GLUTATHIONE DEPLETION BY ANILINE ANALOGS
IN VITRO ASSOCIATED WITH LIVER MICROSOMAL
CYTOCHROME P-450

Kazuo AIKAWA, Tetsuo SATOH, Keiko KOBAYASHI
and Haruo KITAGAWA

Department of Biochemical Pharmacology, Faculty of Pharmaceutical Sciences,
Chiba University, Chiba 280, Japan

Accepted March 1, 1978

Abstract—Enzymic depletion of glutathione (GSH) in vitro by aniline analogs was mostly dependent on the cytochrome P-450 level in liver microsomes. In a case of acetaminophen (AAP), active metabolite of AAP formed through liver microsomal drug metabolizing enzymes consumed GSH. The active metabolite formed binds, at least in part, covalently to liver microsomal proteins. In addition, species differences in the extent of GSH depletion by AAP in vitro was related to the amounts of the active metabolite of AAP bound covalently to liver microsomal protein(s) by experiments using 14C-AAP. Similar depletion of GSH was also seen with other aniline analogs such as aniline itself and p-chloroaniline, but not with acetanilide, in four animal species. These in vitro results obtained here strongly support the well-known findings concerning both GSH depletion and covalent binding in vivo of the active metabolite after AAP treatment.

Numerous drugs and chemicals derived from aniline are known to cause various toxic effects such as methemoglobinemia, hemolysis and hepatotoxicities (1, 2). Acetaminophen (AAP) has been reported to be metabolized by drug metabolizing enzymes in liver microsomes to an active metabolite, presumably N-hydroxy-AAP (3-5). Mitchell et al. (6) have reported that the arylating metabolite of AAP is initially detoxified by reacting preferentially with reduced glutathione (GSH), and active metabolite binds covalently to liver cell protein after GSH was depleted (7). Potter et al. (5) have previously reported the covalent binding level to be an index of the formation of the presumed toxic metabolite of AAP. In addition, they also showed that the severity of hepatic damage in various animal species was correlated directly with the rate of hepatic GSH depletion by AAP. All these papers described only the in vivo correlation between GSH depletion and microsomal drug metabolizing enzyme activity after AAP treatment.

The purpose of the present study was to investigate the in vitro dependency of GSH depletion by aniline analogs such as AAP on the drug metabolizing enzyme activity in liver microsomes.

MATERIALS AND METHODS

Chemicals

NADP was purchased from the Sigma Chemical Co., St. Louis, Mo., and GSH, glucose 6-phosphate (G-6-P) and glucose 6-phosphate dehydrogenase (G-6-PDH) were obtained
from Boehringer, Mannheim, Germany. $^{14}$C-AAP (carbonyl labeled, specific activity 8.04 mCi/mmol) was obtained from New England Nuclear Corporation (Boston, Mass.). The purity was shown to be greater than 99.0% by thin layer chromatography on silicic acid using two solvent systems (ethylacetate alone and chloroform-ethanol 90:10). SKF 525-A was obtained from Smith Kline & French Laboratories, Inc., Montreal, Canada. All other chemicals were purchased from commercial sources.

Animals and treatment

Male mice of CRJ:ICR strain (20–25 g), male rats of Wistar strain (150–180 g), male guinea pigs of Hartley strain (200–250 g), male white rabbits of New Zealand strain (2.0–2.5 kg) were maintained on commercial chow and tap water ad libitum. In some experiments, mice were pretreated with 3-methylcholanthrene (MC, 40 mg/kg, i.p.) for 3 days or were given 0.1% solution of phenobarbital sodium (PB) ad libitum for 3 days. Control animals were given either tap water ad libitum (PB-treated group) or an equivalent volume of corn oil, (MC-treated group).

Preparation of the enzyme source

Animals were fasted for about 18 hr prior to sacrifice, and were decapitated after the final dose of the drug. The livers were perfused with 1.15% KCl solution in situ then homogenized in 3 volumes of ice-cold 1.15% KCl solution in Potter-Elvehjem type homogenizer with a Teflon pestle. The homogenate was centrifuged at 105,000 g for 1 hr. The microsomal pellet was suspended in 1.15% KCl solution and the resultant microsomal suspension was used as the enzyme source. Components of the incubation mixture and details were described in the legends to each figure and table.

Chemical determination

Remaining GSH content in the incubation mixture after incubation was determined by the method of Sedlack and Lindsay (8). Oxidized glutathione (GSSG) was measured by the method of Klotzsch and Bergmeyer (9). The amount of GSH depletion was represented by the values calculated by subtracting GSH depletion in controls without added aniline analogs from those obtained with addition of the chemicals. The amount of GSSG formed during the incubation was not subtracted from the values of total GSH depletion. Cytochrome P-450 content was assayed by the method of Omura and Sato (10). P-aminophenol concentration was determined by the method of Imai et al. (11). Covalent binding level of AAP metabolite to the microsomal protein was determined essentially by the method of Potter et al. (5).

The microsomal protein was determined by the method of Lowry et al. (12). Statistical significance was determined by Student's t-test.

RESULTS

Dependency of AAP-induced GSH depletion on drug metabolizing enzyme activity in vitro

Figure 1 illustrates that little or no GSH depletion was observed when either NADPH or microsomes was absent in the incubation mixture. This suggested that the hepatic
mixed function oxidase system may be involved in the GSH depletion by AAP in vitro. In fact, a significant increase in AAP-induced GSH depletion with microsomes from PB-treated mice (Fig. 2) and, conversely, significant decrease by addition of SKF 525-A (0.5 mM) were

**Fig. 1.** Acetaminophen-induced GSH depletion in the presence or absence of microsomes and NADPH. Incubation mixture was essentially the same as that described in the legend to Table 1. Incubation was carried out in the presence or absence of mouse liver microsomes and NADPH generating system. Each point represents mean ± S.E. for at least 4 trials.

**Fig. 2.** Effect of phenobarbital on acetaminophen-induced GSH depletion in vitro. Microsomes from phenobarbital-pretreated mouse liver were used. Incubation was carried out as described in the legend to Table 1. Each point represents mean ± S.E. for at least 4 trials.

**Fig. 3.** Effect of SKF 525-A on acetaminophen-induced GSH depletion in vitro. SKF 525-A (0.5 mM) was added to the incubation mixture consisting of the same components as described in the legend to Table 1. Each point represents mean ± S.E. for at least 4 trials.
observed (Fig. 3). Moreover, GSH depletion with mouse liver microsomes was significantly inhibited under the carbon monoxide atmosphere (90% CO, 10% O₂), compared with that in air (Table 1). These findings strongly suggest that in vitro depletion of GSH by AAP is mediated through cytochrome P-450-dependent liver microsomal drug metabolizing enzymes.

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>GSH depletion</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>86.74 ± 1.70</td>
<td>100.00</td>
</tr>
<tr>
<td>CO₂/O₂ (90:10)</td>
<td>16.74 ± 0.75*</td>
<td>19.45</td>
</tr>
</tbody>
</table>

Incubation mixture contained 0.1 ml of GSH (0.5 mM); 2 mg of liver microsomal protein; AAP (5 μmol); 0.1 ml of EDTA (0.1 mM); 0.1 ml of NADPH generating system consisting of NADP (0.33 μmol); MgCl₂ (6 μmol), G-6-P (8 μmol) and G-6-PDH (0.045 unit); and 0.2 ml of 0.2 M phosphate buffer, pH 7.4 in a total volume of 1.0 ml. To the control vessels was added 0.1 ml of redistilled water in place of the NADPH-generating system. Reactions were carried out at 37°C for 30 min and stopped by adding 1.0 ml of 10% trichloroacetic acid. Data represents mean ± S.E. from at least 4 trials. *p<0.01 vs air atmosphere.

TABLE 1. Effect of carbon monoxide atmosphere on acetaminophen-induced GSH depletion in mouse liver

There are several kinds of esterases in liver microsomes and some are responsible for the hydrolysis of AAP to p-aminophenol (PAP) (13). As shown in Fig. 4, AAP was deacetylated to form PAP and, PAP formation from AAP during incubation was decreased in the presence of GSH. Figure 4 also showed GSH depletion as a function of concentrations of PAP added (right on the figure). These data demonstrate that PAP formed from AAP during incubation was trapped by GSH added to the incubation mixture.

**P-aminophenol formation and GSH depletion after addition of AAP in vitro in mouse liver**

There are several kinds of esterases in liver microsomes and some are responsible for the hydrolysis of AAP to p-aminophenol (PAP) (13). As shown in Fig. 4, AAP was deacetylated to form PAP and, PAP formation from AAP during incubation was decreased in the presence of GSH. Figure 4 also showed GSH depletion as a function of concentrations of PAP added (right on the figure). These data demonstrate that PAP formed from AAP during incubation was trapped by GSH added to the incubation mixture.

![Fig. 4. Relationship between p-aminophenol formation and GSH depletion by acetaminophen in vitro.](image)

(Left) Enzymatic formation of p-aminophenol (PAP) from acetaminophen was determined in the presence or absence of GSH. Equivalent volume of redistilled water in place of GSH was added to the incubation mixture in the same manner as that described in the legend to table 1. (Right) GSH depletion as a function of p-aminophenol. p-aminophenol (PAP) ranging from 3 to 25 μM was added to the incubation mixture and GSH depletion during incubation was determined as described in Methods. Each points represent mean values of at least 4 trials.
Correlation of GSH depletion, covalent binding level and cytochrome P-450 content in liver microsomes of mice after phenobarbital or 3-methylcholanthrene pretreatment

GSH depletion by AAP in vitro in mouse liver was induced with PB- and MC-pretreatment on the basis of GSH nmoles per mg protein (Table 2). However, there was no significant difference between control and PB-treated group on the basis of GSH nmoles per cytochrome P-450, while, a greater increase was seen in MC-treated group than in the control. These results indicate that the enzyme induction by MC was more susceptible to GSH depletion than PB-treatment. Consequently, it was likely that AAP was me-

**Table 2.** Comparison between phenobarbital and 3-methylcholanthrene pretreatment on GSH depletion, PAP and GSSG formation, and covalent binding level in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSH depletion(1)</th>
<th>PAP formation(2)</th>
<th>GSSG formation()</th>
<th>Covalent binding(4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Control</td>
<td>62.9±2.5</td>
<td>92.1±5.3</td>
<td>10.5±0.6</td>
<td>18.5±2.7</td>
</tr>
<tr>
<td>PB</td>
<td>130.0±9.1</td>
<td>95.7±7.1</td>
<td>11.8±1.0</td>
<td>20.5±3.1</td>
</tr>
<tr>
<td>3-MC</td>
<td>171.1±3.0*</td>
<td>250.5±18.6*</td>
<td>12.5±1.0</td>
<td>25.4±3.6</td>
</tr>
</tbody>
</table>

GSH, GSSG and p-aminophenol concentrations in the incubation mixture consisting of the same constituents as described in the legend to Table 1 were determined by the method described in MATERIALS and METHODS. For experiments of covalent binding the incubation mixture contained MgCl\(_2\) (15 \(\mu\)mol), G-6-P (20 \(\mu\)mol), G-6-PDH (0.1125 unit); \(^{14}C\)-AAP (2.5 \(\mu\)mol) as substrate; 0.2 ml of EDTA (0.25 \(\mu\)mol); 0.8 ml of 0.2 M phosphate buffer, pH 7.4 and redistilled water, to make a total volume of 2.5 ml. To the control vessels was added 0.1 ml of redistilled water in place of the NADPH generating system. Reactions were carried out at 37 C for 30 min and stopped by adding 0.8 ml of 3M trichloroacetic acid. \(1\): A; GSH depleted nmoles/mg protein/30 min.; B; GSH depleted nmoles/nmol p-450/30 min. \(2\): nmoles/mg protein/30 min. \(3\): A; percent of control (nmoles/mg protein/30 min.); B: percent of control (nmoles/nmol p-450/30 min). Data represents mean±S.E. from at least 8 trials. *p<0.01 vs. control.

**Table 3.** Effect of aniline derivatives on GSH depletion and covalent binding level in four animal species

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Species</th>
<th>Mouse</th>
<th>Rat</th>
<th>Guinea Pig</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>[GSH depletion]---</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aniline</td>
<td></td>
<td>43.96±5.26</td>
<td>19.87±1.83</td>
<td>21.54±5.90</td>
<td>13.70±3.42</td>
</tr>
<tr>
<td>p-chloroaniline</td>
<td></td>
<td>49.34±3.30</td>
<td>16.24±1.47</td>
<td>46.76±5.62</td>
<td>49.48±3.39</td>
</tr>
<tr>
<td>Acetanilide</td>
<td></td>
<td>4.20±0.89</td>
<td>6.39±1.77</td>
<td>3.52±2.15</td>
<td>2.72±1.22</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td></td>
<td>75.12±4.00</td>
<td>19.87±2.35</td>
<td>20.31±5.52</td>
<td>13.24±1.03</td>
</tr>
<tr>
<td>[Covalent binding]---</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetaminophen</td>
<td></td>
<td>2.24±0.11</td>
<td>0.38±0.03</td>
<td>0.91±0.11</td>
<td>0.42±0.06</td>
</tr>
</tbody>
</table>

-GSH depleted nmoles/mg protein/30 min. - Covalent binding level (AAP nmoles/mg protein/30 min). Determinations of GSH depletion and covalent binding level were carried out as described in the legends of Tables 1 and 2, respectively. Data represents mean±S.E. from at least 4 trials.
tabolized by cytochrome P-448 which is susceptible to MC induction rather than cytochrome P-450 susceptible to the PB induction. Table 2 also shows that enzymic deacetylation of AAP to PAP was not induced by PB nor MC. Therefore, PAP-evoked GSH depletion was not affected by pretreatment with PB or MC.

**Species difference in GSH depletion by aniline analogs in vitro**

In the present study, aniline analogs were used for the experiments of GSH depletion because these compounds are metabolized through the N-hydroxylation pathway (1, 2, 14). As shown in Table 3, the largest depletion of GSH was seen with AAP in mice among four animal species. The GSH depletion by chloroaniline was larger than aniline in all animal species, except rats. Further, acetanilide-induced depletion of GSH was the smallest among aniline analogs tested in all species.

**DISCUSSION**

In a previous paper we reported that AAP has characteristics of type II substrate for mouse liver microsomal mixed function oxidase and inhibited ethylmorphine N-demethylation in noncompetitive type and aniline p-hydroxylation in competitive one, respectively (15). The present study first demonstrated that GSH depletion by AAP in vitro occurred only when both liver microsomes and NADPH were present in the assay mixture. The fact that enzymic conversion of AAP to its active metabolite occurred with liver cytochrome P-450 is consistent with the in vivo findings reported by the NIH group.

Data presented herein suggest that overall depletion of GSH associated with AAP metabolism was mainly due to formation of the active metabolite, presumably, N-hydroxy AAP, but not PAP because Km value (200 mM) was extremely large. In addition, formation of GSSG from GSH could not be ruled out in control mice liver, however, no increase in the oxidation was seen in mice pretreated with PB or MC.

On the other hand, attempts were made to measure GSH depletion after addition of aniline analogs. The lowest depletion of GSH was observed with acetanilide in all animal species. Such was attributed the fact that the main metabolic pathway was found to be p-hydroxylation of the molecule in liver microsomes (16). The significant depletion of GSH by p-chloroaniline in guinea pigs and rabbits can be explained by that fact that chlorination of acetanilide at the para position produced more N-hydroxy derivatives, which in turn, led to greater depletion of GSH.

With regard to the species difference, Table 3 demonstrates that mice were the most susceptible to both GSH depletion and covalent binding of 14C-AAP metabolite to microsomal protein. This finding is in accordance with the results reported by Davis et al. (17).

**Acknowledgements:** We are grateful for the excellent technical assistance of Miss S. Ura and Mr. F. Ichimura. This work was supported in part by a Grant in Aid for Cancer Research from the Ministry of Health and Welfare.

**REFERENCES**


