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LOSS OF VIABILITY AFTER DISULFIRAM TREATMENT WITHOUT PRECEDING DEPLETION OF INTRACELLULAR GSH

Yasuo OHNO, Kouichi HIROTA, Toru KAWANISHI
and Akira TAKANAKA

National Institute of Hygienic Sciences,
Biological Safety Research Center, Division of Pharmacology,
1-18-1, Kamiyoga, Setagaya, Tokyo 158, Japan

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Abstract.....Effects of disulfiram (DSF) on freshly isolated hepatocytes were examined. Its effects on the cellular reduced form of glutathione (GSH) were triphasic; GSH decreased instantly after the addition of DSF, returned to subnormal levels within 30 min, and then declined gradually. The initial decrease in GSH after DSF treatment and the subsequent recovery of GSH were accompanied by an increase and decrease in the oxidized form of glutathione (GSSG), respectively. Decreases in cell viability brought about by 0.4 mM of DSF were correlated with the later gradual decrease in GSH. The loss of viability by DSF treatment seemed to appear when the initial GSH levels became lower than approximately 5 nmole/ 10^6 cells. Hepatocyte toxicity of DSF was potentiated by diethylmaleate (GSH depletor) and inhibited by N-acetylcysteine (GSH biosynthesis precursor). 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), an inhibitor of GSH reductase, inhibited the GSH recovery and potentiated the toxicity. Respiration of hepatocytes was also inhibited by DSF. Free sulfhydryl groups other than GSH showed similar changes to those of GSH.

From these results, it seemed that DSF reacted with cellular GSH and other free sulfhydryl groups to form diethyldithiocarbamate and GSSG, GSSG was reduced back to GSH by glutathione reductase, and the decrease in the viability was dependent on the initial loss of GSH.

Correspondence : Dr. Yasuo OHNO at the above address.

Key words : Disulfiram, hepatocytes, glutathione.

INTRODUCTION

Disulfiram (DSF) has been used widely as an alcohol deterrent because of its inhibitory action on aldehyde dehydrogenase. The inhibition has been explained to occur due to its competition with NAD^+ (Graham, 1981 ; Deitrich and Hellerman, 1963) or to oxidation of sulfhydryl groups of the enzyme (Kitson, 1978). A great quantity of this kind of chemicals are also used as polymerization accelerators in rubber production.

Neuropsychiatric and gastrointestinal disorders were the main side effects of DSF. Recently, hepatic injury, which was assumed to have been caused by DSF itself, was reported in humans (Hampshire, 1977 ; Kristenson, 1981). Because alcoholic patients often suffer from liver diseases, we examined the effects of DSF on the freshly isolated hepatocytes and found that changes in the cellular reduced form of glutathione (GSH) after DSF treatment were quite distinct from those after treatment with other hepatotoxic substances.

MATERIALS AND METHODS

Male rats (Wistar strain, 9–13 weeks, Nihon rat Co.) were used. Diethyl maleate was dissolved in corn oil (20%) and injected i. p. (0.5 ml/rat) 1 hour before sacrifice.

Isolated hepatocytes were prepared as described by Moldéus et al (1978) and used immediately. The viability of the hepatocytes, determined by trypan blue exclusion, was 92–97%.

Hepatocytes were suspended in Krebs-Henseleit buffer supplemented with 12.5 mM HEPES (10^6 cell/ml, 10ml) and these cells were incubated in rotating round-bottom glass flasks (100ml) at 37°C under continuous gassing with cabogen (95% O_2 , 5% CO_2). DSF was dissolved in dimethyl sulfoxide (DMSO) and other drugs were dissolved in deionized water and added to the hepatocytes suspension. Volume of the drug solution added was less than 0.05 ml/10 ml of suspension. Changes in viability during incubation were determined by measuring the leakage of lactate dehydrogenase from the cells (Högberg and Kristoferson, 1977).

Samples removed from the incubation mixture were centrifuged at 500 rpm for 2 min. The cell pellets thus obtained were washed once with Krebs-Henseleit buffer and resuspended in water. Phosphoric acid (25% solution, 0.2 ml) was added to the cell suspension, which was then mixed and centrifuged at 3,000 rpm for 5 min. Cellular GSH and the oxidized form of glutathione (GSSG) were determined spectrofluorometrically (Hissin and Hilf, 1977). Initial GSH contents of hepatocytes were 48.7 ± 2.6 nmole/ 10^6 cells (mean $\pm$ S. E. M. of 10 different batches of hepatocytes).

Changes in GSH and GSSG content of the hepatocytes were also confirmed by high

Loss of cell viability without preceding depletion of GSH

Changes in GSH and GSSG content of the hepatocytes were also confirmed by high performance liquid chromatography (HPLC) described by Toyo'oka et al (1984). Briefly, cell suspension was centrifuged (8,000 rpm, 5sec) and 1 ml of perchloric acid (3.5%) containing 4 mM EDTA was then added to the pellet and mixed. N-acetyl-L-cysteine was used as an internal standard (10 nmole/tube). The suspension was then centrifuged (15,000 rpm 1.5 min). A few grains of sodium bicarbonate were added to the supernatant and 0.2 ml of neutralized supernatant was mixed with 0.2 ml of 7-fluoro-4-sulfamoyl-2,1,3-benzoxadiazole (ABD-F, 1 ml in 0.2 M potassium borate buffer containing 4 mM EDTA, pH 8.0). These were mixed, heated for 5 min at 50°C, and then cooled on ice. After acidification of the solution by 0.12 ml of 0.1 M-HCl, they were injected into HPLC (Shimadzu LC4A), equipped with a Shiseido C₁₈ column and a spectrofluorometrical detector (Hitachi 650-10S, 18 μ l flow cell, excitation 380 nm, emission 510 nm, slit 10 nm, attenuation 2 sec). The column was perfused with CH₃CN : 0.05M-potassium biphthalate (pH 4.0)=8 : 92 with a flow rate 1 ml/min. The retention time of GSH was 16.5 min.

nitrobenzoic acid (Bellomo et al 1983). Oxygen consumption of hepatocytes was measured by an oxygen electrode (Rank brothers) after 30 min of incubation at 37°C under carbogen gas.

Chemicals

Collagenase, DSF, and ABD-F were purchased from Boehringer Mannheim Yamanouchi (Tokyo, Japan), Wako pure Chemical Industry (Osaka, Japan), and Dojin Chemical Lab. (Kumamoto, Japan), respectively. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) was kindly donated by Bristol-Myers Co. (Syracuse, New York, USA). All other chemicals used were of at least reagent grade and obtained from local commercial sources.

RESULTS

When 0.4 mM of DSF was added to the incubation mixture, viability of the hepatocytes, which was determined by the leakage of LDH, started to decrease after 1 hour of incubation (Figure 1). Viability of the cell was not changed when the dose was lower than 0.2 mM. On the other hand, the degree of GSH decrease caused by DSF was not so pronounced and did not precede the loss of viability. Because the loss of cell viability caused by several hepatotoxic substances was usually preceded by the depletion of cellular GSH, acute effects of DSF on the GSH content of the hepatocytes were also examined. It was found that the decrease in GSH occurred instantly after the addition of DSF. When the doses were lower than about 0.4 mM, GSH levels were recovered to subnormal level after 30 min of incubation, and then they started to decrease again (Figure 1). When the doses were higher than 0.5 mM, GSH levels were not recovered. The initial decrease in GSH was accompanied by the increase of GSSG (Figure 2). These changes in glutathiones were also confirmed by the HPLC method (Table 1).

Fig. 1. Effects of DSF on the GSH content and the viability of hepatocytes.
 ○ : Control (DMSO 40 μ l/10ml of cell suspension)
 × : DSF 0.2 mM
 △ : DSF 0.3 mM
 ● : DSF 0.4 mM
 Left : GSH content of the hepatocytes. Right : Viability of the hepatocytes. Ordinate indicates % of initial values and vertical bars attached to each symbols indicate standard errors of 5–8 different batches of hepatocytes.

Fig. 2. Effects of DSF on the intracellular GSH and GSSG
 ○ : Control (DMSO 40 μ l/10ml of cell suspension)
 × : DSF 0.2 mM
 △ : DSF 0.3 mM
 ● : DSF 0.4 mM
 Left : GSH content of the hepatocytes. Right : GSSG content of the hepatocytes.

Loss of cell viability without preceding depletion of GSH

Figure 3 shows the relation between the initial GSH decrease and the loss of viability after 2 hr of incubation. This figure indicates that cell viability began to decrease later, when the initial GSH level became lower than about 5 nmole/10⁶ cells.

Table 1. Effects of DSF on the GSH and GSSG content of hepatocytes determined by the HPLC method.

	GSH	GSSG*	GSH+GSSG*
	(nmole of GSH eq. / 10 ⁶ cells)		
DSF 0.3mM			
Before	29.6	3.6	33.2
0 min	2.2	28.0	30.2
10 min	13.6	13.7	27.3
DSF 0.6 mM + N-acetyl-L-cysteine 1 mM			
Before	37.2	0.9	38.1
0 min	1.9	35.5	37.4
10 min	20.2	14.2	34.4

Hepatocytes were incubated with or without 1 mM N-acetyl-L-cysteine for 30 min and then 0.02 ml of DSF dissolved in DMSO was added to the incubation mixture.

"0 min" means just after the addition of DSF.

These values indicate the mean of two determinations.

*: Total amount of GSH equivalent (GSH+GSSG) was determined after reduction of the GSSG to GSH by the addition of tri-n-butylphosphine (5 μ l/ml of sample) and GSSG contents were calculated by subtracting the GSH from GSH+GSSG.

Fig. 3. Relationship between initial GSH decrease and the loss of viability by DSF. Viability of hepatocytes was examined 2 hr after DSF addition and intracellular GSH contents were determined just after the addition.

Diethyldithiocarbamate, which was assumed to be formed by the degradation of DSF (Kitson, 1978), had no effects on the hepatocytes (Data not shown).

Cytotoxicity of DSF was potentiated by the previous depletion of cellular GSH by diethylmaleate and prevented by the increase in GSH when pretreated with N-acetylcysteine. The toxicity was also potentiated by the pretreatment of the hepatocytes with BCNU, a glutathione reductase inhibitor (Figure 4). BCNU also inhibited the recovery of GSH after initial depletion (Figure 5).

The amount of free sulfhydryl (SH) groups (including protein, GSH, and other low molecular weight thiol containing substances) in hepatocytes was 175 nmole GSH equivalent/ 10^6 cells. The initial decreases in free SH group and GSH by 0.4 mM of DSF were 98 and 50 nmole/ 10^6 cells, respectively. On the other hand, those by 0.2 mM of DSF were 22 and 19 nmole/ 10^6 cells (Figure 6).

Oxygen consumption of hepatocytes was inhibited to 48.2% of basal level by 0.3 mM of DSF (Table 2), an amount by which cell viability was not changed at all.

Fig. 4. Toxicity of DSF in various hepatocyte preparations.

○: Control hepatocytes

×: Diethylmaleate pretreated hepatocytes

●: Control hepatocytes + N-acetyl-L-cysteine (1 mM)

△: BCNU pretreated hepatocytes

▲: BCNU pretreated hepatocytes + N-acetyl-L-cysteine (1 mM)

Diethylmaleate was treated as described in the "Materials and Methods". N-Acetyl-L-cysteine (1 mM) and BCNU (0.05 mM) were added to the incubation mixture 30 min before the addition of DSF. Viabilities were determined 2 hr after the addition and indicated as % of initial value.

GSH content in diethylmaleate pretreated cells and GSH content in cells replenished by N-acetyl-L-cysteine was 6.9 and 36.5 nmole/ 10^6 cells, respectively.

Fig. 5. Inhibition of GSH recovery by BCNU.
Control hepatocytes (○ : DSF 0 mM, △ : DSF 0.3 mM, □ : DSF 0.4 mM) and BCNU pretreated hepatocytes (● : DSF 0 mM, × : DSF 0.3 mM, ▲ : DSF 0.4 mM) were used. Hepatocytes were treated with BCNU as in Figure 4.

DISCUSSION

DSF showed toxic effects on hepatocytes. Because previous GSH depletion by diethylmaleate potentiated the toxicity and GSH replenishment by N-acetyl-L-cysteine inhibited it, cellular GSH seemed to work as a protective agent against the toxicity of DSF. Johnston (1953) indicated that DSF reacted with GSH nonenzymatically to form diethyldithiocarbamate, which had no effects on the viability and GSH level of hepatocytes. Kitson (1978) suggested that DSF reacts covalently through mixed disulfide formation with enzymic thiol groups converting DSF to diethyldithiocarbamate. Therefore, it seemed that the toxicity of DSF was caused by a nonenzymatic reaction with cellular GSH and macromolecules.

However, leakage of lactate dehydrogenase from the hepatocytes was correlated with the decrease in the cellular GSH. That is, depletion of GSH did not precede to

Fig. 6. Effects of DSF on the free SH content of hepatocytes. Open and dashed symbols indicate the total content of intracellular free SH group and GSH content of hepatocytes, respectively.
 ○, ●: Control
 △, ▲: DSF 0.2 mM
 □, ■: DSF 0.4 mM

Table 2. Effects of DSF on hepatic oxygen consumption.

DSF (μ M)	O ₂ consumptin (% of basal rate)
0*	91.8
250	84.5
300	48.2
400	27.4
500	16.0

Oxygen consumption of hepatocytes was measured by Rank brothers oxygen electrode after about 30 min of incubation at 37°C under carbogen gas (95% O₂, 5% CO₂).

Hepatocytes were allowed to incubate in an incubation vessel (2 ml) without DSF for about 2 min and basal O₂ consumption rates were measured, after which 10 μ l of 100mM-DSF in DMSO was added.

* : Only DMSO was added as solvent (10 μ l/2ml).

Loss of cell viability without preceding depletion of GSH

the loss of cell viability, as observed in several hepatotoxic agents (Thor and Orrenius, 1985 ; Ohno et al., 1985 ; Ohno and Ormstad, 1985 ; Eklow-Låstbom et al., 1986). Instead, in the case of DSF, GSH decreased instantly at the time of its addition, GSH returned to subnormal level rapidly and then it decreased slowly with decrease of cell viability. Loss of viability seemed to depend on the level of initial depletion of cellular GSH and not on the GSH content afterwards. The threshold of initial GSH depletion for the loss of cell viability was around 5 nmole/10⁶ cells.

BCNU, an inhibitor of GSH reductase, inhibited the recovery of GSH and potentiated the toxicity. These results indicated that the GSH reversal seemed to be caused, at least partly, by GSH reductase. Supporting this, GSSG in the cell incubate decreased with the increase of GSH. The inhibition of GSH reversal by BCNU was not complete. This is probably because the inhibition of GSH reductase by BCNU was not complete (Eklow et al., 1984). Free SH groups of other cell constituents were also changed similarly with GSH.

These results indicate that cellular component(s) which is / are critical for cell survival was / were inhibited by DSF at the time of addition and that GSH reductase remained relatively functionable in this condition. Even though most of the oxidized SH groups were reduced during the subsequent incubation, the critical component(s) seemed not to be recovered and the hepatocyte could not retain its viability. Although we do not know the exact reason for the cell death, inhibition of the cell respiration might have some relation to it.

Because the reaction of DSF with cellular SH groups was very rapid, DSF, dosed per os, seemed to react with gastrointestinal and blood constituents and only a very limited amount of DSF reached the liver. We could not observe the increase in serum transaminases after DSF treatment (data not shown). However, the decrease in cyt. P-450 content and p-nitroanisole O-demethylation activity of liver microsomes by *in vivo* treatment of DSF was observed (Lang et al., 1976). DSF also inhibited the induction of microsomal enzymes by phenobarbital (Bartram et al., 1982). The liver microsomal enzymes tended to be decreased by a lower dose of hepatotoxic substances than was necessary to bring about an increase in serum transaminases (Kawanishi et al., 1981). Therefore, even though the possibility of hepatotoxicity seemed to be low, DSF might be able to cause liver injury, if the doses were high and tissue GSH contents were depleted by alcohol injection or by some other drugs.

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Loss of cell viability without preceding depletion of GSH

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