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Effect of *N*-acetylcysteine administration on cysteine and glutathione contents in liver and kidney and in perfused liver of intact and diethyl maleate-treated rats

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Summary. Effect of *N*-acetyl-L-cysteine (NAC) administration on cysteine and glutathione (GSH) contents in rat liver and kidney was studied using intact and diethyl maleate (DEM)-treated rats and perfused rat liver. Cysteine contents increased rapidly, reaching peak at 10 min after intraperitoneal NAC administration. In liver mitochondria it increased slowly, reaching peak at 60 min. GSH content did not change significantly in these tissues. However, in liver and kidney depleted of GSH with DEM, NAC administration restored GSH contents in 60 and 120 min, respectively. Perfusion with 10 mM NAC resulted in 76% increase in liver cysteine content, but not in GSH content. Liver perfusion of DEM-injected rats with 10 mM NAC restored GSH content by 15%. Present findings indicate that NAC is an effective precursor of cysteine in the intact liver and kidney and in the perfused rat liver, and that NAC stimulated GSH synthesis in GSH-depleted tissues.

Keywords: Amino acids – *N*-Acetylcysteine – Cysteine – Glutathione – Diethyl maleate – Perfused rat liver

Introduction

L-Cysteine is a sulfur-containing amino acid which is utilized as an immediate source of glutathione (GSH) and protein biosynthesis and other sulfur-containing compounds, and is finally metabolized to sulfate in animals (Jocelyn, 1972). L-Cysteine is formed from L-methionine through cystathionine pathway or supplied as a component of proteins. In contrast to high concentrations and important protective functions of GSH in mammalian tissues (Meister, 1988), excess cysteine is toxic to animals (Meister et al., 1986). Therefore, less toxic cysteine derivatives such as L-2-oxothiazolidine-4-carboxylic acid (OTC) and *N*-acetyl-L-cysteine (NAC) have been studied as cysteine delivery systems in

animals (Meister et al. 1986; Yamada et al., 1993) and in humans (Borgström et al., 1986; Burgunder et al., 1989; Porta et al., 1991). NAC has been used clinically as a mucolytic agent (Grassi and Morandini, 1976) and an antidote in acetaminophen intoxication (Prescott et al., 1977), and it has been shown to be a precursor of GSH in isolated hepatocytes (Thor et al, 1979) and in intoxicated rats (Lauterburg et al., 1983). However, the in vivo effect of NAC on the tissue cysteine contents has not been fully studied. In the present study, we investigated the effect of NAC administration on cysteine and GSH contents in the liver, kidney and blood in intact and diethyl maleate (DEM)-treated rats. The effect of NAC was further studied using perfused liver of intact and DEM-treated rats.

Materials and methods

Materials

Male Wistar rats weighing 250–350 g were maintained on a laboratory diet, MF, of Oriental Yeast Co., Tokyo, Japan and used in this study. NAC and DEM were obtained from Wako Pure Chemical Ind., Ltd., Osaka, Japan and L-cysteine was from Sigma Chemical Co., St. Louis, MO, U.S.A. GSH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from yeast (grade I), hexokinase (EC 2.7.1.1) from yeast, myokinase (EC 2.7.4.3) from rabbit muscle, pyruvate kinase (EC 2.7.1.40) from rabbit muscle and lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle were obtained from Böhringer-Mannheim, Mannheim, Germany. Glutathione reductase (EC 1.6.4.2) from yeast, NADH, NADP and phosphoenolpyruvate were purchased from Oriental Yeast Co.

General methods

Injections of NAC and DEM were performed to rats fed MF and water ad libitum. NAC (1 mmol/2 ml) was neutralized with sodium hydroxide and injected intraperitoneally at a dose of 5 mmol per kg of body weight. Control rats were injected with the same volume of 0.9% sodium chloride solution. DEM (Boyland and Chasseaud, 1970) was injected intraperitoneally at a dose of 1 g/kg of body weight as an emulsion of 1 g of DEM per 2 ml of corn oil. Control rats received the injection of the same volume of corn oil.

Rats were killed by decapitation after ether anesthesia. Blood was collected in a beaker containing 200 units of heparin and chilled in an ice bath. Blood plasma was separated by centrifugation at 1,200 × g for 10 min at 0° C. Blood and plasma were homogenized in 4 volumes of 5% perchloric acid. Liver and kidney were washed with cold (0° C) 0.9% sodium chloride solution and homogenized as above. Homogenates were centrifuged at 10,000 × g for 30 min at 0° C. Cysteine and GSH in the resulting supernatant were determined immediately after sample preparation as described below.

Liver mitochondria were prepared according to Griffith and Meister (Griffith and Meister, 1985). Protein concentration was determined by biuret method (Layne, 1957). Statistical significance was assessed by Student's t test and a level of $p < 0.05$ was considered significant.

Determination of cysteine and GSH

Cysteine contents in tissue samples were determined by acidic ninhydrin reaction (Gaitonde, 1967). As NAC reacted with the acidic ninhydrin reagent with a color value of 18% of that of cysteine, cysteine and NAC in tissue extracts after the NAC administration were separated by ion-exchange chromatography as follows. Perchloric acid in the tissue extract obtained above was eliminated by titration with potassium hydroxide and centrifugation. One ml of the resulting supernatant was applied to a column of Dowex 50W (× 8, 100–200 mesh, H^+ form, 7 × 100 mm). Elution was performed with 20 ml of water, followed by 24 ml of 2M

hydrochloric acid. NAC was eluted with water. Cystine converted from cysteine during extraction and column chromatographic procedures and cysteine-glutathione mixed disulfide often contained in tissues in small amounts were eluted with 2 M hydrochloric acid. NAC and total cysteine in cystine and cysteine-glutathione mixed disulfide were determined by acidic ninhydrin reaction (Gaitonde, 1967).

Cysteine in liver mitochondria was determined as follows. The mitochondrial fraction obtained as described above was suspended in 3 volumes of 5% perchloric acid and sonicated at 20 KHz for 10 min. Cysteine in the supernatant obtained by centrifugation was determined as above.

Total GSH (GSH and oxidized GSH) was determined according to the method of Tietze (Tietze, 1969).

Cysteine and GSH contents were expressed as μmol/g of wet weight of liver and kidney or ml of blood. In some cases cysteine contents were expressed as mM concentrations as calculated on basis of compartment water space (Wahlländer et al., 1979).

Assay of deacetylation activity

Deacetylation activity of liver and kidney was assayed with homogenates of these tissues. Liver and kidney were homogenized with 2 volumes of 0.15 M potassium phosphate containing 0.15 mM EDTA, pH 7.3. The incubation mixture contained, in a final volume of 3.0 ml, 0.9 ml of the homogenate, 0.1 M potassium phosphate (pH 7.3), 1 mM EDTA and 30 μmol of NAC. After incubation at 37° C for 10 min, the reaction was terminated by the addition of 3.0 ml of 8% perchloric acid. Precipitated protein was centrifuged off and excess perchloric acid was eliminated by neutralizing the resulting supernatant with potassium hydroxide and centrifugation. One ml of the supernatant was applied to a Dowex 50 W column as above and cystine was eluted with 2 M hydrochloric acid. Cysteine was determined by acidic ninhydrin reaction after the reduction of cystine with dithiothreitol (Gaitonde, 1967) and results were expressed as nmol of cysteine formed per mg of protein per min.

Liver perfusion

Liver perfusion was performed using rats fed MF and water ad libitum according to Sugano et al. (Sugano et al., 1978) with some modifications: perfusion was performed *in situ* under pentobarbital anesthesia at a flow rate of 25 ml/min. The perfusion medium warmed at 32° C was continuously oxygenated with 95% O_2-5% CO_2 and introduced to portal vein. When perfusion was performed with a perfusion medium containing 10 mM NAC or cysteine, a solution of 167 mM NAC (neutralized with sodium hydroxide) or cysteine in the perfusion medium was introduced without oxygenation through a T-shaped joint at a flow rate of 1.5 ml/min to the main stream of the perfusion medium which was oxygenated and introduced at a flow rate of 23.5 ml/min.

Viability of the perfused liver was examined by the determination of ATP (Trautschold et al., 1985) and ADP (Jaworek and Welsch, 1985) and by the production of bile (Sugano et al., 1978). ATP and ADP contents in the liver after 60 min of perfusion were 2.91 ± 0.05 and 0.76 ± 0.06 μmol/g of liver, respectively, and thus ATP/ADP ratio was 3.82 ± 0.29. Bile collected during this period was 37 ± 1 μl/60 min/g of liver. These values indicate that the perfused liver was under good conditions (Sugano et al., 1978).

Results

Effect of NAC administration on cysteine and glutathione levels in liver, liver mitochondria, kidney and blood plasma

As shown in Fig. 1, the cysteine contents in the liver, kidney and blood plasma increased rapidly and reached the peak values at 5 to 10 min after the intra-

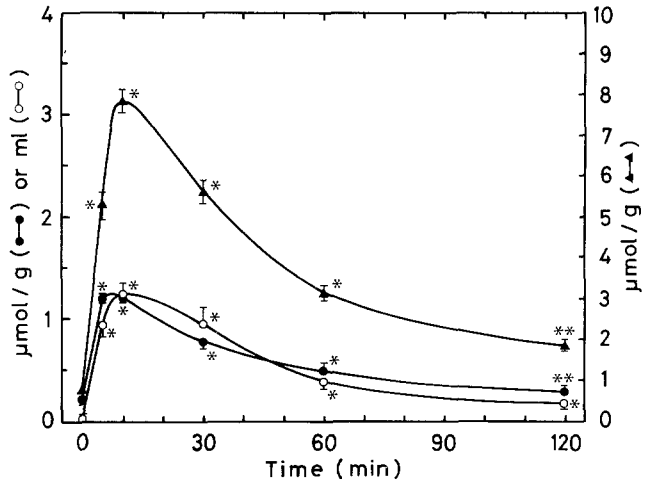

Fig. 1. Cysteine contents in liver, kidney and blood plasma of rats after *N*-acetyl-L-cysteine (NAC) administration. Five mmol of NAC per kg of body weight was intraperitoneally injected to rats at time 0. Cysteine contents in liver (●—●), kidney (▲—▲) and blood plasma (○—○) were determined by acidic ninhydrin reaction as described in Materials and methods section. Values are means ± SD obtained from five rats. Statistical difference from the value at time 0 was assessed by Student's *t* test and shown with asterisks: *, $p < 0.005$; **, $p < 0.01$

peritoneal administration of NAC. The levels in the liver and kidney were 1.20 and 7.81 μmol/g, respectively, and the increases in these tissues were 5.8 and 10.2 times the original level, respectively. NAC was not detected in the liver by the present analytical methods, but in the kidney small amount of NAC was detected. In a typical example, cysteine and NAC contents in liver, kidney and blood plasma at 5 min after NAC administration were 0.90 and 0.00 μmol/g, 3.84 and 0.21 μmol/g , and 0.59 and 0.82 μmol/ml, respectively.

The kinetics of cysteine concentrations in liver and liver mitochondria is shown in Fig. 2. In contrast to the rapid increase in cysteine contents in the liver, its increase in the liver mitochondria was slow, reaching the maximum level and returning to the original level at 60 and 120 min, respectively, after NAC administration.

GSH contents in the liver, liver mitochondria and kidney did not increase by NAC administration as shown in Fig. 3; rather there was a tendency that the GSH level in the liver and kidney decreased by the administration of NAC for the initial 30–60 min. In the blood, there was a slight increase at 60 and 120 min after NAC administration.

Effect of DEM administration on the GSH levels in the liver, kidney and blood after NAC administration

As shown in Figs. 4, 5 and 6, the GSH contents in the liver, kidney and blood decreased significantly to very low levels at 60 min after the administration of DEM. The decrease was especially drastic in the liver and blood. Administration of NAC at 60 min after the DEM administration recovered completely the

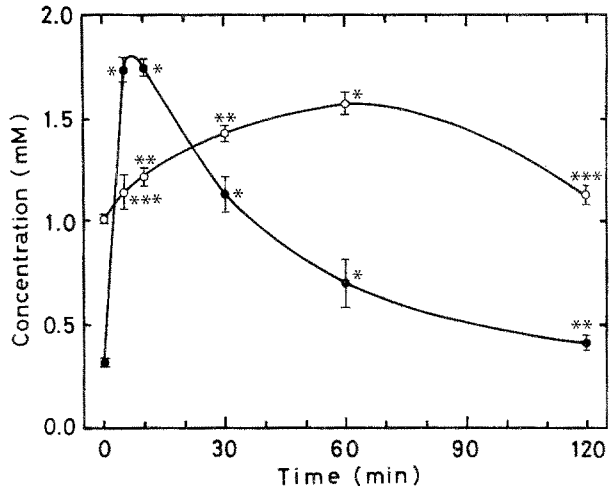

Fig. 2. Cysteine contents in the liver cytosol and mitochondria of rats after *N*-acetyl-L-cysteine (NAC) administration. Cysteine concentrations in liver cytosol (●—●) and mitochondria (○—○) in the same rats as in Fig. 1 were expressed in mM concentrations. Statistical difference was assessed and shown as in Fig. 1. ***, $p < 0.05$

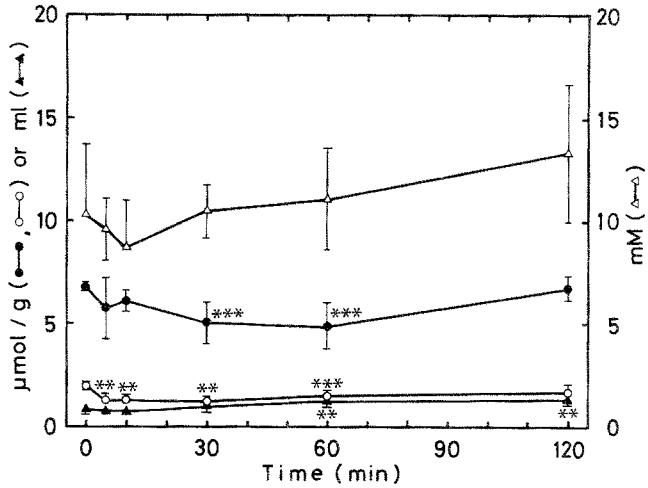

Fig. 3. Total glutathione contents in liver, liver mitochondria, kidney and blood of rats after the administration of *N*-acetyl-L-cysteine (NAC). Five mmol of NAC per kg of body weight was injected intraperitoneally at time 0. Total GSH contents in the liver (●—●), liver mitochondria (△—△), kidney (○—○) and blood (▲—▲) were determined . Values are means ± SD obtained from 5 rats. Statistical difference was assessed and shown as in Figs. 1 and 2

original GSH level in the liver in 60 min as shown in Fig. 4. In the kidney, the complete recovery of the GSH level by NAC administration was attained at 2 h after the NAC administration. The recovery of GSH level in the blood by NAC occurred slowly and was 40% of the original level at 2 h after the NAC administration.

The simultaneous administration of NAC with DEM prevented the drastic decrease in the GSH contents in liver and kidney, and partly in the blood, as

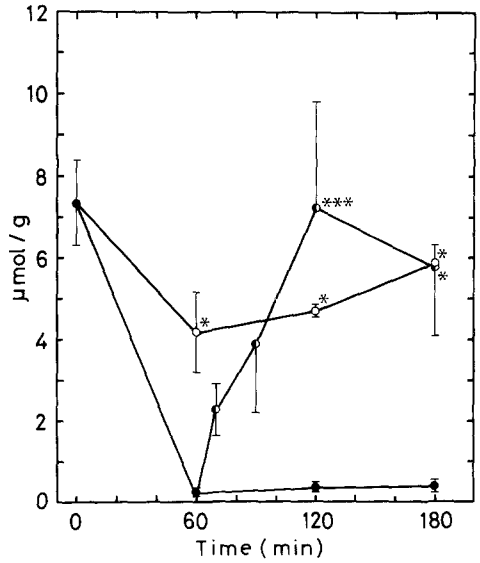

Fig. 4. Decrease in liver glutathione content by diethyl maleate (DEM) and its recovery by *N*-acetyl-L-cysteine (NAC). One gram of DEM and 5 mmol of NAC per kg of body weight were intraperitoneally injected to rats as follows: ●—●, DEM only at time 0; ○—○, DEM at time 0 and NAC at time 60 min; ○—○, DEM and NAC at time 0. Total GSH contents were determined and expressed as means ± SD (n = 5). Statistical difference between the values of DEM and NAC-injected rats at 60, 120 and 180 min and those of DEM-injected rats at corresponding time was assessed and shown as in Figs. 1 and 2

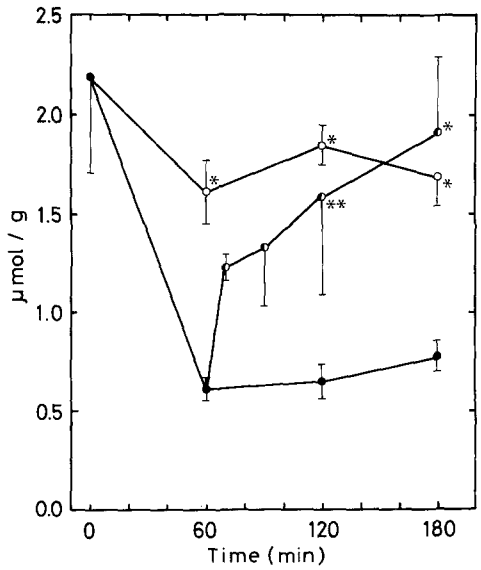

Fig. 5. Decrease in kidney glutathione content by diethyl maleate (DEM) and its recovery by *N*-acetyl-L-cysteine (NAC) in the same rats as in Fig. 4. Statistical difference was assessed and shown as in Fig. 4

shown in Figs. 4, 5 and 6. The preventive effect in the liver, kidney and blood at 60 min were 57, 74 and 21%, respectively.

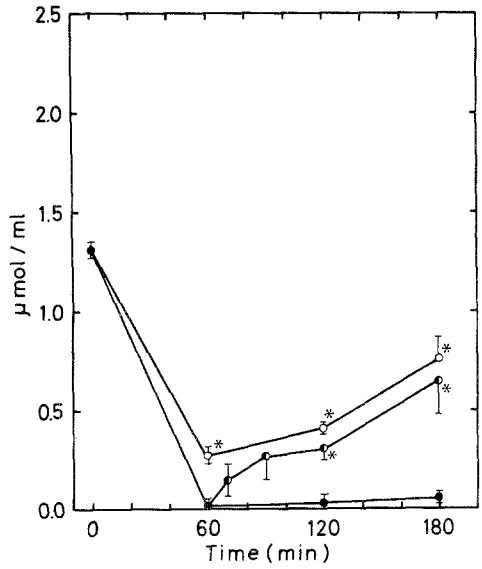

Fig. 6. Decrease in blood glutathione content by diethyl maleate (DEM) and its recovery by *N*-acetyl-L-cysteine (NAC) in the same rats as in Fig. 4. Statistical difference was assessed and shown as in Fig. 4

Cysteine and GSH levels in rat liver perfused with a perfusion medium containing NAC or L-cysteine

In the present perfusion experiment, the liver was perfused for 30 min with the perfusion medium (Sugano et al., 1978), then perfused for 10 min with the perfusion medium containing 10 mM NAC or L-cysteine. The liver was finally perfused for 10 min with the perfusion medium without the addition compounds in order to wash out NAC or cysteine in the perfusion medium remained in the blood vessels.

Cysteine and GSH contents in the control liver after the perfusion for 50 min with the perfusion medium without added compounds were 0.21 ± 0.01 and 7.21 ± 0.41 μmol/g, respectively, as shown in Table 1. The cysteine content increased significantly to 0.93 ± 0.19 and 0.37 ± 0.04 μmol/g after perfusion for 10 min with the perfusion medium containing 10 mM L-cysteine or 10 mM NAC, respectively. NAC was not detected in the perfused liver. In contrast to the cysteine content, the GSH content in the perfused liver did not exhibit any significant change as was the case in the *in vivo* experiment.

Effect of NAC on the cysteine and GSH levels of the perfused liver of rats injected with DEM

Liver perfusion was performed using the liver of a rat injected with 1 g of DEM per kg of body weight at 60 min before the start of perfusion. In this experiment, perfusion with the perfusion medium containing 10 mM NAC or L-cysteine was continued for 30 min after the initial perfusion for 30 min. After washout with the perfusion medium without added compounds for 10 min, cysteine and GSH were determined.

Table 1. Cysteine and glutathione (GSH) contents in rat liver perfused with perfusion medium containing *N*-acetyl-L-cysteine (NAC) or L-cysteine (Cys)

Group	Addition to medium	Contents (μmol/g)	
		Cysteine	GSH
A	None	0.21 ± 0.01	7.21 ± 0.41
B	NAC	0.37 ± 0.04	6.93 ± 0.75
C	Cys	0.93 ± 0.19	6.12 ± 0.35

Liver was perfused with a bicarbonate-buffered solution (perfusion medium) at 32° C for 30 min. Then, perfusion was continued for 10 min with the perfusion medium containing 10 mM NAC (group B), 10 mM Cys (group C) or no addition (group A). Finally, the liver was perfused for additional 10 min with the perfusion medium, and Cys and GSH contents were determined as described under Materials and methods. Results are means ± SD of 5 experiments. Statistical difference in cysteine contents was assessed by Student's *t* test: A versus B, A versus C and B versus C, $p < 0.005$. No significant difference was present among GSH contents.

Table 2. Cysteine and glutathione (GSH) contents in the liver of diethyl maleate (DEM)-injected rat after perfusion with perfusion medium containing *N*-acetyl-L-cysteine (NAC) or L-cysteine (Cys)

Group	DEM	Addition to medium	Contents (μmol/g)	
			Cysteine	GSH
A	−	None	0.21 ± 0.01	6.68 ± 0.09
B	+	None	0.04 ± 0.00	0.34 ± 0.02
C	+	NAC	0.32 ± 0.01	1.21 ± 0.29
D	+	Cys	1.08 ± 0.11	1.63 ± 0.24

Liver perfusion was performed with rats injected with 1 g of DEM/kg of body weight. At 60 min after the injection, perfusion was started and continued for 30 min as in Table 1. Then perfusion was continued for 30 min with the perfusion medium containing NAC, Cys or none for 30 min. After perfusion with the perfusion medium for additional 10 min, cysteine and GSH contents were determined. Results are means ± SD of 3 experiments. Statistical difference in cysteine contents was assessed by Student's *t* test: A versus B, B versus C, B versus D and C versus D, $p < 0.005$ and that in GSH contents was done as above: A versus B, $p < 0.005$; B versus C, $p < 0.025$; B versus D, $p < 0.005$; C versus D, $p > 0.05$.

As shown in Table 2, cysteine and GSH in the perfused liver from DEM-injected rats were 19 and 5%, respectively, of the control liver. Perfusion with NAC-containing medium resulted in 8-fold increase in the cysteine content (C

versus B), which was 1.5-fold higher than that of the non-DEM control (C versus A) and this value was comparable to that of non-DEM experiment (B of Table 1). The recovery of the GSH content was 3.6-fold (C versus B), which was 18% of the non-DEM control (C versus A).

Perfusion with the L-cysteine-containing medium resulted in 27-fold increase in the cysteine content (D versus B), which was 5-fold increase as compared to the non-DEM control (D versus A) and this value was also comparable to that of non-DEM experiment (C of Table 1). The recovery of the GSH content was 4.8-fold (D versus B), which was 24% of the non-DEM control (D versus A).

Deacetylation of NAC by liver and kidney homogenates

As shown in Table 3, the activity of the deacetylation of NAC in the kidney homogenate was 5-fold higher than that in the liver homogenate.

Table 3. Deacetylation of *N*-acetyl-L-cysteine (NAC) by homogenates of rat liver and kidney

Homogenate	Cysteine formed (nmol/mg of protein per min)
Liver	4.8 ± 1.0
Kidney	25.3 ± 0.5

Liver and kidney homogenates were incubated with 10 mM NAC at 37° C for 10 min. Cysteine formed was determined by acidic ninhydrin reaction after separation with a Dowex 50 W column as described under Materials and methods. Results are means ± SD of 3 experiments.

Discussion

It has been shown that NAC is utilized for the biosynthesis of GSH (Thor et al., 1979; Lauterburg et al., 1983). As shown in the present study, intraperitoneal administration of NAC to rats resulted in rapid increase in cysteine contents in liver, kidney and blood plasma, indicating NAC was rapidly converted to cysteine. The increase was especially high in the kidney, being 6.5-fold higher than that in the liver. This is in accordance with the higher deacetylation activity in the kidney than that in the liver as shown in Table 3.

The mean cysteine concentration in liver mitochondria of untreated rats was 1.01 ± 0.01 mM as shown in Fig. 2. This result agrees with our previous report (Ubuka et al., 1992) and is much higher than that in the liver cytosol. The mitochondrial cysteine content increased 1.6-fold at 60 min and returned to the original level at 2 h after the NAC administration. As reported previously (Ubuka et al., 1990 and 1992), cysteine is metabolized actively in mitochondria to sulfate via 3-mercaptopyruvate pathway (MP pathway) and the role of MP pathway has been proposed to supply bivalent sulfur for the biosynthesis of iron-sulfur cluster. Therefore, it may be assumed that the cysteine concentration

in mitochondria is maintained at a relatively high level (around 1 mM), which is much higher than that in the cytosol (around 0.1 mM). Moreover, the kinetics of cysteine increase after NAC administration seems to indicate that cysteine formed from NAC is actively taken up by mitochondria.

In contrast to cysteine, GSH contents in liver, liver mitochondria, kidney and blood did not change remarkably by NAC administration. Those in liver and kidney exhibited rather slight decreasing tendency after the NAC administration as reported by others (Viña et al., 1980), and then returned to the original levels. Thus, GSH levels seems to be maintained in relatively narrow ranges.

It has been shown that administration of DEM resulted in the depletion of liver GSH (Boyland and Chasseaud, 1970) and that GSH depleted by acetaminophen (Lauterburg et al, 1983) and phorone (Traber et al., 1992) was recovered by NAC administration. In the present study, effect of NAC administration on the GSH contents in the liver of DEM-injected rats was examined. As shown in Fig. 4, liver GSH content at 60 min after the injection of 1 g/kg of DEM was 0.27 ± 0.11 μmol/g of liver, i.e. only 3% of that before DEM injection, and this low value continued even at 2 h after the DEM injection, which is in agreement with other reports (Plummer et al., 1981). Intraperitoneal injection of 5 mmol of NAC per kg of body weight at 60 min after the DEM injection resulted in the increase in GSH contents, which returned to the original level at 60 min after the NAC injection. The simultaneous administration of NAC with DEM prevented the drastic drop of GSH contents produced by the injection of DEM alone. These findings indicate that NAC administration stimulated GSH biosynthesis and rapid recovery of GSH level.

In the kidney, the GSH contents decreased to 28% of the original level following the DEM administration. This low level continued at least for 2 h as seen in the liver. By the administration of NAC the GSH content recovered to the original level in 2 h after the NAC injection. Simultaneous injection of NAC with DEM prevented the rapid decrease in the GSH level as that in the liver. Thus, the action of DEM and NAC on the renal GSH level is apparently somewhat less effective than that on the hepatic level. The reason is unknown, but it might be related with the difference in the intracellular distribution and/or turnover rate of GSH in these tissues.

The GSH content in the blood decreased strikingly from the original level of 1.31 ± 0.39 μmol/ml to 0.02 ± 0.02 μmol/ml by DEM injection. Injection of NAC recovered GSH level as in the liver and kidney, but the effect was lower than that in those tissues. The slow recovery of the GSH level in the blood seems to reflect the slower rate of GSH synthesis in the red blood cells than that in the liver and kidney.

Experiments with perfused liver of untreated and DEM-treated rats were performed in order to confirm the effect of NAC on the cysteine and GSH contents in the whole body. In perfused liver of intact rats, cysteine contents increased 4.4-fold when perfusion was performed for 10 min with the perfusion medium containing 10 mM L-cysteine. Perfusion with 10 mM NAC under the same conditions resulted in 1.8-fold increase in cysteine contents in the perfused liver. These results indicate that cysteine was taken up more effectively than

NAC. In contrast to cysteine contents, GSH contents did not exhibit significant change as the case in the whole body experiments.

In perfused liver of rats injected with DEM, the increase in cysteine contents following perfusion with a medium containing 10 mM L-cysteine or NAC was similar to the results obtained in the perfused liver of intact rats.

GSH contents in the liver of rats injected with DEM increased 4.8- and 3.6-fold when perfusion was performed for 30 min with perfusion medium containing 10 mM L-cysteine and NAC, respectively. Although NAC was less effective on the increase in cysteine contents in the liver, its effect on the increase in GSH content was comparable to that of L-cysteine. These findings seem to indicate that GSH synthesis was more rate-limiting than the availability of L-cysteine on the GSH level under the present conditions.

Although it is unknown where NAC is deacetylated *in vivo* (Meister et al., 1986), the perfusion experiments in the present study show that the liver has activity to utilize NAC for the formation of cysteine and GSH in the liver tissue. However, as shown in Table 3, kidney homogenate was 5-fold more active than liver homogenate. It has been reported that rat kidney contains high acylase activity (Birnbaum et al., 1952). As described above, blood plasma contained a relatively high concentration of NAC and kidney contained an appreciable amount of NAC, but it was not detected in the liver. Fig. 1 shows that the kinetics of cysteine increase in the kidney and blood was similar. These findings seem to suggest that a part of cysteine in the liver, which increased after the intraperitoneal injection of NAC, derived from the cysteine which was formed in the kidney, transported to and taken up by the liver.

In vivo and perfusion experiments in the present study clearly show that NAC is effective on the elevation of tissue cysteine levels and on the stimulation of GSH synthesis in tissues, in which GSH demand increased.

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