Effects of Phenobarbital on Aniline Metabolism in Primary Liver Cell Culture of Rats with Ethionine-Induced Liver Disorder

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Abstract: In experiment 1, the amount of aniline (AN) metabolites in the primary cell culture medium of the liver cells obtained from ethionine (ET)-treated rats was compared with that of the control (normal) rats. Although the metabolites detected in both groups were p-aminophenol (p-AP), N-acetyl-p-AP (AAP), acetoanilide (AAN), AAP-glucuronide (AAGP), phenylhydroxyamine sulfate (PHAS) and p-AP-glucuronide (p-APG), the amount of AAP was lower and that of p-APG was markedly higher in the ET-treated rats than in the control rats. In experiment 2, phenobarbital (PB) was orally administered to the ET-treated and control rats at a dose of 100 mg/kg. The time course changes in AN metabolites in the primary cell culture medium of liver cells obtained at 2 or 48 hr after PB treatment were compared with those without PB treatment. In the ET-treated rats, the amount of PHAS was slightly higher at 2 hr after PB treatment, and that of AAP was lower and that of p-APG was higher at 48 hr after PB treatment as compared with those without PB treatment. In the control rats, the amounts of AAP, AAN, p-AP and p-APG at 2 hr after PB treatment remained lower than those without PB treatment, and that of AAP was markedly lower and that of p-APG was higher at 48 hr after PB treatment as compared with those without PB treatment. These findings indicated greater detoxication in the primary liver cell culture in the ET-treated rats than in the control rats. Furthermore, detoxication was greater in the primary cell culture of liver cell obtained from the ET-treated rats after PB treatment than from those without PB treatment, because the production of acetylates (AAP) decreased and p-APG increased (induction of conjugated enzyme) in the PB treatment group.

Key words: ethionine, liver disorder, phenobarbital, primary liver cell culture, rats

Introduction

Many drugs are metabolized as water soluble substrates by drug metabolizing enzyme (DME) in phase I and phase II reactions in the liver [2, 3]. Metabolites such as N-acetylate derivatives have been estimated to possibly induce toxicity and mutagenicity in the liver and kidneys [16, 17]. On the other hand, synthetic

(Received 26 July 1995 / Accepted 16 November 1995)
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derivatives such as conjugates have been considered to be the most important metabolites in detoxication and to be almost non-toxic [9, 25]. The amount of conjugates is dependent on glucuronosyltransferase, sulfotransferase or other conjugate enzyme activity and the concentration of substrates such as ATP, UDP-glucose, UDP glucuronic acid or sulfate in the liver.

Abnormalities of the drug metabolic system, prolonged elimination and accumulation of drug are observed in the disordered liver [19]. Previously, we reported prolonged elimination of phenobarbital (PB) in rats with experimental liver disorder [24]. In patients with liver disorders, accumulation of metabolites (non-synthetic or N-acetylate derivatives) may induce toxicity and mutagenicity in the liver.

Hepatic DME consists of a different family of isoenzymes, is partially overlapped in substrate specificities, and is induced by PB [6, 12, 29]. PB, a sedative and antiepileptic drug [13], is also administered to epilepsy patients with liver disorders. The hepatic DME induced by PB treatment also affects the metabolism of other drugs.

Detoxification in patient with liver disorders is influenced by liver enzyme activities capable of detoxifying non-synthetic derivatives, the content of glutathione in the liver, or the quantitative balance between non-synthetic and synthetic metabolites in the liver. As for the balance, if the amount of conjugates is higher than that of N-hydroxy and N-acetylates, hepatotoxicity is may be low, but if this phenomenon is reversed, the hepatotoxicity may be high. Such a quantitative balance of the metabolites in the liver is considered to be changeable by the effects on the liver. Normal animals treated with an inducer such as PB show a liver protecting effect, but when PB is administered to animals with liver injury, it is not always clear whether the metabolism is similar to that in normal animals.

In this study, to clarify the quantitative balance of metabolites in the liver in rats with ethionine-induced liver disorders, the primary cell cultures of liver cells obtained from these rats were incubated with aniline (AN), and the amount of metabolites of AN in medium was determined. Furthermore, the amount of metabolites in that from rats after treatment with PB was also determined by the same experimental system.

### Materials and Methods

1. **Chemicals**
   Aniline (AN), sulfate, aminophenol (AP) and acetoaminophenol (AAP) were purchased from Katayama Chemical Industries. DL-ethionine (ET) was obtained from Nacalai Tesque Ltd (Kyoto, Japan). Sodium phenobarbital (PB) and collagenase were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All reagents were of special grade.

2. **Animals**
   Female Sprague-Dawley (Slc: SD) rats (Japan SLC) weighing 135 to 160 g were used at approximately 6 weeks of age. The rats were given rodent chow (CRF-1, Oriental Yeast Co.) and tap water ad libitum. All animals were individually housed in a stainless steel cage and the animal room was maintained at a temperature of 23°C, with a relative humidity of 55 ± 5% and a 12-hr light: 12-hr dark cycle (lights on from 6:30 to 18:30). Each experimental group consisted of five rats.

3. **Experimental design**
   ET and PB were dissolved in physiological saline. ET was intraperitoneally administered to female rats at a dose of 500 mg/kg/day for four consecutive days. PB was orally administered to ET-treated rats at a dose of 100 mg/kg 24 hr after the final treatment with ET. PB was also administered to the control (normal) rats. At 2 and 48 hr after treatment with PB, the rats were anesthetized with sodium pentobarbital and liver specimens were taken. Table 1 shows the experimental design.

<table>
<thead>
<tr>
<th>Group</th>
<th>Phenobarbital</th>
<th>Sampling of liver</th>
<th>No. of rats</th>
</tr>
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<tbody>
<tr>
<td>Control rats</td>
<td>No treatment</td>
<td>2 hr after PB treatment</td>
<td>5</td>
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<tr>
<td></td>
<td>Treatment</td>
<td>48 hr after PB treatment</td>
<td>5</td>
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<tr>
<td>ET-treated rats</td>
<td>No treatment</td>
<td>2 hr after PB treatment</td>
<td>5</td>
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<tr>
<td></td>
<td>Treatment</td>
<td>48 hr after PB treatment</td>
<td>5</td>
</tr>
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</table>
4. Preparation of isolated rat hepatocytes

Rats were anesthetized with sodium pentobarbital, and the liver cells were isolated by perfusion of the liver with collagenase-containing solution by the method of Seglen [26]. The liver cells were washed two times, and suspended and incubated in a Williams E medium containing 10% fetal calf serum, 10^{-6} M insulin and 10^{-3} M dexamethasone. The final density of the liver cell suspension prepared was 1 × 10^6 cells/ml. Three ml of the liver cell suspension was seeded on a collagen-coated culture dish (five dishes per group, CELLTIGHT C-1, &U35 mm, SUMITOMO BAKELITE Co., Ltd.); cultured for 24 hr at 37°C under a 95% O_2-5% CO_2 atmosphere; and the culture medium was exchanged for a new medium containing 1 mM each of AN and sodium sulfite (Na_2SO_3). The medium of the primary liver cell culture in all groups was taken at 0.5, 1, 1.5, 2 and 3 hr after changing medium. Fig. 1 shows the study design.

5. Detection of conjugates

To detect glucuronide and sulfate conjugates in the medium, β-glucuronidase (pH 6.2) or arylsulfatase (pH 5.2) was added to the culture medium, the mixture was incubated for about 24 hr at 37°C, and the peaks before and after incubation were examined by HPLC.

![Fig. 1](image)

Fig. 1. The process of sampling of the medium containing aniline metabolites.

6. Assay

The sample of culture medium was centrifuged, and the supernatant obtained was stored at −40°C. In the HPLC method, the column used was CAPCELL PAK 5C18 AG (5 μm, 4.6 mm φ × 150 mm + precolumn 35 mm, Shiseido, Tokyo, Japan) and kept at 20°C. An SPD-6A UV spectrophotometric detector (Shimadzu, Kyoto, Japan) was used to detect the difference between the properties of the samples and those of the mobile phase [methanol (MeOH): 0.15 M ammonium acetate buffer, pH 7.5 = 1.9] at 280 nm. The flow-rate was kept at 1 ml/min at ambient temperature. The individual peak areas were calculated with a C-R6A Chromatopac (Shimadzu). The amount of each metabolite was described as the ratio to that of the metabolite 0.5 hr after incubation in control rats without PB treatment.

An eluent of the mobile phase (trifluoroacetic acid-ammonia buffer, pH 7.5) was used for separation of each fraction of HPLC. Samples of each fraction and a mixed sample (incubation mixture) were measured with a mass spectrometer (M-1000 LC/API-MS, HITACHI, Tokyo, Japan) with an electrospray pressure ionization source operating in the positive-ion mode. The apparatus conditions were as follows: flow rate, 1 ml/min; column temperature, 40°C; mobile phase, 1.0 M ammonium acetate and MeOH (9:1); all mass range, M/Z 5 to 500 in 4 sec; and dwell time, 0.5 sec. Sensitivity was measured by selected ion monitoring (SIM) in flow-injection analysis. The nebulizer temperature was set at 330 or 399°C. The drift voltage varied in the range 120–170 V.

7. Statistical analysis

The values are the means ± S.D. Statistical analysis was done by parametric analysis of variance with a Statistical Analysis Package (Y.K.D. Co., Ltd.) and an NEC PC-9801; MUSCOT computer program. The values with P≤0.05 were considered to be significant.

--- Experiment 1 ---

1. Detection of AN metabolites

1-1. Conjugates

Figs. 2 and 3 show the peak and resolved peak before and after the enzyme treatment in the medium.
Fig. 2. Chromatograms in the medium with or without β-glucuronidase treatment. (A) No β-glucuronidase treatment. (B) Beta-glucuronidase treatment.

Fig. 3. Chromatograms of fraction M₄ in the medium with or without sulfatase treatment. (A) Fraction M₄ in an incubation mixture. (B) Fraction M₄ in an incubation mixture with sulfatase treatment.

Fig. 4. Mass spectrum of aniline metabolites in the medium of primary liver cell cultures.
The peaks of M₃ and M₄ were glucuronic and sulfate conjugates, respectively.

1-2. Other metabolites (Fig. 4)

The major metabolites in the medium were acetanilide (AAN), p-aminophenol (p-AP), phenylhydroxyamine sulfate (PHAS), p-AP-glucuronide (p-APG) and aniline (AN). The peak of M₁ consisted of AAP including a trace of N-acetyl-p-aminophenol-glucuronide (AAPG).

2. AN metabolites in the primary liver cell culture medium of ET-treated rats (Table 2)

The amount of AN metabolites in the medium for 0.5 hr incubation in the ET-treated rats was compared with that in the control rats. The amount of AAP was significantly lower and that of p-APG was significantly higher in the medium in the ET-treated rats than in the control rats. There was no change in the amount of AAN, p-AP or PHAS.

The amount of p-AP for 0.5 hr after incubation in the control rats was about 300 nmol/10⁶ cell/0.5 hr. Although the amounts of other metabolites were 2 to 10 times those of p-AP, the amount of AAPG was very small (judged from the peak area in the HPLC analysis at λ=280 nm).

--- Experiment 2 ---

1. M₁, (AAP) (Fig. 5)

ET-treated group: The amount of AAP in the medium in the group 2 hr after PB treatment indicated a pattern of time-course increase similar to that without PB treatment. AAP was low in the group 48 hr after

| Table 2. Aniline metabolites after incubation for 0.5 hr in primary cell culture of liver cells from ethionine (ET)-treated rats |
|-----------------|----------------|-----------------|-----------------|-----------------|
| Group           | AAP            | AAN             | p-AP            | PHAS            | p-APG           |
| Control group   | 1.00 ± 0.32    | 1.00 ± 0.07     | 1.00 ± 0.04     | 1.00 ± 0.44     | 1.00 ± 0.11     |
| ET-treated group| 0.21 ± 0.02**  | 0.98 ± 0.05     | 1.02 ± 0.02     | 1.17 ± 0.02     | 1.45 ± 0.09**   |

AAP: N-acetyl-p-aminophenol, AAN: Acetanilide, p-AP: p-aminophenol, PHAS: Phenylhydroxyamine-sulfate, p-APG: p-aminophenol-glucuronide. The quantity of each metabolite in the ET-treated group is shown as the ratio to the level of metabolite 0.5 hr after incubation in the control group. **: Significantly different (P<0.01 from control group. Values are the means ± S.D.

**Fig. 5. Time-course change in N-acetyl-p-aminophenol (AAP) in primary hepatocytes cultures of the ethionine (ET)-treated and control (normal) group after PB treatment. Animals were killed 2 or 48 hr after dosing with phenobarbital (PB). The ET-treated and control group without dosing with PB (●), the ET-treated and control group 2 hr after dosing with PB (○), and the ET-treated and control group 48 hr after dosing with PB (△). Means ± S.D. (n=5).**
PB treatment; that is, the increase in AAP induced by PB treatment was slight.

**Control group:** The amount of AAP in the medium 2 hr after PB treatment was lower than that without PB treatment. AAP 48 hr after PB treatment was slightly increased and the amount was small.

2. *M*₂(AAN) and *M*₃(p-AP) (Figs. 6 and 7)

**ET-treated group:** The amounts of AAN and p-AP in the medium reached a plateau level after 0.5 hr incubation with and without PB treatment. There was no time-course difference in the amounts of any group.

**Control group:** The amounts of AAN and p-AP in the medium with and without PB treatment reached a plateau level for 0.5 hr incubation. There were no time-course differences between the groups at 48 hr with and without PB treatment, but the amounts 2 hr after PB treatment remained slightly smaller than those.

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**Fig. 6.** Time-course change in acetoamidone (AAN) in primary hepatocytes cultures of the ethionine (ET)-treated and control (normal) group after PB treatment. Animals were killed 2 or 48 hr after dosing with phenobarbital (PB). The ET-treated and control group without dosing with PB (○), the ET-treated and control group 2 hr after dosing with PB (●), and the ET-treated and control group 48 hr after dosing with PB (△). Means ± S.D. (n=5).

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**Fig. 7.** Time-course change in p-aminophenol (p-AP) in primary hepatocytes cultures of the ethionine (ET)-treated and control (normal) group after PB treatment. Animals were killed 2 or 48 hr after dosing with phenobarbital (PB). The ET-treated and control group without dosing with PB (●), the ET-treated and control group 2 hr after dosing with PB (○), and the ET-treated and control group 48 hr after dosing with PB (△). Means ± S.D. (n=5).
in the other groups.

3. M₄ (PHAS) (Fig. 8)

**ET-treated group:** The amount of PHAS in the medium reached a plateau level for 0.5 hr incubation with and without PB treatment. There was no difference in the amount between the groups at 48 hr with and without PB treatment but, PHAS 2 hr after PB treatment remained slightly higher than that in the other groups.

**Control group:** The amount of PHAS in the medium of the groups with and without PB treatment reached a plateau level after 0.5 hr incubation. There was no difference in the amount of PHAS between the groups at 48 hr with and without PB treatment, but the amount 48 hr after PB treatment remained slightly smaller than that without PB treatment.

4. M₅ (p-APG) (Fig. 9)

**ET-treated group:** The amount of p-APG in the medium in group 2 hr after PB treatment and without PB

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**Fig. 8.** Time-course change in phenylhydroxylamine sulfate (PHAS) in primary hepatocytes cultures of the ethionine (ET)-treated and control (normal) group after PB treatment. Animals were killed 2 or 48 hr after dosing with phenobarbital (PB). The ET-treated and control group without dosing with PB (●), the ET-treated and control group 2 hr after dosing with PB (○), and the ET-treated and control group 48 hr after dosing with PB (△). Means ± S.D. (n=5).

**Fig. 9.** Time-course change in p-aminophenol glucuronide (p-APG) in primary hepatocytes cultures of the ethionine (ET)-treated and control (normal) group after PB treatment. Animals were killed 2 or 48 hr after dosing with phenobarbital (PB). The ET treated and control group without dosing with PB (●), the ET-treated and control group 2 hr after dosing with PB (○), and the ET-treated and control group 48 hr after dosing with PB (△).
treatment reached a plateau level after 0.5 hr incubation and showed no time-course difference. The amount of p-APG in the group 48 hr after PB treatment increased for 1.5 hr, reached a plateau level, and was sustained at a higher level than that in the group 2 hr after PB treatment and without PB treatment.

**Control group:** The amount of p-APG in the medium of the groups 2 and 48 hr after PB treatment and without PB treatment reached a plateau level after 0.5 hr incubation. The amount of p-APG 48 hr after PB treatment remained relatively higher than that in the other groups, but its amount in the group 2 hr after PB treatment was low.

**Discussion**

The present study consisted of in vitro studies with the primary cell culture of liver cells obtained from rats with ET-induced liver disorders. Although in vitro studies with healthy animals have been reported for some drugs [4, 8, 18], a few on animals with liver disorders have been reported [1, 7, 10]. The examination of the amount of drug metabolites is useful for the evaluation of the elimination and toxicity of drugs in the disordered liver.

In experiment 1, the kinds of metabolites detected (AAP, AAN, p-AP, PHAS, AAPG and p-APG) in the culture medium were similar for the ET-treated and control groups. The conjugated metabolites were PHAS, AAPG and p-APG, and their results were in accordance with the findings obtained on AN urine metabolites in the report by Kao et al. [11]. The amount of AAP was significantly low in the ET-treated rats. These results suggest that the degree of hepatotoxicity induced by accumulated AAP may be small under the ET treatment. Increased p-APG in the ET-treated rats was considered to be due to the low activity of UDP-glucuronosyltransferase (UDPGT), because the content of UDP-glucuronic acid (UDPGA) was relatively high and unchanged in various pathological conditions despite the decreased amount of liver glycogen. Such a change in the p-APG of the ET-treated rats was in accordance with that of various abnormal states [2, 7, 28]. Since the amounts of AAN and PHAS in the ET-treated rats were similar to those in the control rats, N-acetyltransferase (ATase) and sulfotransferase (ST, PHAS-producing enzyme) activities to AN were considered to be similar in the ET-treated and control rats.

In experiment 2, when PB was administered to normal rats, the amounts of AAP, AAN, p-AP and p-APG in the medium were decreased in the group 2 hr after PB treatment and the p-APG in the medium was increased in the group 48 hr after PB treatment. These changes in the medium suggested that hydroxylation, acetylation and glucuronidation were slightly decreased in the early stage, and that glucuronidation of p-AP was markedly increased 48 hr after PB treatment. This was closely correlated with the enzymal changes in the previous study [15, 23]. When PB was administered to ET-treated rats, the amount of AAP in the group 2 hr after PB treatment was similar to that without PB treatment, but that in the group 48 hr after PB treatment was markedly decreased. This indicated that the amount of AAP was unchanged as acetylation of p-AP and conjugation of AAP proceeded to the same degree at an early stage, but that the amount of AAP was decreased in a late stage by the marked formation of the conjugates. This phenomenon may be associated with an increase in p-APG (conjugate) mentioned below.

A slight increase in the amount of PHAS is considered to be due to an increase in sulfate conjugation to PHA at an early stage. In the ST reaction, 3'-phosphoadenosine sulfate produced from ATP was used as the substrate. Since the level of liver ATP reported in the ET-treated mice [27] and alcohol-treated baboons [14] is sufficient for the ST reaction, low ATP in the liver is not related to low PHAS. Accordingly, the cause of the slight rise in PHAS in spite of high ST activity in an early stage of PB treatment in the ET-treated rats is considered to be due to the low Km [19] of ST in relation to PHAS. Since decreases in AAN, p-AP and AAP in the control rats at an early stage of PB treatment were not seen in the ET-treated rats, the induction of DME in these reactions in the ET-treated rats was little influenced by PB treatment.

The difference between the ET-treated and control rats in the time-course change in p-APG and PHAS after PB treatment indicated that the regulating-process of glucuronidation and sulfation of AN metabolites varied in these two groups, and this suggested a relationship with the changes in DME in the liver referred to in a previous report [23].

In conclusion, the findings obtained with the liver culture indicated that AN is first hydroxylated and then
conjugated. The metabolites in the ET-treated rats were identified as metabolites similar to those in the control rats, but their amounts in the ET-treated rats were different from those in the control rats. When PB was administered to the ET-treated rats, acetylates were decreased, and glucuronyl conjugates (at a late stage) and sulfated conjugates (at an early stage) were increased. Such changes in both groups are attributed to a detoxicating action.

Acknowledgments

We wish to express our thanks to Dr. Hiroshi Morishita, General Manager of Safety Research Laboratory, and Dr. Mamoru Matsumoto, Senior Scientist of Lead Optimization Research Laboratory, Tanabe Seiyaku, for their many important suggestions and encouragement during this work, and our thanks to Messrs. Koki Tanaka and Tatsuya Fujitsuka for expert technical assistance.

References


