH assay

GSH was assayed in the homogenates (centrifuged at 700×g for 10 minutes) by using the method of Higash (1988). For the determination of nonprotein SH, homogenate (100 μl) was treated with trichloroacetic acid and centrifuged. The supernatant (100 μl) was mixed with 0.05 mL 0.01 M $NaNO_2$, and 0.45 mL 0.2 N H_2SO_4, and was permitted to stand for 5 minutes. Afterward 0.5% sulfamic acid ammonium solution (0.2 mL), 0.1 mL 1% $HgCl_2$, 0.9 mL 3.4% sulfanilamide and 1 mL 0.1% N-naphthylethylenediamine (in 0.4 N HCl) were added to the supernatant. The mixture was kept for 5 minutes and detected at 540 nm. The standard used was 125 nM GSH solution.

Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard (Lowry *et al.*, 1951).

7. Enzyme assay

ADH activity was measured by the method of Crow *et al.* (1997) using 50 μl ethanol following the appearance of NADH at 340 nm. Reaction solution (1.0 mL) contained 1.25 mM NAD^+, 6.25 μM rotenone, 50 μl S9 fraction and 50 mM phosphate buff-

er (pH 7.4). Activity was calculated by molecular absorbance constant 6.22 $cm^{-1}mM^{-1}$ and represented unit/mg protein.

ALDH activity was determined by the method of Tottman *et al.* (1973) using 6.25 mM propanal following the appearance of NADH at 340 nm. Reaction solution (1.0 mL) contained 1.25 mM NAD^+, 0.125 mM pyrazole, 6.25 μM rotenone, 2 mM 2-mercaptoethanol, 50 μl S9 fraction and 50 mM phosphate buffer (pH 7.4). Activity was calculated by molecular absorbance constant 6.22 $cm^{-1}mM^{-1}$ and represented unit/mg protein. The concentration of pyrazole inhibited ADH activity but didn't affect ALDH activity. Rotenone was used to inhibit the activity of mitochondrial NADH oxidase and 2-mercaptoethanol was added to protect the enzyme.

III. RESULTS and DISCUSSION

The detoxification mechanism of aliphatic alcohols including 1-decyl alcohol, 1-nonanol, 1-heptanol, 1-hexanol, and 1-propanol was investigated using the rat normal liver cell, Ac2F. When the cells were incubated with various concentrations of the alcohols for 4 hrs and 24 hrs, the cell viability was significantly decreased in a dose and time dependent manner(Table 1). The greatest toxic effect was exhibited by 1-decyl alcohol, followed by 1-nonanol, 1-heptanol, 1-hexanol and n-propyl alcohol on the Ac2F cells. There seemed to be a correlation between the carbon chain length and toxicity on these cells.

4-MP reported to be an inhibitor of ADH. The inhibitory effect of 4-MP on ADH in Ac2F cells is shown in Table 2. Compared to the control cells which showed ADH activity as 4.94×10^{-3} unit/mg protein, cells treated with 0.1 mM 4-MP for 2 hours showed a decrease in ADH activity to 1.85×10^{-3} unit/mg prot.(37.5% of the control). The activity of ADH in cells decreased more as the incubation time increased. However, after 32 hrs of incubation, the activity was almost restored to control level.

Table 1. The IC_{50} (mM) of aliphatic alcohols on the Ac2F cell

	4 hr	24 hr
1-decyl alcohol (n=10)	0.88	1.05×10^{-3}
1-nonanol (n=9)	2.07	5.25×10^{-3}
1-heptanol (n=7)	8.51	30.9×10^{-3}
1-hexanol (n=6)	11.22	3.47
n-propyl alcohol (n=3)	279.90	25.7

Table 2. The inhibitory effect of 4-MP on the ADH activity in the Ac2F cell

Concentration (mM)	Time (hr)				
	2	4	8	16	32
0.02	43.7	18.4	N.D.	N.D.	95.8
0.1	37.5	26.9	N.D.	N.D.	29.0
0.5	N.D.	N.D.	N.D.	N.D.	33.2

4-MP was dissolved in the culture medium and added to the culture medium to final 0.02, 0.1, and 0.5 mmol/L for each incubation time. At the end of incubation, the cells were collected into PBS and sonicated. The ADH activity in the supernatant of the S9 fraction (centrifuged at 14,240×g for 15 minutes) was measured by the method of Crow. The ADH activity are expressed as percentages for activity of 4-MP free cell (normal cell). Data are shown as mean of duplicate experiments. N.D. means not detected.

Table 3 demonstrates the inhibitory effect of PG, a known ALDH inhibitor, on ALDH in Ac2F cells. ALDH activities in the cells treated with 0.02 mM PG for 2 hrs and for 4 hrs were significantly reduced to 4.02×10^{-3} and 1.0×10^{-3} unit/mg protein, respectively (60.6 and 15.1 % of the control). The ALDH level was restored to the control level after 32 hrs of incubation. With 0.1 mM PG, ALDH activities were reduced to 37.4% of the control and after 4 hrs of incubation, the activities were virtually zero.

BSO is a potent and specific inhibitor of γ-glutamyl cysteine synthetase. It is reported that administration to animals or incorporated into tissue

Table 3. The inhibitory effect of PG on the ALDH activity in the Ac2F cell

Concentration (mM)	Time (hr)				
	2	4	8	16	32
0.02	60.6	51.1	N.D.	30.6	100
0.1	37.4	N.D.	N.D.	N.D.	14.8
0.5	N.D.	N.D.	N.D.	N.D.	29.6

For measurement of ALDH activity after exposure to PG, the cells were incubated with PG at 0.02, 0.1, and 0.5 mmol/L for the indicated hours. After the culture medium in the dish had been removed, the cells were washed twice with PBS. From each culture dish a 1 ml cell suspension was removed and placed in a 1.5 ml tube. The collected cells were sonicated and centrifuged at 14,240×g for 15 minutes. The measurement of ALDH activity was used by Tottman method. The ALDH activity are expressed as percentages for activity of PG free cell (normal cell). Values represent mean of duplicate experiments. N.D. means not detected.

Table 4. The inhibitory effect of BSO on the cellular content of GSH in the Ac2F cell

Concentration (mM)	Time (hr)				
	2	4	8	16	32
0.1	51.8	48.5	41.0	18.2	10.0
0.5	51.2	42.4	44.9	16.2	9.7
2.5	43.8	53.2	39.9	12.6	9.6

Cells treated with DL-BSO (0.1, 0.5, 2.5 mmol/L) were incubated for varying hours. Cells were washed and disrupted using a sonicator, and GSH was assayed in the homogenates (centrifuged at 700×g for 10 minutes) by using the method of Higash. The GSH contents are expressed as percentages for activity of BSO free cell (normal cell). Data are shown as mean of duplicate experiments.

culture media of BSO causes an inhibition of GSH biosynthesis and a depletion of cellualar GSH levels (Griffith, 1982). The cellular content of GSH in Ac 2F cells also decreased in a time-dependent fashion upon addition of BSO (Table 4). At a concentration of 0.1 mM BSO, GSH was less than 30.48 nmol/mg protein after 2 hrs (51.8% of control).

Based on these studies, to investigate the roles of ADH, ALDH and GSH on the various alcohol's toxic effects, the cells were pretreated with 0.1 mM 4-MP, 0.1 mM PG and 0.5 mM BSO for 30 minutes (Table 5). With these conditions, the cell viabilities were not altered from that of untreated cells. The alcohol-induced cytotoxicities were not affected by 4-MP pretreatment while an occasional increasement of the susceptibility to the alcohol-induced cytotoxicity was observed with the PG pretreatment. However, the extent of toxicity in BSO pretreated cells was enhanced which suggested that GSH may have a greater effect in detoxifying alcohols.

This tendency seemed to be more striking in allyl alcohol (Fig. 1). Allyl alcohol is used in various chemical industries as a synthetic intermediate (Patel *et al.*, 1980). When administered to animals, allyl alcohol produces periportal hepatic necrosis (Reid, 1972), and ADH, which is located mainly in the periportal areas of the liver lobule (Rees and Tarlow, 1967), catalyzed the formation of the aldehyde acrolein from allyl alcohol (Jose *et al.*, 1989). In this study we used the normal rat hepatic cell, Ac2F, and the rate of cellular viability loss increased along with the concentration of allyl alcohol during incubation. The IC_{50} was calculated as 52.5 mM in 2 hrs incubation.

Although ADH, ALDH activities and GSH content in the cell were reduced by 4-MP (0.1 mM), PG (0.1 mM) and BSO (0.5 mM) respectively, the cell viabilities were not altered from that of untreated cells at these concentrations during the 2 hrs incubation period. However with the addition of allyl alcohol, although 4-MP pretreatment did not have an effect, PG pretreatment significantly influenced cell viability at concentrations from 20 to 50 mM. The pronounced effect of BSO was again observed

Table 5. Effects of 4-MP, PG and BSO pretreatment on the susceptibility of the Ac2F cell to aliphatic alcohols

	Concentration (mM)	Control	4-MP	pargyline	DL-BSO
1-decyl alcohol (n=10)	1.6×10^{-3}	46±2	43±1**	41±2**	36±4**
	8.0×10^{-3}	31±3	28±1	25±2*	23±1**
	40×10^{-3}	16±1	16±1	14±0*	14±0**
1-nonanol (n=9)	1.6×10^{-3}	65±2	66±4	62±3	59±3**
	8.0×10^{-3}	42±3	42±7	41±2	39±1*
	40×10^{-3}	27±2	26±3	26±0	22±1**
1-heptanol (n=7)	16×10^{-3}	94±2	97±2	91±1*	82±3***
	80×10^{-3}	71±2	71±2	66±2**	62±2***
	400×10^{-3}	46±1	45±1	43±1**	39±2***
1-hexanol (n=6)	1	67±4	71±3	66±2	65±1
	5	59±2	55±4	54±2**	49±1***
	25	3±0	3±0	3±0	3±0
n-propyl alcohol (n=3)	1	89±2	95±4	98±4	86±3
	5	84±3	89±5	85±5	84±2
	25	76±3	79±2	77±2	75±2

The cells were preincubated with 4-MP (0.1 mmol/L), PG (0.1 mmol/L) and DL-BSO (0.5 mmol/L) 30 minutes before the subsequent alcohols exposure and were then incubated for 24 hrs after adding alcohols. Survivals estimated by MTT assay are expressed as percentages for survival of the normal cells. Data are the mean±S.D. (n=4). Significantly different *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. control.

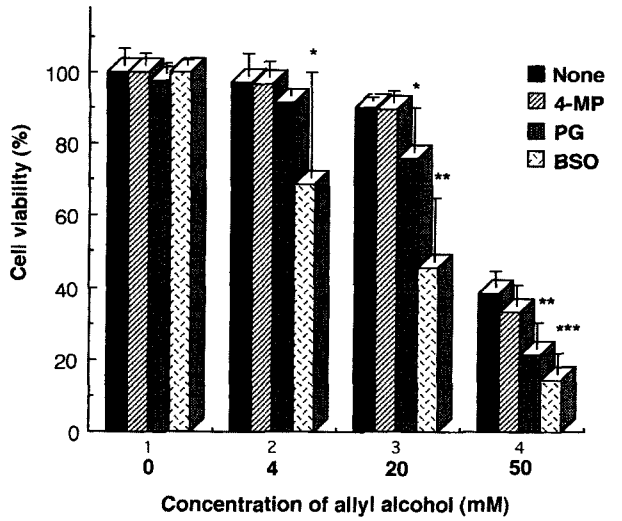

Fig. 1. Effects of 4-MP, PG and BSO on the allyl alcohol-induced cytotoxicity. Cells were treated with allyl alcohol for 2 hrs. Survivals estimated by MTT assay are expressed as percentages for survival of the normal cells. Results are the mean±S.D. (n=4). Significantly different *p<0.05, **p<0.01 and ***p<0.001 vs. control.

in allyl alcohol induced cell toxicities, and the effect of inhibitor was clearly potentiated with the increasing in concentration of allyl alcohol from 4 mM to 50 mM. At a concentration of 50 mM allyl alcohol, cell viability was reduced to 38.3% of untreated cells, whereas it was reduced to 14.2% of untreated cells (37.0% of 50 mM allyl alcohol treated cells) by the pretreatment with BSO.

A number of aldehydes formed during the normal biological process. ALDH has been suggested to play a major role in the detoxification of aldehydes generated by alcohol and biogenic amine metabolism and or detoxification of the reactive aldehyde (Olivares *et al.*, 1997, Iwao *et al.*, 1997). In the present studies, the susceptibility to aliphatic and allyl alcohol was markedly enhanced by the pretreatment of BSO. Although the exact mechanism of the role of GSH is still not clear from this studies, the results suggest that intracellular GSH plays an important role in the detoxification of aliphatic alcohol.

ACKNOWLEDGEMENT

This paper was supported by NON DIRECTED RESEARCH FUND, Korea Research Foundation, 1996.

REFERENCES

Crow, K.E., Cornell, N.W. and Veech, R. L. (1977): Alcohol and aldehyde metabolizing system (Thurman, R.G., Williamson, J.R., Drott, H.R. and Chance, B. ed.), (Academic Press), Vol. III, 335.

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.

Higash, T. (1988): Critical review on the determination of glutathione in biological preparations, *Proteins, Nucleic Acid and Enzyme*, **33**, 1370.

Iwao, K., Hajime, H., Soichiro, M., Naoyuki, W., Ryota, H., Masahiko, H., Masaaki, T., Sigeyuki, Z., Tetsuya, N., Hirotoshi, E., Shinzo, K. and Hiromoasa, I. (1997): Oxidative stress-mediated apoptosis of hepatocytes exposed to acute ethanol intoxication, *Hepatology*, **25**, 368-378.

Sliva, J.M. and O' Brien, P.J. (1989): Allyl alcohol- and acrolein-induced toxicity in isolated rat hepatocytes, *Arch. Biochem. Biophys.*, **275**, 551-558.

Lieber, C.S. (1988): Metabolic effects of acetaldehyde, *Biochem. Soc. Trans.*, **16**, 241-247.

Lowry, O.H., Rosebrough, N.R., Farr, A.L. and Randall, R.J. (1951): Protein measurement with the folin phenol reagent, *J. Biol. Chem.*, **193**, 265-275.

Olivares, I.P., Bucio, L., Souza, V., Carabez, A. and Gutierrez-Ruiz, M.C. (1997): Comparative study of the damage produced by acute ethanol and acetaldehyde treatment in a human fetal hepatic cell line, *Toxicology*, **120**, 133-144.

Patel, J.M., Wood, J.C. and Leibman, K.C. (1980): The biotransformation of allyl alcohol and acrolein in rat liver and lung preparations, *Drug Metab. Dispos.*, **8**, 305-308.

Rees, K.R. and Tarlow, M.J. (1967): The hepatotoxic action of allyl formate, *Biochem. J.*, **104**, 757-761.

Reid, W.D. (1972): Mechanism of allyl alcohol-induced hepatic necrosis, *Experientia*, **9**, 1058-1061.

Sorell, M.F. and Tuma, D.J. (1987): The functional implications of acetaldehyde binding constituents, *Ann. NY. Acad. Sci.*, **429**, 50-62.

Tim, M. (1983): *J. Immunol. Method*, **15**, 5363-5370.

Tottman, S.O.C., Petterson, H. and Kiessling, K.H. (1973), *Biochem. J.*, **135**, 577.

Tuma, D.J., Hoffmann, T. and Sorell, M.F. (1991): The chemistry of acetaldehyde-protein adducts, *Alcohol Alcoholism [Suppl]*, **1**, 271-276.

Wang, T.T., Farres, J. and Weiner, H. (1989): Liver mitochondrial aldehyde dehydrogenase in vitro expression, *in vitro* import, and effect of alcohols on import, *Biochem. Biophys.*, **272**, 440-449.