METABOLISM AND HEPATOTOXICITY OF ACETAMINOPHEN IN MICE FED ON A LIQUID GLUCOSE DIET

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ABSTRACT

The role of dietary factors in the toxicity and metabolism of acetaminophen (APAP) was examined using male ICR mice. A 5% (w/v) liquid glucose solution replaced a regular lab chow and water exactly for 24 or 48 hr prior to measurement of hepatic glutathione (GSH) contents at 8 am, 2 pm, or 8 pm of a day. The characteristic temporal variation in hepatic GSH contents disappeared in mice provided with the glucose diet for 24 hr or longer. The microsomal p-nitrophenol hydroxylase and ethoxyresorufin O-deethylase activities, most responsible for metabolic activation of this drug, were examined in mice fed on the glucose diet for 24 hr. There were no differences in either enzyme activity between 8 am and 8 pm. Mice were injected with a 200 mg/kg dose of APAP, ip, 24 hr following initiation of the glucose diet supply and the plasma concentrations of this drug and its metabolites were monitored for 3 hr. There were no significant differences in plasma level of APAP or the major metabolites, APAP-glucuronide and APAP-sulfate, between the mice treated with this drug at 8 am and at 8 pm. However, the plasma concentrations of metabolites generated via the conjugation reaction with GSH were lower in mice treated with APAP at 8 pm. The APAP-induced hepatotoxicity was determined by increases in serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities and by decreases in hepatic glucose-6-phosphatase (G-6-Pase) activity 24 hr following the treatment. Significant differences in the AST/ALT and G-6-Pase activities were observed between 8 am, 2 pm and 8 pm in mice fed on the glucose solution for 24 hr. However, supply of the glucose diet for 48 hr abolished the time-dependent differences in serum AST/ALT and hepatic G-6-Pase activities resulting from a dose of APAP. The results indicate that supply of a glucose diet for 24 hr may eliminate the temporal variation in hepatic GSH contents, but the glucose diet supply should continue at least for 48 hr to effectively diminish the fluctuation in the toxicity of chemicals dependent on the availability of this endogenous tripeptide.

KEY WORDS: Circadian rhythm, Temporal variation, Acetaminophen, Hepatotoxicity, Glucose diet

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INTRODUCTION

Acetaminophen (APAP), a widely used analgesic-antipyretic, is safe at therapeutic doses, but upon large overdoses severe hepatotoxicity may result in experimental animals and human. At low dose levels this drug is detoxified rapidly through formation of sulfate and glucuronide conjugates (1). As the dose increases, however, APAP is increasingly metabolized into a reactive metabolite, N-acetyl-p-benzoquinonimine (NAPQI), by cytochrome P450-mediated reactions in which cytochrome P450 1A2 (CYP1A2) and 2El (CYP2El) play critical roles (2,3). The reactive, electrophilic metabolites are normally detoxified by conjugation with hepatic glutathione (GSH). When the generation of reactive metabolites exceeds the availability of hepatic GSH for conjugation, covalent binding of the reactive metabolite to macromolecules may result, an event that correlates with hepatic necrosis. Therefore, the hepatotoxicity of APAP is mostly dependent on the metabolic activities responsible for activation of this drug and effectiveness of GSH conjugation reaction.

It has been well known that hepatic GSH concentrations show distinct circadian rhythms or variations. Such variations appear to be related to changes in hormonal level, dietary intake, lighting schedule, etc. (4,5,6,7). Since GSH plays a critical role in detoxification of electrophilic toxicants, it is suspected that the toxicity of xenobiotics induced by generation of electrophilic reactive metabolites would demonstrate a temporal variation in a manner opposite to that of this endogenous tripeptide. In fact it has been noted that the toxicity of acetaminophen and chloroform, both detoxified mainly by GSH-dependent metabolic reactions, shows remarkable circadian variations (8,9,10). In a recent study conducted in this laboratory it was suggested that a variation in enzyme activities responsible for activation of APAP would also significantly contribute to the circadian variation in hepatotoxicity of this drug (11).

The objective of the present study was to examine the role of dietary factors in the time-dependent variation in hepatic GSH concentrations by limiting protein intake to mice. Since the hepatic GSH content plays a critical role in the disposition of APAP in animals, it was of interest to investigate the metabolism and toxicity of this analgesic in mice fed on a liquid glucose solution.

MATERIALS AND METHODS

Animals and Treatments

Adult male ICR mice (Animal Breeding Center, Seoul National University) weighing 30 - 35 g were used. Animals were housed in temperature (22 ± 2°C) and humidity (55 ± 5 %) controlled rooms with a 12 hr light:dark cycle (light: 0800-2000). Regular lab chow and tap water, allowed ad libitum before experiment, were replaced by a 5 % (w/v) liquid glucose diet exactly 24 or 48 hr before APAP (200 mg/kg, ip) administration made at either 8 am, 2 pm or 8 pm of a day. Acetaminophen (APAP) was injected as a saturated solution in saline (20 ml/kg) prepared by heating the APAP solution to approximately 60°C followed by cooling to 37°C right before administration. After the APAP treatment mice were provided with tap water only till sacrifice.

Chemicals

Acetaminophen, D-glucose, NADH, β-NADPH, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), glutathione (GSH), GSH reductase, 4-nitrocatechol, 7-ethoxyresorufin, resorufin and aminopyrine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). p-Nitrophenol
was obtained from Aldrich-Chemie (Steinheim, Germany). Standard metabolites of APAP, such as APAP-glucuronide, APAP-sulfate, APAP-mercapturate, APAP-cysteine and APAP-GSH, were all kindly donated by McNeil Consumer Products (Fort Washington, PA, U.S.A.). All other chemicals and solvents were reagent grade or better.

Assays

The hepatotoxicity of APAP was estimated by measuring serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities (12). Hepatic total GSH content was determined using an enzymatic recycling method (13). Glucose-6-phosphatase (G-6-Pase) activity in liver homogenates was determined using the method of Traiger and Plaa (14). The plasma concentrations of APAP and its metabolites were measured using HPLC (15). Plasma samples were mixed with an aliquot of acetonitrile containing theophylline as an internal standard. After extraction and centrifugation, the resulting supernatant was evaporated to dryness under nitrogen. The residues were diluted with distilled water as necessary before being injected into a Shimazu HPLC system (Kyoto, Japan) consisting of Model 7125 injector, LC-9A pump, and SPD-6AV UV detector equipped with C-R6A integrator. Parent APAP and its metabolites, ie., APAP-GSH, APAP-cysteine, APAP-mercapturate, APAP-glucuronide and APAP-sulfate were separated in a reverse phase C18 μ Bondapack column (Waters Associates, Milford, MA, U.S.A.; 30 cm × 3.9 mm). APAP and metabolites were eluted with 1.5 % aqueous acetic acid-methanol (90:10) at a flow rate of 1.5 ml/min. The elution of APAP and metabolites was monitored at 254 nm.

For the measurement of microsomal enzyme activities, the liver was homogenized in an ice-cold buffer of 0.154 M KCl/50 mM Tris-HCl in 1 mM EDTA, pH 7.4. The homogenate was centrifuged at 12,000 g for 20 min, and the supernatant fraction was further centrifuged at 104,000 g for 60 min. The microsomal pellet was resuspended followed by recentrifugation at 104,000 g for 60 min. The concentration of cytochrome P450 was estimated from CO difference spectrum (16). Protein content was measured using the method of Lowry et al. (17). p-Nitrophenol hydroxylase activity was determined by measuring the formation of p-nitrocatechol (18). The reaction mixture contained 0.1 mM p-nitrophenol, 1.0 mM ascorbic acid, 0.1 ml microsomal suspension, and 1 mM NADPH in 0.1 M potassium phosphate buffer, pH 6.8, in a final volume of 1.0 ml. The temperature was maintained at 37°C. Reaction was terminated by addition of ice-cold 10 % perchloric acid after a 3 min incubation period. After centrifugation 10 N NaOH was added to the supernatant and the absorption at 546 nm was measured. Ethoxyresorufin O-deethylase activity was measured by monitoring the rate of increasing fluorescence of resorufin (19). The reaction mixture contained 10 μg of microsomal protein, 1 mM ethoxyresorufin, and 0.1 M Tris buffer, pH 7.8, in a total volume of 1.8 ml. The reaction was started by addition of 50 μl of 10 mM NADPH. The reaction rate was measured directly by monitoring increases in fluorescence of the reaction mixture. Excitation and emission wave lengths were set at 530 and 585 nm, respectively.

RESULTS AND DISCUSSION

In this study the role of protein intake in the time-dependent variation of GSH contents and also of the response to a hepatotoxic dose of APAP was examined. The remarkable time-dependent variation in GSH contents in normal animals disappeared in mice fed on a liquid glucose diet for 24 hr or longer (Table 1). There was no significant difference in GSH contents measured at 8 am, 2 pm, and 8 pm on the second day and also on the third day of the glucose diet supply suggesting that protein intake plays a pivotal role in the temporal variation in the hepatic
contents of this endogenous tripeptide.

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Regular diet</th>
<th>Glucose diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr</td>
<td>48 hr</td>
</tr>
<tr>
<td>8 am</td>
<td>8.36 ± 0.30</td>
<td>3.49 ± 0.28</td>
</tr>
<tr>
<td>2 pm</td>
<td>7.11 ± 0.36*</td>
<td>3.15 ± 0.15</td>
</tr>
<tr>
<td>8 pm</td>
<td>6.20 ± 0.22***</td>
<td>3.43 ± 0.26</td>
</tr>
</tbody>
</table>

Mice were provided with a liquid glucose diet for 24 or 48 hr prior to sacrifice. Each value represents mean ± SE for 9 to 11 mice. Asterisks indicate significant differences from the value measured at 8 am (Student's t-test, *; p < 0.05, ***; p < 0.001).

APAP is conjugated with glucuronic acid or sulfate, or is activated by cytochrome P450 into a reactive metabolite, NAPQI, which may either bind to hepatic GSH, or to hepatic macromolecules; the latter binding being associated with liver injury induced by this drug (20,21,22). Therefore, the induction of APAP hepatotoxicity is mostly dependent on hepatic GSH content and cytochrome P450 activity.

It has been reported that CYP1A2 and CYP2E1 are primary catalysts for APAP in human and rodents (2,3). In the present study hepatic microsomal drug metabolizing activities mediating the activation of APAP were measured at 8 am and 8 pm, exactly 24 hr following initiation of the glucose diet supply (Table 2). Total cytochrome P450 contents determined at 8 am and 8 pm of a day did not show differences. Either p-nitrophenol hydroxylase or ethoxyresorufin O-deethylase activity, which is associated with CYP2E1 or CYP1A2, respectively, also did not show a time-dependent variation. Therefore, it is suggested that the two important factors determining the metabolism and toxicity of APAP were not different between 8 am and 8 pm in mice provided with a glucose diet for 24 hr.

APAP and its metabolites were measured in plasma of mice fed on the glucose diet for 24 hr before the drug injection (Table 3). The parent compound, APAP, was rapidly eliminated from the plasma reaching negligible levels in 3 hr. The disappearance of APAP and the major metabolite, APAP-glucuronide, from the plasma seemed to be delayed in mice treated at 8 pm compared to those treated at 8 am, although the differences were not statistically significant. The APAP metabolites generated through conjugation reactions with GSH, that is, APAP-GSH, APAP-mercapturate and APAP-cysteine, were lower in the plasma of mice treated with this drug at 8 pm. Therefore, the above results imply that the effectiveness of GSH conjugation reactions is not directly proportional to either hepatic GSH contents or metabolic activity needed for the reactions.

The hepatotoxicity of APAP was estimated by changes in serum ALT/AST and hepatic G-6-Pase activities. In mice provided with a glucose diet exactly for 24 hr the elevations in the serum enzyme parameters were increased as the time of APAP injection was delayed (Table 4). The G-6-Pase activities were also not equal among the mice treated with this drug at either 8 am, 2 pm, or 8 pm of a day, indicating that the toxic potential of APAP was not identical at the time points chosen in this study. However, supply of the glucose diet for 48 hr abolished the difference in the
ALT/AST and G-6-Pase activities in mice challenged with this analgesic at 8 am, 2 pm or 8 pm.

### TABLE 2
Hepatic Microsomal Enzyme Activities in Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Time of Measurement</th>
<th>Cytochrome P450 (nmol/mg protein)</th>
<th>$p$-Nitrophenol Hydroxylase (nmol/mg protein/min)</th>
<th>Ethoxyresorufin O-Deethylase (pmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular diet</td>
<td>8 am</td>
<td>1.09 ± 0.05</td>
<td>3.44 ± 0.11</td>
<td>32.6 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>8 pm</td>
<td>1.28 ± 0.07</td>
<td>3.20 ± 0.14</td>
<td>34.1 ± 2.4</td>
</tr>
<tr>
<td>Glucose diet</td>
<td>8 am</td>
<td>0.93 ± 0.03</td>
<td>5.85 ± 0.30</td>
<td>39.6 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>8 pm</td>
<td>1.01 ± 0.05</td>
<td>5.55 ± 0.14</td>
<td>37.3 ± 2.2</td>
</tr>
</tbody>
</table>

Mice were provided with a liquid glucose diet exactly for 24 hr prior to sacrifice at 8 am or 8 pm. Each group consisted of 12 mice. Value given represents mean ± SE for 6 pooled samples each made of livers from 2 mice.

### TABLE 3
Time Courses of APAP and Its Metabolites in Plasma

<table>
<thead>
<tr>
<th>Time of APAP Administration</th>
<th>APAP (µg/ml)</th>
<th>APAP-Glucuronide (µg/ml)</th>
<th>APAP-Sulfate (µg/ml)</th>
<th>APAP-GSH (µg/ml)</th>
<th>APAP-Cysteine (µg/ml)</th>
<th>APAP-Mercapturate (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 am</td>
<td>44.4 ± 5.4</td>
<td>323.1 ± 22.0</td>
<td>14.4 ± 2.1</td>
<td>11.0 ± 1.1</td>
<td>14.7 ± 1.3</td>
<td>9.4 ± 0.7**</td>
</tr>
<tr>
<td>8 pm</td>
<td>61.3 ± 6.6</td>
<td>325.8 ± 34.2</td>
<td>19.9 ± 1.1</td>
<td>12.7 ± 1.1</td>
<td>9.6 ± 1.7</td>
<td>9.3 ± 1.0</td>
</tr>
</tbody>
</table>

Mice were provided with a liquid glucose diet exactly for 24 hr prior to APAP treatment (200 mg/kg, ip) at 8 am or 8 pm. Values are means ± SE for 4 to 6 mice. Asterisks indicate significant differences from the mice treated with APAP at 8 am (Student's t-test, **; p < 0.01).

Glutathione (GSH), γ-glutamyl cysteinyl glycine, is present in relatively high concentrations as reduced form in most mammalian tissues. This endogenous tripeptide constitutes a major protective mechanism against toxic consequences resulting from xenobiotics and normal oxidative products of cellular metabolism. GSH acts as a nucleophilic scavenger for numerous toxic chemicals or their metabolites converting the electrophilic centers to thioether bonds, and
also as a substrate in the reduction of hydroperoxides mediated by glutathione peroxidases. Thus GS II depletion to 20 - 30 % of normal levels may significantly compromise the defense mechanism of an organism leading to severe cell injury and death (23).

<table>
<thead>
<tr>
<th>TABLE 4</th>
<th>Hepatotoxicity of APAP in Mice Fed on a Glucose Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hr</td>
<td></td>
</tr>
<tr>
<td>Time of Measurement</td>
<td>AST (units/ml)</td>
</tr>
<tr>
<td>8 am</td>
<td>1407 ± 180</td>
</tr>
<tr>
<td>2 pm</td>
<td>2595 ± 447*</td>
</tr>
<tr>
<td>8 pm</td>
<td>2918 ± 624*</td>
</tr>
<tr>
<td>48 hr</td>
<td></td>
</tr>
<tr>
<td>8 am</td>
<td>1407 ± 180</td>
</tr>
<tr>
<td>2 pm</td>
<td>2595 ± 447*</td>
</tr>
<tr>
<td>8 pm</td>
<td>2918 ± 624*</td>
</tr>
</tbody>
</table>

Value given is mean ± SE for 9 to 16 mice. In mice fed on regular diet the ALT/AST activities at 8 am, at 2 pm, and at 8 pm, were measured to be 37 ± 8 / 79 ± 2, 40 ± 4 / 79 ± 5, and 40 ± 8 / 60 ± 4, respectively. Asterisks indicate significant differences from the mice treated with APAP at 8 am (Student's t-test, *; p < 0.05, **; p < 0.01).

It has been well known that tissue concentrations of GSH exhibit characteristic circadian rhythms in experimental animals. The factors responsible for the temporal variation in hepatic GSH concentrations are still unclear. However, it is generally accepted that dietary factors play a major role in fluctuation of hepatic GSH level in rodents (8). Because of their nocturnal nature of behavior consumption of diet at night would result in an increase in the hepatic GSH levels in the morning, whereas a gradual reduction in GSH is anticipated during afternoon and till evening. The characteristic time-dependent fluctuation in hepatic GSH concentration was abolished in mice when the animals were fed on a 5 % liquid glucose diet exactly for 24 or 48 hr. Also there were no differences in the hepatic GSH content among the mice fed on the glucose diet for either 24 or 48 hr. This result suggests that the initial rapid decline of GSH level induced by limiting the supply of S-containing amino acids is slowed or halted, and the level of this tripeptide in liver is stabilized during the time periods.

In mice fed on the glucose diet for 24 hr the metabolites generated via conjugation reactions with GSH were significantly lower when treated with APAP at 8 pm. Also the hepatotoxicity of APAP was greater in mice treated with this drug at this time period. These observations suggest that the efficiency of GSH conjugation reactions was decreased in this group of animals although the amount of substrate, reduced GSH, was not altered. The metabolic activity responsible for generation of reactive metabolites from APAP could affect the formation of GSH-conjugates. But neither the p-nitrophenol hydroxylase nor ethoxyresorufin deethylase activity, which represents CYP2E1 and IA2 activity, respectively, was different between the two time points. Glutathione S-transferase (GST) activity could play a role in changes in GSH conjugation reaction. The activities of this enzyme were not measured in this study, but it has been reported that there were no time-related differences in the activity of GST (11), and accordingly, the role of this enzyme activity in dissimilar response of mice to APAP at 8 am and 8 pm appeared to be minimal, if at all. The reason for altered effectiveness of GSH conjugation reaction with APAP in spite of identical hepatic GSH contents and metabolic activity for the enzyme reaction remains unclear. Alterations in availability or supply of hepatic GSH under increased demand for this tripeptide could be a possibility. However, the GSH contents measured 3 hr following the APAP challenge did not
demonstrate a difference between 8 am, 2 pm and 8 pm (data not shown) in mice fed on the glucose diet exactly for 24 hr.

In the present study the role of protein intake in the time-dependent variation in the toxicity of acetaminophen was examined by feeding a glucose diet to mice. It is suspected that supply of a glucose diet would have advantages over simple fasting of animals in that the former may provide essential energy for maintenance of normal physiological functions and, more importantly, maintain the glucuronide conjugation reaction by supplying the essential substrate, uridine-diphosphate glucuronic acid (UDPGA), needed for the formation of a major detoxified metabolite of APAP. In summary the present study indicates that the hepatic GSH contents in mice could be leveled down by limiting the protein intake for a relatively short period. But it was suggested that inhibition of GSH contents should be sustained for a sufficiently longer period to eliminate the time-dependent variation in the response to drugs and chemicals of which degradation is largely dependent on conjugation reactions with this endogenous tripeptide.

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REFERENCES


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