Acetaminophen-Derived Activation of Liver Microsomal Glutathione S-Transferase of Rats

Masato Yonamine, Yoko Aniya*, Takahiro Yokomakura, Tomoyuki Koyama, Tatsumi Nagamine and Hisaharu Nakanishi

Laboratory of Physiology and Pharmacology, School of Health Sciences, Clinical Laboratory of the University Hospital, Faculty of Medicine, University of the Ryukyus, Okinawa 903-01
Tropical Technology Center Limited, Okinawa 904-22, Japan

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ABSTRACT—Effect of acetaminophen on glutathione (GSH) S-transferase and related drug metabolizing enzymes was studied in vivo. Rats were given acetaminophen (250 mg/kg, i.p.) 24 hr after the treatment with 3-methylcholanthrene (25 mg/kg, i.p.) and killed by decapitation at indicated times. Liver microsomal GSH S-transferase activity was increased to 331%, 193% and 158% of the control level at 3, 6 and 12 hr, respectively, after the administration of acetaminophen, while GSH content in the liver was markedly decreased at 3 and 6 hr. The increase in the transferase activity was not recovered by the treatment with dithiothreitol. Microsomal GSH peroxidase activity was significantly enhanced at 3 hr. Cytosolic GSH S-transferase and aniline hydroxylase in microsomes were gradually decreased with the increase in the time after administration of acetaminophen. Vmax values of both GSH S-transferase and GSH peroxidase activities in microsomes were increased at 3 hr. Two Km values were obtained for the peroxidase in the control, while only one was observed after the acetaminophen treatment. These results indicate that acetaminophen is converted via cytochrome P-450 to the reactive intermediate N-acetyl-p-benzoquinone imine, which binds to microsomal GSH S-transferase, resulting in the activation of the enzyme.

Keywords: Acetaminophen, Microsome, Glutathione S-transferase, Glutathione peroxidase, Enzyme activation

Pharmacological action and toxicity of drugs depend, at least in part, on the drug-metabolizing enzyme activity in the liver. The phase II enzyme of drug metabolism glutathione (GSH) S-transferase catalyzes the conjugation of reactive metabolites with GSH and thereby plays an important role for the detoxication of toxic metabolites. GSH S-transferases are present not only in cytosol but also in microsomes and mitochondria. Since many xenobiotics are transformed to reactive and toxic metabolites by microsomal cytochrome P-450, it could be suggested that microsomal GSH S-transferase has a more critical role for the detoxication of metabolites than that of the cytosol. Furthermore, the microsomal transferase also acts as a Se-independent GSH peroxidase and can detoxify organic hydroperoxides including fatty acid and lipid hydroperoxides (1). Liver microsomal GSH S-transferase contains one cysteine residue per subunit and is activated by various ways such as alkylation of the sulfhydryl group (2), thiol/disulfide exchange (3), limited proteolysis (4, 5), heating (6) and oxidative stress (7-9). Thus, judging from the reactivity of the sulfhydryl group in the enzyme, it was expected that toxic metabolites of drugs derived from biotransformation by cytochrome P-450 may bind to the microsomal GSH S-transferase and activate the enzyme. Indeed, the activation of microsomal GSH S-transferase by metabolites of xenobiotics has been reported (10). However, these studies were carried out in vitro, and the whole site of the enzyme modulation has not been studied.

Acetaminophen, a widely used analgesic/antipyrretic drug, is very safe at therapeutic doses; However, in overdoses, it causes hepatic necrosis (11-13). The hepatotoxicity of acetaminophen is associated with biotransformation to a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), by the cytochrome P-450 system. NAPQI is normally detoxified by conjugation with glutathione; However, when GSH is depleted, it reacts with protein thiol(s) forming a 3-(cystein-S-y1) adduct.

To whom correspondence should be addressed.
Acetaminophen also causes oxidative stress and oxidizes protein thiols (16, 17). Thus it was assumed that NAPQI binds to the sulfhydryl group of microsomal GSH S-transferase or generates a mixed disulfide bond with GSH, resulting in an increase in the activity. To clarify these possibilities, we examined whether microsomal GSH S-transferase is activated by acetaminophen in vivo.

MATERIALS AND METHODS

Chemicals

Reagents used were as follows: Reduced glutathione, cumene hydroperoxide (CuOOH), glutathione reductase, dithiothreitol (DTT) and acetaminophen were from Sigma Chemicals (St. Louis, MO, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) and 2,4-dinitrofluorobenzene were from Wako Pure Chemicals (Osaka). 3-Methylcholoranthrene (3-MC) was from Kanto Chemicals (Tokyo). All other reagents were of analytical grade.

Animal treatment

Male Sprague Dawley rats (170–250 g) from Nihon SLC Co. (Shizuoka) were given acetaminophen (250, 180, 75 mg/kg) intraperitoneally 24 hr after the administration of a cytochrome P-450 inducer, 3-MC (25 mg/kg, i.p.). Acetaminophen was dissolved with 30% dimethyl sulfoxide in saline. Control rats were given the vehicle. The rats were killed by decapitation at the indicated times after starvation for 15 hr, and blood was collected from the stump. The liver was removed after perfusion with 1.15% potassium chloride solution in situ and homogenized in 2 vol. of the same solution with a teflon-glass homogenizer. The cytosol and microsomes were prepared as described previously (8).

Measurement

GSH content in the liver homogenate was measured by high performance liquid chromatography (Toyo Soda, Tokyo) by the method of Reed et al. (18). Activities of GSH S-transferase for CDNB and GSH peroxidase for CuOOH were determined by the methods of Habig et al. (19) and Reddy et al. (20), respectively. Aniline hydroxylase activity in microsomes was measured by measuring p-aminophenol (21). Serum glutamic-oxaloacetic transaminase (GOT) activity was measured using Assay Kit (Kainos, Tokyo). For the in vitro experiments, microsomes (1.6–2.8 mg/ml) obtained from 3-MC treated rats were incubated with acetaminophen in 0.05 M Tris-HCl buffer (pH 7.4) at 37°C for 15 min in the presence of an NADPH generating system followed by centrifugation at 105,000 × g for 60 min. The thus obtained precipitate was suspended in the same buffer, and GSH S-transferase activity was measured. The NADPH generating system consisted of NADP (0.33 mM), glucose 6-phosphate (8 mM), magnesium chloride (6 mM) and glucose 6-phosphate dehydrogenase (0.2 unit). Protein concentration was measured by the method of Lowry et al. (22).

Statistical analyses

Data are expressed as the mean ± S.D. The significance of difference was calculated by Student's t-test, where P values < 0.05 were taken as significant.

RESULTS

Time course of acetaminophen effect on GSH content and drug metabolizing enzymes

As shown in Fig. 1A, the GSH content in liver homogenates decreased significantly at 1 hr after the administration of acetaminophen and reached its minimum level at 3 hr (6% of control) and then increasing to 248% at 12 hr and 346% at 24 hr. Microsomal GSH S-transferase activity markedly increased at 3 hr (331% of control) and 6 hr (193%); Even after 12 hr, a significant increase in the activity was still observed. GSH peroxidase activity in the microsomes was enhanced at 3 hr but was decreased after 12 hr. On the contrary, at 3 hr after the treatment with acetaminophen, the activities of GSH S-transferase and GSH peroxidase in the cytosol were decreased to 86% and 54% of the control, respectively, and the aniline hydroxylase activity in the microsomes was decreased to 82% of the control level (Fig. 1B). Serum GSH S-transferase and GOT activities gradually increased with an increase in the time after administration of acetaminophen, reaching the maximum level after 24 hr (Fig. 2). Microsomal GSH S-transferase activity was also increased when microsomes were incubated with acetaminophen in vitro (Fig. 3).

Measurement of kinetic parameters of GSH S-transferase and GSH peroxidase in microsomes

Eadie-Hofstee plots for GSH S-transferase and peroxidase activities in liver microsomes obtained from the control and acetaminophen-treated rats are presented in Fig. 4. The K_m value of GSH S-transferase activity for CDNB was increased from 0.023 to 0.047 mM by acetaminophen treatment and the V_max was twice that of the control. For GSH peroxidase, the Eadie-Hofstee plots were not linear, and two K_m values were obtained in the control rats. However, after acetaminophen treatment, the Eadie-Hofstee plots were linear with only one K_m value. The V_max value of the peroxidase activity was also increased in acetaminophen-treated liver. K_m and V_max values from the control and acetaminophen-treated rats are summarized in Table 1.
Enzyme Activation by Acetaminophen

**Fig. 1.** Time course of acetaminophen effect on GSH content and drug metabolizing enzyme activities in liver. Acetaminophen (250 mg/kg, i.p.) was given to rats 24 hr after 3-MC (25 mg/kg, i.p.) treatment. At the indicated times, GSH content and each enzyme activity in the liver were measured as described in Materials and Methods. Enzyme activity and GSH content in the control are as follows: A: GSH content (0, 11.94±4.67-15.61 ± 2.82 nmol/mg protein), microsomal GSH S-transferase (0, 0.043±0.004-0.074±0.007 pmol/mg protein/min), microsomal GSH peroxidase (0, 0.037±0.001-0.067±0.013 pmol/mg protein/min). B: cytosolic GSH S-transferase (0, 1.014±0.170-1.471 ±0.240 pmol/mg protein/min), cytosolic GSH peroxidase (0, 0.320±0.060-0.488±0.033 pmol/mg protein/min) and aniline hydroxylase (0, 9.3±1.7-12.6±2.7 nmol/mg protein/min). Each point represents the mean ±S.D. for 3 to 6 rats. *P<0.05, **P<0.01, ***P<0.001 vs control.

**Fig. 2.** Time course of acetaminophen-induced liver toxicity. GOT and GSH S-transferase activities in the serum, which were obtained from the same rats used in the experiments shown in Fig. 1, were measured. Each point represents the mean of 3 to 6 rats. *P<0.05, **P<0.01 vs control. □, GOT; ○, GSH S-transferase.

**Fig. 3.** Effect of acetaminophen on liver microsomal GSH S-transferase activity in vitro. Microsomes (1.8-2.8 mg/ml) from 3-MC treated rats were incubated with various concentrations of acetaminophen (AAP) at 37°C for 15 min in the presence of the NADPH generating system, and then GSH S-transferase activity was measured as described in Materials and Methods. Each point represents the mean of duplicate incubations.

**Dose-dependent effect of acetaminophen on drug metabolizing enzymes**

An increase in GSH S-transferase (176% of control) and GSH peroxidase (126% of control) activities in microsomes and a marked decrease in GSH content (13% of control) were observed at 3 hr after the treatment of rats with acetaminophen at a dose of 180 mg/kg. Cytosolic GSH S-transferase, GSH peroxidase and aniline hydroxylase activities were decreased to 53%, 46% and 96%, respectively. At a dose of 75 mg/kg of acetaminophen...
minophen, there was no change in the activity of these enzymes.

Effect of DTT on acetaminophen-induced increase in microsomal GSH S-transferase activity

We examined whether the increase in microsomal GSH S-transferase activity seen after treatment with acetaminophen is reversed by DTT, which is a reductant of disulfide bonds. As shown in Table 2, GSH S-transferase activity was increased to 139% of the control at 1 hr, 331% at 3 hr and 193% at 6 hr after acetaminophen treatment; and it was 139%, 277% and 181% of the control, respectively, when DTT was added. Thus the acetaminophen-derived increase in microsomal GSH S-transferase activity was slightly depressed by DTT, showing that the increase in the activity caused by acetaminophen is mostly due to an irreversible change of the enzyme.

Table 1. Alteration of kinetic parameters of microsomal GSH S-transferase and GSH-peroxidase activities after acetaminophen (AAP) treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>GST</td>
<td>0.520</td>
<td>62.5</td>
</tr>
<tr>
<td></td>
<td>CDNB</td>
<td>0.023</td>
<td>85.2</td>
</tr>
<tr>
<td></td>
<td>GSH-Px</td>
<td>0.050</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>CuOOH</td>
<td>0.055</td>
<td>57.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.400</td>
<td>142.9</td>
</tr>
<tr>
<td>AAP</td>
<td>GST</td>
<td>0.609</td>
<td>142.8</td>
</tr>
<tr>
<td></td>
<td>CDNB</td>
<td>0.047</td>
<td>174.1</td>
</tr>
<tr>
<td></td>
<td>GSH-Px</td>
<td>0.660</td>
<td>287.0</td>
</tr>
<tr>
<td></td>
<td>CuOOH</td>
<td>0.055</td>
<td>157.1</td>
</tr>
</tbody>
</table>

$K_m$ and $V_{max}$ values of GSH S-transferase (GST) and GSH peroxidase (GSH-Px) for each substrate were calculated from Eadie-Hofstee plots as shown in Fig. 4.

Fig. 4. Eadie-Hofstee plots for microsomal GSH S-transferase (A) and GSH peroxidase (B) activities. GSH S-transferase for CDNB (0.015 - 1.0 mM) and GSH peroxidase for CuOOH (0.03 - 1.2 mM) activities in liver microsomes obtained from the control and acetaminophen (AAP, 250 mg/kg, i.p., 3 hr)-treated rats were measured in the presence of a fixed concentration of GSH. Each point represents the mean of triplicate assays.

Table 2. Effect of DTT on microsomal GSH S-transferase activity of acetaminophen (AAP)-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GST activity (μmol/mg/min)</th>
<th>DTT (-)</th>
<th>DTT (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.043±0.004</td>
<td>0.033±0.004</td>
</tr>
<tr>
<td>AAP (1 hr)</td>
<td></td>
<td>0.060±0.013</td>
<td>0.046±0.007</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.074±0.006</td>
<td>0.060±0.006</td>
</tr>
<tr>
<td>AAP (3 hr)</td>
<td></td>
<td>0.245±0.024 ***</td>
<td>0.167±0.036 **</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.071±0.004</td>
<td>0.059±0.002</td>
</tr>
<tr>
<td>AAP (6 hr)</td>
<td></td>
<td>0.137±0.029 **</td>
<td>0.107±0.023 **</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.061±0.010</td>
<td>0.061±0.007</td>
</tr>
<tr>
<td>AAP (12 hr)</td>
<td></td>
<td>0.104±0.024 **</td>
<td>0.096±0.023 **</td>
</tr>
</tbody>
</table>

Microsomes from the control or AAP (250 mg/kg, i.p.) treated rats were incubated with DTT (10 mM) at room temperature for 10 min, and then GSH S-transferase activity was measured. Values represent means±S.D. for 3 to 6 rats. ** P<0.01, *** P<0.001 vs control.
in rats that were given 3-MC to induce cytochrome P-450 and were starved overnight before killing to reduce the GSH content, thereby acetaminophen metabolism and toxicity were accelerated. Under these conditions, microsomal GSH S-transferase and GSH peroxidase activities were increased at 3 hr after acetaminophen administration, when the GSH content in the liver was minimum. Since acetaminophen is converted to the reactive intermediate NAPQI, which covalently binds to protein thiols when GSH is depleted (13–15), this suggests that NAPQI binds covalently to the cysteine residue of microsomal GSH S-transferase, resulting in the activation of the transferase. GSH content rapidly decreased during the first 6 hr and markedly increased 12 hr after acetaminophen administration. This means that reserved GSH was consumed for the conjugation of NAPQI at the beginning and then GSH synthesis was stimulated. The fact that GSH S-transferase activity was increased at 12 hr regardless of a marked increase in GSH content suggests that once formed, the NAPQI-adduct of the transferase is stable for a long time and is not reversed by excess GSH. Since acetaminophen treatment can produce a protein-glutathione mixed disulfide (16, 17) or reactive oxygen species (23), it was assumed that microsomal GSH S-transferase is activated by oxidative modification of the sulfhydryl group of the enzyme. To clarify the possibility, the microsomes from acetaminophen-treated rats were incubated with DTT. The acetaminophen-induced increase in GSH S-transferase activity was only slightly depressed by the addition of DTT. Therefore, it is clear that microsomal GSH S-transferase is mostly activated by the covalent binding of the intermediate NAPQI to the enzyme thiol, but not by oxidation of the cysteine residue.

Although microsomal GSH S-transferase functions as a Se-independent GSH peroxidase, a significant increase in peroxidase activity was observed only at 3 hr after acetaminophen injection. Dissociation of GSH S-transferase (increase to 158%) and GSH peroxidase (decrease to 74%) activities as seen at 12 hr after acetaminophen treatment has also been observed under various conditions (24, 25). Peroxidase activity did not always increase in correspondence to the increase in its transferase activity. Microsomal GSH peroxidase, not the transferase, activity is regulated by membrane lipids and the activation of the peroxidase is observed only under the conditions such as the removal of lipid or disturbance of membrane interactions (unpublished data, Y. Aniya). Thus both activities seem to be regulated differently. To gain a better understanding of the activation of both GSH S-transferase and GSH peroxidase in microsomes, kinetic parameters for the activities at 3 hr after acetaminophen administration were determined: the $V_{\text{max}}$ value of GSH S-transferase was increased twofold as compared to that of the control. GSH peroxidase showed two $K_m$ values in control rats, while after acetaminophen treatment, the $V_{\text{max}}$ value was increased with only one $K_m$ value. Since purified microsomal GSH peroxidase has only one $K_m$ (unpublished data, Y. Aniya), it was suggested that the GSH peroxidase in microsomes may be present in two forms with different affinity for CuOOH and that removal of membrane lipids during purification steps may result in the apparent one form of enzyme. Thus it is likely that membrane lipids have been damaged by acetaminophen treatment, resulting in the change of the enzyme affinity for CuOOH. The fact that $K_m$ and $V_{\text{max}}$ values of GSH peroxidase for GSH were markedly increased by acetaminophen treatment also shows that GSH peroxidase has been modified to the active form. Microsomal GSH S-transferase and peroxidase can detoxify 4-hydroxyalkenals and fatty acid hydroperoxides, respectively, which are products from lipid peroxidation (1, 26), and acetaminophen is known to cause lipid peroxidation (27). It could be, therefore, concluded that in addition to the conjugation of NAPQI with GSH, the activated microsomal GSH S-transferase may detoxify such alkenals and hydroperoxides produced by acetaminophen.

In contrast to the marked increase in microsomal GSH S-transferase activity, cytosolic GSH S-transferase activity was decreased after acetaminophen treatment, showing the release of the enzyme into serum as seen previously (28). Although microsomal aniline hydroxylase activity was decreased by acetaminophen, it may reflect a disturbance of the association of the cytochrome P-450 complex (29, 30).

Covalent binding of NAPQI to microsomal GSH S-transferase was demonstrated in rat hepatocytes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a thiol specific fluorescent agent (31), and acetaminophen-induced increase in the activity was also shown in mice at 6 hr after the treatment (32). Taken together, all these data provide the explanation that when rats were overdosed with acetaminophen, the P-450-mediated reactive metabolite NAPQI, which escaped from the conjugation with GSH, binds covalently to liver microsomal GSH S-transferase, resulting in the activation of the transferase and peroxidase. This means that microsomal GSH S-transferase functions as an NAPQI binding protein and simultaneously becomes the active form that may detoxify not only NAPQI but also toxic metabolites of lipid peroxidation and thereby play an important role for protection against cellular damages. The proposed mechanism of the activation of microsomal GSH S-transferase (GSTm-SH) is as follows:
In summary, liver microsomal GSH S-transferase is activated by the electrophilic metabolite NAPQI by covalent binding when the GSH content in the liver is decreased, suggesting that microsomal GSH S-transferase functions not only as a binding protein of the toxic metabolite NAPQI but also a detoxification enzyme of other metabolites.

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REFERENCES


