Xenobiotic Tolerance of Primary Cultured Hepatocytes in Rats Fed a High-Fat or High-Protein Diet

Hitoshi ASHIDA, Reiko NAKAI, Kazuki KANAZAWA and Gen-ichi DANN\*O

Department of Biofunctional Chemistry, Faculty of Agriculture, Kobe University, Kobe 657-8501, Japan

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Summary The diet and nutritional status dominate a tolerance to environmental xenobiotics. In this study, the cytotoxic action of carbon tetrachloride (CCl\(_4\)) and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), one of the dietary carcinogens, was investigated using primary cultured hepatocytes from rats fed a high-fat (23% corn oil) or high-protein (50% casein) diet for three weeks. Both chemicals showed strong cytotoxicity to hepatocytes, which was judged by measurement with the MTT-test and lactate dehydrogenase leakage test. A dietary effect on cytotoxicity was observed; hepatocytes from rats fed the high-protein diet were more susceptible to cytotoxicity than the cells from rats fed a standard diet. On the other hand, ureogenesis, as a cellular function of hepatocytes, was markedly decreased in the cells from rats fed the high-fat diet. These activities were affected in the CCl\(_4\)-treated cells but not in the Trp-P-1-treated cells. The same trend of both diet and chemical effects was observed in gluconeogenesis from fructose. We conclude that the hepatocytes from rats fed a high-protein diet have high susceptibility to the cytotoxicity of CCl\(_4\) and Trp-P-1, but cytotoxicity was not related to the reduction of cellular functions.

Key Words high-fat diet, high-protein diet, Trp-P-1, cytotoxicity, primary cultured hepatocytes

Our diet and nutritional status are important determinants for individual variability in drug metabolism (1) and they can influence cancer formation. The role of dietary protein is to supply amino acids, but a high-protein diet may enhance the risk of cancer through certain environmental xenobiotics to which humans are exposed (2, 3). The intake of a high-fat diet has been correlated epidemiologically with the incidence of breast and colon cancers (1, 4-6). In addition, our diet should contain the lowest concentration of environmental xenobiotics as possible. It is known that smoking, drinking and eating charcoal broiled food are sources of

*To whom correspondence should be addressed.
environmental xenobiotics that may enhance cancer formation (1, 7). These results show that the diet and nutritional status dominate a tolerance to environmental xenobiotics; however, it is difficult to obtain the experimental evidence of tolerance to xenobiotics under different diets because of the number of animals required to conduct experiments of this kind.

Recently, many cell lines are used for the screening of potential carcinogens instead of experimental animals. However, since these cells often lack some functions of original tissues or organs, data from cell lines are difficult to apply to living animals, whereas primary cultured cells from tissues or organs usually maintain their original functions and the past nutritional status of the animal. For example, isolated lymphocytes reflect the nutritional history of the organism, and in vitro culturing of these cells may enable the identification of both previous deficits and present metabolic patterns (8, 9). Among tissues and organs, the liver is the most dominant tissue for detoxification of xenobiotics because incorporated xenobiotics are mainly metabolized in the liver cytochrome P450 system. Therefore, primary cultured hepatocytes are a suitable model for the assessment of the toxicity of xenobiotics under different nutritional conditions.

In this study, we first survey the cytotoxicity of eight chemicals to primary cultured hepatocytes from rats fed a standard diet. Among the chemicals, carbon tetrachloride (CCl₄) and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) showed strong cytotoxicity to hepatocytes. Then, we compared their cytotoxicity to the hepatocytes from rats fed high-fat or -protein diets. Finally, we investigated effects of these chemicals on ureogenesis and gluconeogenesis using the cells from rats fed different diets.

**MATERIALS AND METHODS**

**Materials.** Tryptophan pyrolysis products, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). For the culturing of hepatocytes, fetal bovine serum (FBS) and William's medium E were purchased from Life Technologies, Inc. (Tokyo, Japan). Supplements for the medium were obtained from Wako Pure Chemical Industries. Other chemicals were of the highest purity grade available.

**Animals and diets.** Male Wistar rats (ST, SPF: Japan SLC, Inc., Shizuoka, Japan), 3-weeks-old and each weighing about 50g, were housed in stainless steel cages in a temperature-controlled (24±1°C) room with 60±5% humidity and a 12-h light-dark cycle. Rats were divided into three groups at random, and received water and either a standard, high-fat or high-protein diet (Table 1) ad libitum. The standard diet was prepared according to the American Institute of Nutrition (AIN)-76 semipurified diet (10, 11).

**Isolation and culture of hepatocytes.** After feeding the diets for 3 weeks, rats were subjected to the isolation of hepatocytes by in situ perfusion of collagenase.
Table 1. Composition of the experimental diets.

| Ingredient (g/kg diet) | Standard | High-fat | High-protein |
|-----------------------|----------|----------|--------------|
| Casein                | 200      | 200      | 500          |
| Sucrose               | 500      | 320      | 200          |
| Corn starch           | 150      | 150      | 150          |
| Corn oil              | 50       | 230      | 50           |
| Cellulose powder      | 50       | 50       | 50           |
| Mineral mixture*      | 35       | 35       | 35           |
| DL-Methionine         | 3        | 3        | 3            |
| Choline bitartrate    | 2        | 2        | 2            |
| Vitamin mixture*      | 10       | 10       | 10           |

*Prepared according to AIN-76 formulation.

according to the method of Tanaka et al (12). Isolated hepatocytes were suspended at the concentration of $5 \times 10^5$ cells/mL in the William’s medium E containing 5% FBS, kanamycin (60 mg/L), aprotinin (5 kIU/L), 1 nM insulin and 1 nM dexamethasone. The cells were seeded on plastic dishes or multi-well plates (Sumitomo Bakelite Ltd., Tokyo, Japan) precoated with collagen, and then cultured under 95% air–5% CO$_2$ at 37°C for 6 h. After the medium was removed, the cells were washed twice with phosphate-buffered saline (PBS) and used as zero-time cells. The zero-time cells were cultured into serum-free medium and submitted to the following experiments.

*Treatment of chemicals to hepatocytes.* The zero-time cells were treated with the following chemicals: Benzene, carbon tetrachloride (CCl$_4$) and ethanol were used at 5 mM (final); and other chemicals, methyl viologen (PQ), phenobarbital (PB), 3-methyl-cholanthrene (MC), benzo[a]pyrene (B[a]P) and Trp-P-1, were used at 30 nM (final). Each chemical dissolved in DMSO was pre-mixed in culture medium (1:1,000, v/v). Control cells were given only DMSO as a vehicle. After treatment with each chemical for 12 h, hepatocytes were washed twice with ice-cold PBS and subjected to the following experiments.

*Measurement of cytotoxicity.* The cytotoxicity of chemicals to hepatocytes was judged by measuring cell viability and membrane damage. Cell viability was estimated using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium-bromide (MTT) assay (14, 15) with a slight modification: 96-well plates with hepatocytes in 0.1 mL medium. After the cells were treated with each chemical, 10 μL of MTT solution (0.5 mg/mL) was added to the medium and incubated for 4 h. Then, a 0.04 M HCl-isopropanol solution (100 μL) was added to dissolve formed MTT-formazan, and the optical densities at 570 and 630 nm were measured by a microplate reader. For assessment of membrane damage, the cells were cultured on 12-well plates with 0.7 mL medium, and lactate dehydrogenase (LDH) activity into the medium was measured by the reduction in absorbance at 340 nm following...
NAD\(^+\) formation from NADH (16). Intracellular LDH activity was also determined after the cells were homogenized with 0.05 M sodium-potassium phosphate buffer (pH 7.4) by ultrasonication. The LDH leakage into the medium was expressed as a percent of the sum of activity in medium and cell homogenate.

**Activity of serine dehydratase, malic enzyme and glucose-6-phosphate dehydrogenase (G6PDH) in rat liver and primary cultured hepatocytes.** To determine whether or not primary cultured hepatocytes maintain their past nutritional status, the activities of serine dehydratase, malic enzyme and G6PDH were measured in rat liver (before isolation of hepatocytes) and in the zero-time cells. Liver was perfused with ice-cold PBS and homogenized with 3 volumes of PBS. In the case of cultured hepatocytes (100 mm dishes with 10 mL medium), the cells were washed, harvested and homogenized with ice-cold PBS. Both homogenates were centrifuged at 16,000 \(\times g\) for 20 min, and the supernatant was used as a crude enzyme source. For the measurement of serine dehydratase activity, the supernatant was incubated at 37°C for 20 min in a reaction mixture (0.3 mL) consisting of 0.5 M sodium phosphate buffer, pH 7.5, and 0.1 M L-serine as the substrate. The reaction was terminated by the addition of 10% trichloroacetic acid (0.15 mL) and kept in an ice-water bath for 20 min. After centrifugation at 1,000 \(\times g\) for 10 min, the supernatant (0.3 mL) was mixed with 0.033% 2,4-dinitrophenyl-hydrazine (0.3 mL) and let stand at room temperature for 5 min. The mixture was alkalized with 2 M NaOH (2.6 mL), and then absorbance at 520 nm was measured. The activities of malic enzyme and G6PDH were measured as the increase in absorbance at 340 nm following NADPH formation from NADP\(^+\) according to the methods of Ochoa (17) and Glock and McLean (18), respectively.

**Content of cytochrome P450 and the activities of drug-metabolizing enzymes in the microsomal fraction of rat liver.** Liver was submitted to the preparation of microsomal fraction. After perfusion with ice-cold 1.15% KCl, liver was homogenized with 3 volumes of KCl. The homogenate was centrifuged at 9,000 \(\times g\) for 20 min, and the supernatant was further centrifuged at 105,000 \(\times g\) for 60 min. The precipitate was suspended in distilled water with a Potter homogenizer. The resultant suspension was stored at \(-80^\circ C\) and used as the microsomal fraction within 2 to 3 d. The contents of cytochrome P450 and cytochrome \(b_5\) in the microsomal fraction were determined by the method of Omura and Sato (19). The activities of NADPH-cytochrome \(c\) reductase (20), cytochrome \(b_5\) reductase (21), aniline hydroxylase (22), and aminopyrine N-demethylase (23, 24) were also measured in the microsomal fraction.

**Measurement of ureogenesis and gluconeogenesis.** Ureogenesis and gluconeogenesis, liver-specific functions, were measured in chemical-treated hepatocytes on 24-well plates with 0.35 mL medium as follows. For the measurement of ureogenesis, the cells were washed with Earle’s buffer twice and incubated with the same buffer solution containing 5 mM ammonium chloride as the substrate for 2 h. Urea concentration in cell-free buffer was determined by the method of Marsh et al (25). For the measurement of gluconeogenesis, the cells were washed with glucose-free
Earle’s buffer twice and incubated with the same buffer containing 5 mM fructose or 5 mM pyruvate as the substrate for 30 min. The amount of released glucose was quantified with a glucose assay kit (Wako Pure Chemical Ind.).

*Measurement of protein content.* Protein contents in the homogenate and subcellular fractions were measured by the method of Lowry et al (26).

*Statistical analysis.* Data were expressed as M±SE. Statistical analysis was performed by computer using Stat View (ver. 4.0). Data for the cytotoxicity of various chemicals to hepatocytes from rats fed a standard diet were analyzed using Student’s t-test, and the 0.05 level of probability was used as the criterion of significance. Data from the hepatocytes of rats fed different diets were analyzed by two-way fractional ANOVA, and a separate analysis was carried out to determine the significance of chemicals in each diet group by one-way fractional ANOVA with multiple comparison tests using Scheffe’s method as a post-hoc test.

**RESULTS**

*Cytotoxicity of xenobiotics to primary cultured hepatocytes from rats fed a standard diet*

We first surveyed the cytotoxicity of eight chemicals that acted as pro-oxidants in our previous in vivo study (13). The results are shown in Fig. 1. Among them, CCl₄ and Trp-P-1 reduced cell viability as measured by the MTT test and also

![Graph](image)

**Fig. 1.** Effects of xenobiotics on cell viability and lactate dehydrogenase (LDH) leakage in primary cultured hepatocytes from rats fed the standard diet. Hepatocytes were cultured for 6 h and separately treated with eight kinds of pro-oxidative xenobiotics for another 12 h. Benzene, ethanol, and carbon tetrachloride (CCl₄) were used at 5 mM, and paraquat (PQ), phenobarbital (PB), methylchlorantrane (MC), benzo[a]pyrene (B[a]P) and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) were incubated with the cells at 30 µM. Control cells were treated with the same volume of dimethyl sulfoxide (DMSO) as a vehicle. The cell viability, as judged by MTT-test and LDH-leakage, was measured as described in Materials and Methods. Data are M±SE from six animals. Asterisks indicate significant differences from control cells (p<0.01, Student’s t-test).
caused LDH leakage. Other chemicals, benzene, ethanol, PQ, PB, MC and B[a]P, did not change either cell viability or LDH leakage. The cytotoxic action of Trp-P-1 to hepatocytes has not been known, whereas CCl₄ is a well-known chemical for inducing necrotic cell death in hepatocytes at 5 mM (27). Thus, LDH leakage was measured after treatment with Trp-P-1 and related compounds (Fig. 2). Both tryptophane pyrolysates, Trp-P-1 and Trp-P-2, caused LDH leakage at 30 and 150 µM, but tryptophane itself and other related chemicals did not cause LDH leakage even at 150 µM. Only 2-acetyl aminofluorene, which is chemically related to heterocyclic amine, showed a significant increase in leakage at 150 µM. Thus, we used CCl₄ and Trp-P-1 in the following experiments.

Effect of past nutritional status on primary cultured hepatocytes

To determine the effects of nutritional status on the cytotoxicity of CCl₄ and Trp-P-1, we attempted to isolate and culture hepatocytes from rats fed a standard, high-fat or high-protein diet for three weeks. The body-weight gain of rats fed the high-fat and high-protein diets was identical to that of rats fed the standard diet. In addition, diet did not affect the yield of isolated cells, their viability as judged by the trypan blue exclusion test or the levels of protein and DNA in cultured hepatocytes after 12 h (data not shown). To determine whether or not the past nutritional status of animals is maintained in these primary cultured cells, we measured the activities of serine dehydratase, malic enzyme and glucose-6-phosphate dehydrogenase in the zero-time hepatocytes from rats fed each diet. As shown in Table 2, serine dehydratase activity in the cells from rats fed the high-protein diet

Fig. 2. Effects of Trp-P-1 and related compounds on lactate dehydrogenase (LDH) leakage in primary cultured hepatocytes from rats fed the standard diet. Hepatocytes were treated with Trp-P-1 and related compounds for 12 h at indicated concentrations in panel shown in Fig. 1. Control cells were treated with the same volume of DMSO as a vehicle. The LDH leakage was measured in hepatocytes as described in Materials and Methods. Data are M±SE from four animals. Asterisks indicate significant differences from control cells (p<0.01, Student’s t-test).
Table 2. Effect of the diet on the activities of serine dehydratase, malic enzyme and glucose-6-phosphate dehydrogenase in rat liver and primary cultured hepatocytes.

| Diet           | Serine dehydratase (nmol/min/mg protein) | Malic enzyme (nmol/min/mg protein) | Glucose-6-phosphate dehydrogenase (nmol/min/mg protein) |
|----------------|------------------------------------------|-----------------------------------|-------------------------------------------------------|
| Activity in rat liver | 189 ± 33<sup>a</sup> | 47.1 ± 3.2<sup>a</sup> | 115 ± 11<sup>a</sup> |
| Standard       | 120 ± 24<sup>a</sup> | 27.4 ± 2.5<sup>b</sup> | 41.2 ± 4.7<sup>b</sup> |
| High-fat       | 891 ± 73<sup>b</sup> | 45.9 ± 3.5<sup>a</sup> | 134 ± 9<sup>a</sup> |
| Activity in 6 h-cultured hepatocytes | 2.2 ± 1.0<sup>a</sup> | 31.0 ± 9.3<sup>a</sup> | 63.7 ± 17.9<sup>a</sup> |
| Standard       | 2.4 ± 1.2<sup>a</sup> | 13.3 ± 3.0<sup>b</sup> | 29.1 ± 5.1<sup>b</sup> |
| High-protein   | 22.1 ± 6.0<sup>b</sup> | 29.1 ± 5.1<sup>a</sup> | 48.9 ± 8.4<sup>a</sup> |

Rats were fed a standard, high-fat or high-protein diet for 3 weeks, and liver or isolated hepatocytes was prepared from these rats. Livers and hepatocytes (6 h-cultured) were each homogenized with phosphate-buffered saline, and the homogenates centrifuged at 16,000 x g for 20 min. The activities of serine dehydratase, malic enzyme and glucose-6-phosphate dehydrogenase were measured in the supernatant as described in Materials and Methods. Data are M ± SE (n=12 for rat livers or n=6 for hepatocytes) and means in the same column having different superscript letters are significantly different (p < 0.05).

was about 10-fold higher than that in the cells from rats fed other diets. An increase in serine dehydratase activity was also observed in the livers of rats fed the high-protein diet, although there was a marked difference in the absolute activities between the cultured hepatocytes and those in the liver. This difference is due to the difficulty of maintaining serine dehydratase in cultured hepatocytes (28, 29).

The activities of malic enzyme and glucose-6-phosphate dehydrogenase in the hepatocytes from rats fed the high-fat diet were less than 50% of those in the cells from rats fed other diets. These enzyme activities in the livers of rats fed the high-fat diet also showed significant decreases. These data indicate that cultured hepatocytes maintained the past nutritional status of the rats in this experiment.

Effect of diets on the contents of cytochrome P450 and cytochrome b<sub>5</sub>, and the activities of drug-metabolizing enzymes in the microsomal fraction of rats

After the feeding of each diet for 3 weeks, the effect of diet on the drug-metabolizing enzyme system was examined, and the results obtained are shown in Table 3. The high-fat diet did not affect the contents of cytochrome P450 and cytochrome b<sub>5</sub>, or the activities of the drug-metabolizing enzymes tested here. The activity of cytochrome b<sub>5</sub> reductase was significantly increased in the microsomal fraction of rats fed the high-protein diet. The content of cytochrome b<sub>5</sub> also tended to increase without significant difference.

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Table 3. Effect of the diet on the content of cytochrome P450 and the activities of drug-metabolizing enzymes in the microsomal fraction of rat liver.

| Diet        | Content (nmol/mg protein) | Activity (µmol/min/mg protein) | Activity (nmol/min/mg protein) |
|-------------|---------------------------|-------------------------------|--------------------------------|
|             | Cytochrome P450 | Cytochrome b<sub>5</sub> | Cytochrome P450 reductase | Cytochrome b<sub>5</sub> reductase | Aniline hydroxylase | Aminopyrine N-demethylase |
| Standard    | 0.479 ± 0.035<sup>a</sup> | 0.264 ± 0.025<sup>a</sup> | 0.243 ± 0.041<sup>a</sup> | 4.86 ± 0.32<sup>a</sup> | 0.220 ± 0.043<sup>a</sup> | 4.33 ± 0.58<sup>a</sup> |
| High-fat    | 0.484 ± 0.036<sup>a</sup> | 0.279 ± 0.021<sup>a</sup> | 0.222 ± 0.036<sup>a</sup> | 4.15 ± 0.32<sup>a</sup> | 0.168 ± 0.023<sup>a</sup> | 4.91 ± 0.76<sup>a</sup> |
| High-protein| 0.472 ± 0.031<sup>a</sup> | 0.351 ± 0.039<sup>a</sup> | 0.262 ± 0.039<sup>a</sup> | 6.18 ± 0.33<sup>b</sup> | 0.194 ± 0.038<sup>a</sup> | 4.49 ± 0.66<sup>a</sup> |

Rats were fed a standard, high-fat or high-protein diet for 3 weeks. A hepatic microsomal fraction was prepared by centrifugation, and the contents of cytochrome P450 and b<sub>5</sub>, and the activities of enzymes were measured as described in Materials and Methods. Results are M ± SE from at least 8 rats, and means in the same column having different superscript letters are significantly different (p < 0.05).
Effect of past nutritional status on \( \text{CCl}_4 \)- and Trp-P-1-induced cytotoxicity

Figure 3 shows the cytotoxicity of 0.5 mM \( \text{CCl}_4 \) or 30 \( \mu \text{M} \) Trp-P-1 to hepatocytes from rats fed different diets. A dietary effect on the reduction of cell viability by treatment with both chemicals was detected. It is worth noting that the reduction of cell viability and increase in LDH leakage in the cells from rats fed the high-protein diet were greater than those in the cells from rats fed other diets, indicating that the high-protein diet is susceptible to \( \text{CCl}_4 \) or Trp-P-1 poisoning in liver cells. In the Trp-P-1-treated hepatocytes, interaction was observed between diet and chemical while no interaction was seen in the \( \text{CCl}_4 \)-treated cells (Fig. 3A). With regard to LDH leakage, not only a dietary effect but also interaction between diet and chemical was detected for both chemicals (Fig. 3B).

Effect of past nutritional status on ureogenesis and gluconeogenesis in \( \text{CCl}_4 \)- and Trp-P-1-treated hepatocytes

In the next series of experiments, we measured the liver functions, namely ureogenesis and gluconeogenesis in the hepatocytes from rats fed different diets.
Fig. 4. Effect of past nutritional status on ureogenesis in carbon tetrachloride (CCl₄)- and Trp-P-1-treated hepatocytes. Hepatocytes from rats fed standard (open bars), high-fat (hatched bars) and high-protein (closed bars) diets were treated with 5 mM CCl₄ or 30 μM Trp-P-1 as mentioned in Fig. 1. Ureogenesis in hepatocytes was measured as described in Materials and Methods. Data are M±SE from six animals. Data were analyzed by ANOVA, the same as in Fig. 3.

Fig. 5. Effect of past nutritional status on gluconeogenesis in carbon tetrachloride (CCl₄)- and Trp-P-1-treated hepatocytes. Hepatocytes from rats fed standard (open bars), high-fat (hatched bars) and high-protein (closed bars) diets were treated with 5 mM CCl₄ or 30 μM Trp-P-1 as mentioned in Fig. 1. Gluconeogenesis in hepatocytes was measured from fructose (panel A) and pyruvate (panel B) as described in Materials and Methods. Data are M±SE from six animals. Data were analyzed by ANOVA, the same as in Fig. 3.
Dietary effects on ureogenesis were detected in both CCl₄- and Trp-P-1-treated hepatocytes; the high-fat diet tended to reduce ureogenic activity while the high-protein diet increased activity as compared to that of the standard diet (Fig. 4). Chemical effects were detected in CCl₄-treated cells but not in Trp-P-1-treated cells. Diet also affected gluconeogenesis when fructose was used as substrate (Fig. 5A); the gluconeogenic activity in the hepatocytes from rats fed the high-fat diet showed lower activity than that in the cells from rats fed other diets. On the other hand, no dietary effect was detected when pyruvate was used as the substrate (Fig. 5B). CCl₄ decreased gluconeogenesis from either fructose or pyruvate markedly, but Trp-P-1 decreased slightly.

**DISCUSSION**

In this study, we have demonstrated that diets affect the cytotoxicity of CCl₄ and Trp-P-1 to primary cultured hepatocytes; hepatocytes from rats fed the high-protein diet were more susceptible to cytotoxicity induced by both chemicals as compared to the cells from rats fed the standard diet. We have also shown that diet affects liver-specific functions; the high-fat diet tended to reduce ureogenesis and gluconeogenesis from fructose in cultured hepatocytes.

Among the eight chemicals tested in this study, CCl₄ and Trp-P-1 showed strong cytotoxicity. The cytotoxicity of CCl₄ to hepatocytes in this study met our expectations; CCl₄ is well known as a chemical that induces liver injury by the formation of a center radical via cytochrome P450 monooxygenases (30), and it induces cell death by necrosis. On the other hand, the cytotoxic action of Trp-P-1 to cultured mammal cells is not well known. Trp-P-1 has been shown to be mutagenic in bacterial cells, and also to be carcinogenic in rats and/or mice when administered in a diet (31). The mutagenicity of Trp-P-2, the analog compound of Trp-P-1, is known to be due to active oxygen radicals generating from its activated forms (32, 33). From these results, Trp-P-1-induced cytotoxicity may be due to radical reaction. However, other radical sources, such as PQ and B[a]P, did not show any cytotoxicity to hepatocytes in this study (Fig. 1). There arises a question how Trp-P-1 acts on hepatocytes and induces cytotoxicity? One clue to solve this question is that Trp-P-2, which showed the same cytotoxicity to hepatocytes as Trp-P-1 and 2-acetyl aminofluorene, also had moderate toxicity. Heterocyclic amine-type chemicals seem to have the potency to induce cell death in hepatocytes.

To determine whether cultured hepatocytes maintained in vivo nutritional status, we measured the activities of three enzymes for nutritional markers in cultured hepatocytes and compared the results to those in the livers of rats. Hepatocytes from rats fed different diets also maintained their in vivo nutritional status before chemical exposure. In addition, the hepatocytes from rats fed the high-protein diet showed higher ureogenic activity than those from the rats fed the standard diet even after chemical exposure. Iritani et al (34, 35) reported these lipogenic enzymes were downregulated by high-fat diets. Ogawa et al (36) reported that hepatic serin...
dehydratase activity and its mRNA level showed parallel increases with increasing protein content in a diet. Our results using the livers of rats were identical with these previous results, indicating that three weeks of feeding was enough to alter nutritional status in vivo. Thus, the hepatocytes used in this study maintained their in vivo nutritional status during the experiments.

To obtain information about the effect of nutritional status on susceptibility to Trp-P-1, we used cultured hepatocytes for the measurement of cytotoxicity of Trp-P-1 and compared it to that of CCl₄. Our results showed that the high-protein diet is susceptible to liver injury by treatment with these chemicals. Under the condition without chemicals, the high-protein diet showed low cell viability and a trend of high LDH leakage as compared to the standard diet. We suggest that the high-protein diet has the ability to injure liver cells, and this injury would be enhanced when the cells receive chemical exposure. It is difficult to explain the relationship between susceptibility to foreign chemicals and the drug-metabolizing system, since we only detected an increase in cytochrome b₅ reductase activity in the microsomal fraction of rats fed the high-protein diet. We suggest that both chemicals acted in different ways for the induction of cytotoxicity and the reduction of liver-specific functions.

Although diets affected ureogenesis after treatment with both CCl₄ and Trp-P-1, Trp-P-1 did not affect ureogenesis while CCl₄ suppressed it. We have suggested that CCl₄ may drop these liver functions unanimously by necrotic action such as inflammation. On the other hand, Trp-P-1-induced cell death may occur suddenly in the selected cells because surviving hepatocytes have sound functions. Therefore, the toxic mechanism of Trp-P-1 to hepatocytes is different from the necrotic cell death induced by CCl₄. Cell death can be classified by either necrosis or apoptosis (37). It is interesting whether or not Trp-P-1 can induce apoptosis in hepatocytes, and we are progressing in this investigation now.

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REFERENCES

1) Waltersack I, Klotz U. 1996. Influence of diet and nutritional status on drug metabolism. Clin Pharmacokinet 31: 47–64.
2) Hietanen E. 1980. Dietary components and cancer. J Toxicol Environ Health 6: 963–969.
3) Chow W-H, Gridley G, McLaughlin JK, Mandel JS, Wacholder S, Blot WJ, Niwa S, Fraumeni JF, Jr. 1994. Protein intake and risk of renal cell cancer. J Natl Cancer Inst 86: 1131–1139.
4) Freedman LS, Clifford C, Messina M. 1990. Analysis of dietary fat, calories, body weight, and the development of mammary tumors in rats and mice: A review. Cancer Res 50: 5710–5719.
5) Erickson KL, Hubbard NE. 1990. Dietary fat and tumor metastasis. *Nutr Rev* **48**: 6–14.
6) Statland BE. 1992. Nutrition and Cancer. *Clin Chem* **38**: 1587–1594.
7) Lieber CS. 1990. Interaction of alcohol with other drugs and nutrients: Implication for the therapy of alcoholic disease. *Drugs* **40** (Suppl 3): 23–44.
8) Das KC, Herbert V. 1978. The lymphocytes as a marker of past nutritional status: Persistence of abnormal lymphocyte deoxyuridine (dU) suppression test and chromosomes in patients with past deficiency of folate and vitamin B12. *Br J Haematol* **38**: 219–233.
9) James SJ, Swendsie M, Makinodan T. 1987. Macrophage-mediated depression of T-cell proliferation in zinc-deficient mice. *J Nutr* **117**: 1982–1988.
10) American Institute of Nutrition. 1977. Report of the American Institute of Nutrition Ad Hoc Committee on standards for nutritional studies. *J Nutr* **107**: 1340–1348.
11) American Institute of Nutrition. 1980. Second report of the Ad Hoc Committee on standards for nutritional studies. *J Nutr* **110**: 1726.
12) Tanaka K, Sato M, Tomita Y, Ichihara A. 1978. Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. *J Biochem* **84**: 937–946.
13) Ashida H, Kanazawa K, Danno G. 1994. Hepatic phosphoglucomutase activity as a marker of oxidative stress induced by pro-oxidative drugs. *Biosci Biotech Biochem* **58**: 55–59.
14) Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Meth* **65**: 55–63.
15) Oka M, Maeda S, Koga N, Kato K, Saito T. 1992. A modified colometric MTT assay adapted for primary cultured hepatocytes: Application to proliferation and cytotoxicity assays. *Biosci Biotech Biochem* **56**: 1472–1473.
16) Bergmeyer HU, Graßl M, Walter H-E. 1983. Reagents for enzymatic analysis: biochemical reagents for general use: enzymes. In: Methods of Enzymatic Analysis, 3rd ed (Bergmeyer HU, Bergmeyer J, Graßl M, eds), Vol 2, p 126–328. Verlag Chemie, Weinheim.
17) Ochoa S. 1955. “Malic” enzyme. In: Methods in Enzymology (Colowick SP, Kaplan NO, eds), Vol 1, p 739–753. Academic Press, New York.
18) Glock GE, McLean P. 1953. Further studies on the properties and assay of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. *Biochem J* **55**: 400–408.
19) Omura T, Sato R. 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378.
20) Omura T, Takesue S. 1970. A new method for simultaneous purification of cytochrome b5 and NADPH-cytochrome c reductase from rat liver microsomes. *J Biochem* **67**: 249–257.
21) Takesue S, Omura T. 1970. Solubilization of NADH-cytochrome b5 reductase from rat liver microsomes by lysosomal digestion. *J Biochem* **67**: 259–266.
22) Imai Y, Ito A, Sato R. 1966. Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. *J Biochem* **60**: 417–428.
23) Hiramatsu N, Kishida T, Natake M. 1987. Effect of autoxidized linoleic acid on the contents of cytochrome P-450 and cytochrome b5, and drug-metabolizing enzyme activities in rat liver. *J Nutr Sci Vitaminol* **33**, 37–48.
24) Nash T. 1953. The colorimetric estimation of formaldehyde by means of the Hantzch reaction. *Biochem J* **55**: 416–421.
25) Marsh WH, Fingerhut B, Miller H. 1965. Automated and manual direct methods for the determination of blood urea. *Clin Chem* **11**: 624–627.

26) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the folin phenol reagent. *J Biol Chem* **193**: 265–275.

27) Nakamura T, Fuji T, Ichihara A. 1985. Enzyme leakage due to change of membrane permeability of primary cultured rat hepatocytes treated with various hepatotoxins and its prevention by glycyrrhizin. *Cell Biol Toxicol* **1**: 285–295.

28) Noda C, Shinjyo F, Nakamura T, Ichihara A. 1983. Requirement of prolonged presence of a high intracellular level of cyclic AMP for induction of serine dehydratase in primary cultured rat hepatocytes. *J Biochem* **93**: 1677–1684.

29) Noda C, Yakiyama M, Nakamura T, Ichihara A. 1988. Requirement of both glucocorticoids and glucagon for activation of transcription of the serine dehydratase gene in cultured rat hepatocytes. *J Biol Chem* **263**: 14764–14768.

30) Guengerich FP, Shimada T. 1991. Oxidation of toxic and carcinogenic chemicals by human P-450 enzymes. *Chem Res Toxicol* **4**: 168–179.

31) Wakabayashi K, Nagao M, Esumi H, Sugimura T. 1992. Food derived mutagens and carcinogens. *Cancer Res* **52**: 2092s–2098s.

32) Wakata A, Oka N, Hiramoto K, Yoshioka A, Negishi K, Wataya Y, Hayatsu H. 1985. DNA strand cleavage in vitro by 3-hydroxyamino-1-methyl-5H-pyrido[4,3-b]indole, a direct-acting mutagen formed in the metabolism of carcinogenic 3-amino-1-methyl-5H-pyrido[4,3-b]indole. *Cancer Res* **45**: 5867–5871.

33) Wataya Y, Yamane K, Hiramoto K, Ohtsuka Y, Okubata Y, Negishi K, Hayatsu H. 1988. Generation of intracellular active oxygens in mouse FM3A cells by 3-hydroxyamino-1-methyl-5H-pyrido[4,3-b]indole, the activated Trp-P-2. *Jpn J Cancer Res* **79**: 576–579.

34) Iritani N. 1993. Regulation of gene expression in fat synthesis. *Nippon Eiyo Shokuryou Gakkaishi (J Jpn Soc Nutr Food Sci)* **46**: 379–386.

35) Fukuda H, Katsurada A, Iritani N. 1992. Nutritional and hormonal regulation of mRNA levels of lipogenic enzymes in primary cultures of rat hepatocytes. *J Biochem* **111**: 25–30.

36) Ogawa W, Fujiooka M, Su Y, Kamamoto R, Pitot HC. 1991. Nutritional regulation and tissue-specific expression of the serine dehydratase gene in rat. *J Biol Chem* **266**: 20412–20417.

37) Kerr JF, Wyllie AH, Currie AR. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Brit J Cancer* **26**: 239–257.