

Alteration of Acetaminophen-induced Cytotoxicity in Mouse Hepatocytes during Primary Culture

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The present study investigated issues regarding cytotoxicity tests using mouse hepatocytes in primary culture. The results indicated that the cytotoxicity of acetaminophen (APAP) markedly varied depending on culture period for up to 5 days. P450 isoforms involved in the activation of APAP, such as CYP1A2, CYP2E1, and CYP3A, showed a marked decrease in expressions of respective mRNA. Their expression levels were a few percent within 24 hr after the start of the culture, and remained low throughout. Meanwhile, the mRNA expression of enzymes involved in the detoxification of APAP, UGT1A6 and SULT1A1, declined moderately and then recovered to approximately 40% of the *in vivo* level later in the culture. Treatment with APAP at non-cytotoxic concentrations increased expressions of CYP1A2, CYP2E1, CYP3A11 and CYP3A41 mRNA, while it decreased that of SULT1A1 mRNA. Using the lactate dehydrogenase (LDH) assay, the cells treated with 25-mM APAP for 24–48 or 48–72 hr showed higher cytotoxicity values than those for 72–96 or 96–120 hr, but the values after 50-mM treatment were completely inverted. By employing the methylthiazolyl tetrazolium (MTT) assay, culture period-dependent cytotoxicity was also observed on 25-mM APAP treatment, but this was not clear at 50 mM. Lower LDH or higher MTT activities than the control were observed in cells treated with lower concentrations of APAP, becoming the more prominent the longer the culture period. The observations suggested that, since the expression of metabolizing enzymes alters markedly during the primary culture and the range of cytotoxic concentrations of a test compound varies depending on the culture period, it is necessary to take account of these fluctuating parameters when assessing the cytotoxic character of a compound.

Key words—mouse hepatocyte, primary culture, cytotoxicity, detoxification enzyme, cytochrome P450, acetaminophen

INTRODUCTION

Several assay-based methods for evaluating toxicity have been developed for drugs, food additives, dietary supplements, pesticides, cosmetics, etc. Cell culture systems have been employed for such testing. Since human tissues are of limited availability, the tissues of animals, especially rodents, are widely used. Biotransformation is necessary for most compounds to exert toxic effects on cells. Therefore, hepatocyte cultures have been considered to show

several advantages over other tissue-derived cultures, since the liver is a major organ metabolizing xenobiotics. However, in reality, there is no hepatocyte culture system in which the expression of metabolizing enzymes is comparable to that in the liver. Hence, the advantages of cultured hepatocytes for toxicity screening have not been well elucidated. It is necessary to understand moot issues, even if some cannot be reasonably explained, when a primary culture system employing hepatocytes is introduced for the assay of hepatotoxic compounds.

Acetaminophen (APAP), an analgesic and antipyretic drug widely available over the counter, can cause hepatotoxicity.¹⁾ The hepatotoxicity of APAP is suggested to result from a depletion of glutathione (GSH), the binding of proteins to metabolites of APAP, increased oxidative stress, or a loss of the mitochondrial membrane potential.^{2,3)} APAP

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is primarily metabolized by conjugation enzymes strongly expressed in the liver: UDP-glucuronosyl transferase, UGT1A6,⁴⁻⁶⁾ or sulfonyletransferase, SULT1A1,^{7,8)} in humans and rodents. When APAP is taken at high doses, these pathways are saturated and large proportions of the drug proceed to be oxidized by CYP2E1, 1A2, and 3A4 in humans.⁹⁻¹¹⁾ In knock-out mice, CYP2E1 and CYP1A2 have been demonstrated to convert APAP to *N*-acetyl-*p*-benzoquinone imine (NAPQI), and CYP2E1 was shown to play a more important role in the bioactivation of APAP.^{12,13)} The oxidized metabolite, NAPQI, binds to GSH, and the depletion of GSH leads to unconjugated NAPQI becoming a harmful hepatotoxic compound.^{14,15)}

This paper studies the cytotoxicity of APAP during the primary culture of mouse hepatocytes by employing lactate dehydrogenase (LDH) and methylthiazolyl tetrazolium (MTT) assays. The results indicated that cytotoxic concentrations of APAP after 24-hr treatment varied depending on the starting point during a 5-day culture. Further observations whereby the mRNA expression of metabolizing enzymes involved in either activation or detoxification processes changed markedly might partly explain the varying cytotoxicity of APAP.

MATERIALS AND METHODS

Chemicals — Materials for culturing mouse hepatocytes were purchased from Wako Pure Chemical Industries (Osaka, Japan), Sigma-Aldrich (St. Louis, MO, U.S.A.), and Invitrogen Corp. (Carlsbad, CA, U.S.A.). Percoll was obtained from GE Healthcare UK Ltd. (Buckinghamshire, U.K.). APAP was obtained from Wako Pure Chemical Industries. All other laboratory chemicals were of the highest quality and available purity from commercial suppliers.

Preparation of Primary Cultures of Mouse Hepatocytes — The isolation and culture of hepatocytes were performed as described.¹⁶⁾ Briefly, the livers of 8-week-old male ddY mice (Sankyo Laboratories, Tokyo, Japan) were perfused with collagenase-containing Hanks' solution, and then viable hepatocytes were isolated by Percoll iso-density centrifugation. The cells were cultured in Waymouth MB 752/1 medium supplemented with 0.5 mg/l of insulin, 0.5 mg/l of transferrin, 0.5 µg/l of selenium, 10⁻⁷-M dexamethasone, and 2 g/l of bovine serum albumin. The hepatocytes were

seeded in collagen-coated (for monolayer cultures) or uncoated (for spheroid cultures) dishes at a concentration of 5 × 10⁵ cells/ml and incubated in a humidified atmosphere at 37°C in 5% CO₂. The medium was replaced everyday. The seeding time of the hepatocytes on culture dishes was set at 0 hr of observation. The cells were allowed to stand for 3 hr (for monolayer cultures) or overnight (for spheroid cultures) without moving to allow them to attach to the dish. APAP at specific concentrations was dissolved in the medium by sonication for 3 hr, and then the medium was sterilized by filtration. Treatment with APAP and/or a P450 inducer was started simultaneously with the change of medium at the indicated time points, and the medium or cells were harvested 24 hr later for the LDH and MTT assays or preparation of RNA.

The inducers employed were as follows: 1 mM of isoniazid (INH) as the CYP2E1 inducer,¹⁷⁾ 0.01 mM of mifepristone (RU486) as the CYP3A inducer,^{18,19)} and 13 µM of benz[*a*]anthracene (Benz) as the CYP1A inducer.²⁰⁾ Dimethyl sulfoxide (DMSO), a vehicle for the inducers, was employed at a maximum concentration of 0.5%, due to the low solubility of some inducers, but LDH values did not differ from those of the DMSO-treated and untreated groups.

Determination of LDH Activity — The level of activity of the LDH released into the culture medium by the hepatocytes over the course of the experiments was measured using an LDH-Cytotoxic test Kit Wako Pure Chemical Industries (Osaka, Japan). Briefly, the medium was centrifuged at 4000 rpm for 10 min and 25 µl of sequentially diluted supernatant was incubated with 25 µl of coloring agent at 37°C for a further 30 min. The reaction was terminated by the addition of 50 µl of 1 N HCl. The absorbance at 540 nm of the resulting solution was read using an ImmunoMini NJ2300 (Nalge Nunc International Japan, Tokyo, Japan) 96-well microplate reader. The absorbance from 0.3% Tween80-treated cells was taken as 100% cytotoxicity.

MTT Cytotoxicity Assay — The MTT assay was conducted according to the method of Cedillo-Rivera *et al.*²¹⁾ Briefly, after the removal of the culture medium, each dish was incubated for 4 hr with 1 mg/ml MTT in fresh medium. Subsequently, the produced dye was dissolved in lysis buffer. The optical density of the resulting homogenous solution was measured photometrically at 540 nm. The absorbance in APAP-untreated cells was taken as

100% viability.

Real-time RT-PCR — Total RNA was extracted from hepatocytes by employing the method of Chomczynski and Sacchi.²²⁾ The mRNA expression of the specified genes was verified by quantitative real-time RT-PCR as follows. Total RNA was reverse-transcribed using random hexamer primers and cDNA was synthesized as recommended in the TaKaRa[®] RT-PCR kit (Perfect Real Time). Polymerase chain reactions were performed using an Applied Biosystems 7000 Sequence Detection System with ABI Prism[®] 7000 SDS software (Applied Biosystems; Foster City, CA, U.S.A.). Specific probes for the TaqMan[®] Gene Expression Assay were: for *Cyp1a2* (Mm 00487224_m1), *Cyp2e1* (Mm 00491127_m1), *Cyp3a11* (Mm 00731567_m1), *Ugt1a6* (Mm 01967851_s1), and *Sult1a1* (Mm 00467072_m1). A specific TaqMan[®] MGB Gene Expression Detection kit was used for *Cyp3a41* (in which the forward primer, reverse primer, and probe were 5'-GCC AAA GGG ATT TTA AGA GTT GTT GAC T-3', 5'-GGT GTC AGG AAT GGA AAA AGT ACA-3', and 5'-FAM[™]-ATC CTT TGG TCT TCT CAG-MGB-3', respectively) and the SYBR[®] Green PCR Master Mix was used for GAPDH (in which the forward and reverse primers were 5'-TCC ACT CAC GGC AAA TTC AAC G-3' and 5'-TAG ACT CCA CGA CAT ACT CAG C-3', respectively). The conditions for the amplification were as follows: denaturation at 95°C for 5 sec, and annealing and extension at 60°C for 1 min (50 cycles). The fluorescence resulting from the reporter dye (FAM[™]) was directly monitored from the amplification of *Cyp1a2*, *Cyp2e1*, *Cyp3a11*, *Cyp3a41*, *Ugt1a6*, and *Sult1a1* cDNAs. The increase in SYBR[®] Green binding to double-stranded DNA amplified by PCR was monitored for each annealing/extension cycle for GAPDH. Standard curves were generated for each gene. Data are presented as the relative expression compared to GAPDH mRNA as a standard. The specificity of the amplification of cDNAs was confirmed by polyacrylamide gel electrophoresis (all genes) and the dissociation curve of the product (GAPDH).

RESULTS

Alteration of APAP-induced Cytotoxicity During Culture of Mouse Hepatocytes

Mouse hepatocytes were cultured as monolay-

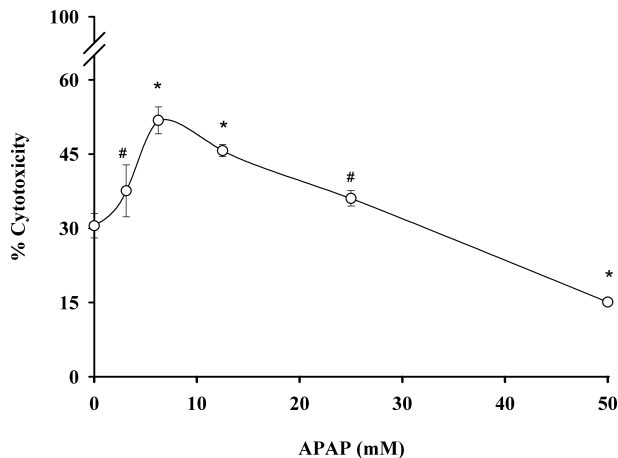


Fig. 1. Cytotoxicity of APAP Early in the Primary Culture of Mouse Hepatocytes

Mouse hepatocytes in monolayer cultures were treated with APAP at 0, 3.125, 6.25, 12.5, 25, or 50 mM from 3 to 27 hr after the seeding of cells. The activity of LDH released from hepatocytes into the medium was determined. LDH activity after the treatment of hepatocytes with 0.3% Tween80 was taken as 100% cytotoxicity. Each point shows the mean \pm S.D. ($n = 9$). Significance was calculated using ANOVA with the Student-Newmann-Keuls, post-hoc testing method between untreated and APAP-treated hepatocytes. #, $p < 0.05$, *, $p < 0.01$.

ers or spheroids and treated with APAP at up to 50 mM. Figure 1 shows that APAP at 3.125 mM was significantly cytotoxic in monolayer-cultured hepatocytes treated from 3 hr, when the first change of medium took place, based on the LDH activity determined 24 hr later. However, the cytotoxicity did not increase with the concentration of APAP. In contrast, the level of LDH activity at 50 mM was lower than the control values. In Fig. 2, the treatment was started at 24, 48, 72, or 96 hr of culture, and the cytotoxicity was assessed 24 hr later. In control cells, the LDH activity showed 5–30% cytotoxicity throughout the observation period. These values imply several kinds of damage to hepatocytes during the isolation and cultivation procedures. A concentration-dependent cytotoxic effect of APAP was observed in both culture systems, but the responsiveness of the hepatocytes to the treatment differed on a daily basis. Cytotoxicity, namely the LDH value being significantly higher than the control, was observed in the cells treated with 12.5-mM APAP for 24–48 hr, or 25-mM APAP for 24–48 or 48–72 hr, and concentration-dependent cytotoxicity was not demonstrated by treatment with 50-mM APAP during these periods. The MTT assay invertibly reflected this culture time-dependency at 25 mM APAP, but not at 50 mM, suggesting saturated toxic activity at this concentration. At lower concentrations of APAP, the levels of LDH activity

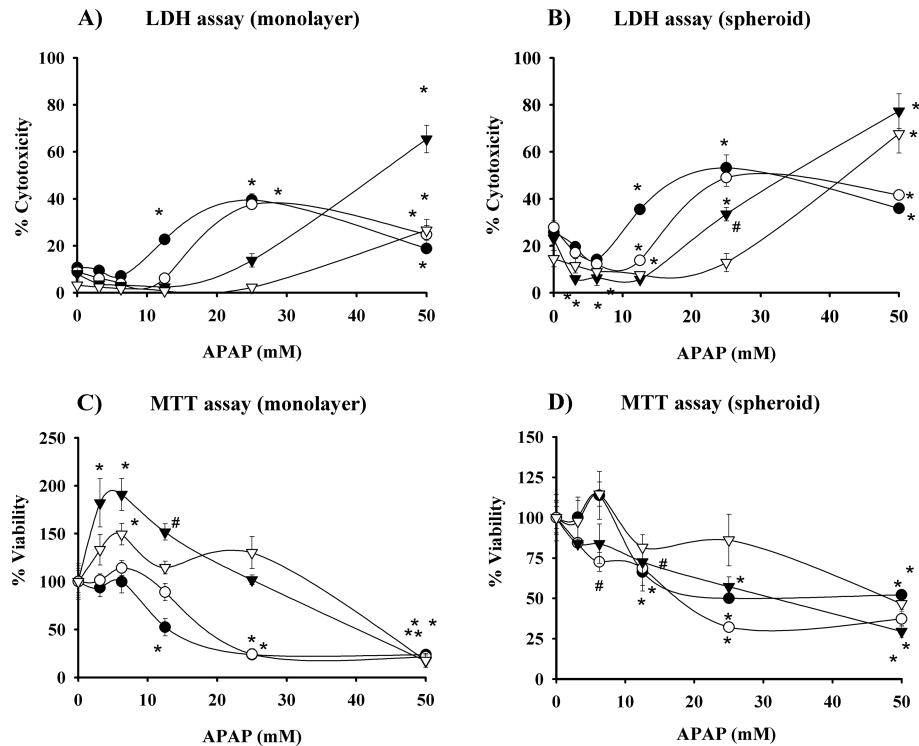


Fig. 2. Cytotoxicity of APAP in Primary Cultures of Mouse Hepatocytes

Mouse hepatocytes in primary cultures were treated with APAP at 0, 3.125, 6.25, 12.5, 25, or 50 mM for the following periods: treatment from 24 to 48 hr (●), from 48 to 72 hr (○), from 72 to 96 hr (▼), or from 96 to 120 hr (△). The activity of LDH released from hepatocytes into the medium was determined in (A) monolayer or (B) spheroid cultures. LDH activity after the treatment of hepatocytes with 0.3% Tween80 was taken as 100% cytotoxicity. The formation of the colored product formazan from MTT by mitochondrial enzyme was assessed in (C) monolayer or (D) spheroid cultures. Activity in untreated cells was taken as 100% viability. Each point shows the mean \pm S.D. ($n = 9$). Significance was calculated using ANOVA with the Student-Newmann-Keuls, post-hoc testing method between untreated and APAP-treated hepatocytes in the same period. #, $p < 0.05$, *, $p < 0.01$.

were always lower than the control values. Additionally, the MTT assay showed nearly a two-fold increase in the monolayer-cultured cells treated at lower concentrations of APAP. In the present system, the MTT assay reflects not cell proliferation but vitality, since DNA synthesis starts at 72 hr with a peak at 96 hr in a very small proportion of monolayer-cultured cells.²³⁾ As the cytotoxicity of APAP is known to be caused by its metabolites, the expression of enzymes involved in the activation or detoxification of APAP was examined to determine the possible role of each enzyme.

mRNA Expression of Enzymes Involved in Activation and Detoxification of APAP in Mouse Hepatocytes in Primary Culture

The expression of mRNAs related to the metabolism of APAP was examined using spheroid cultured cells, since CYP1A2 mRNA levels in hepatocytes were higher in spheroid than monolayer cultures.²⁴⁾ However, it was less than 2% of the initially isolated cells after 24 hr (Fig. 3). Low levels of expression were also found for mRNAs of CYP2E1,

CYP3A11, and CYP3A41, which are conceivable P450s for the activation of APAP. The expression of these mRNAs did not recover at all during the observation period. The expression of the mRNAs of UGT1A6 and SULT1A1, the major phase II enzymes responsible for the metabolism of APAP, was decreased during the culture, but, compared with the expression of P450 mRNAs, the decline was moderate (Fig. 4). The expression of these mRNAs increased in the later observation period and approached approximately 40% of that at 0 hr.

Alteration of APAP-induced Cytotoxicity in Hepatocytes Treated with Specific Enzyme Inducers

The effects of P450 inducers on the cytotoxicity of APAP were then examined. APAP (25 mM) and each inducer were added to the medium and cytotoxicity was measured 24 hr later (Fig. 5). The addition of either 1-mM INH, 10- μ M RU486, or 13- μ M Benz between 24–48 hr after the beginning of the culture did not alter the cytotoxicity of APAP. However, INH enhanced the cytotoxicity observed in the

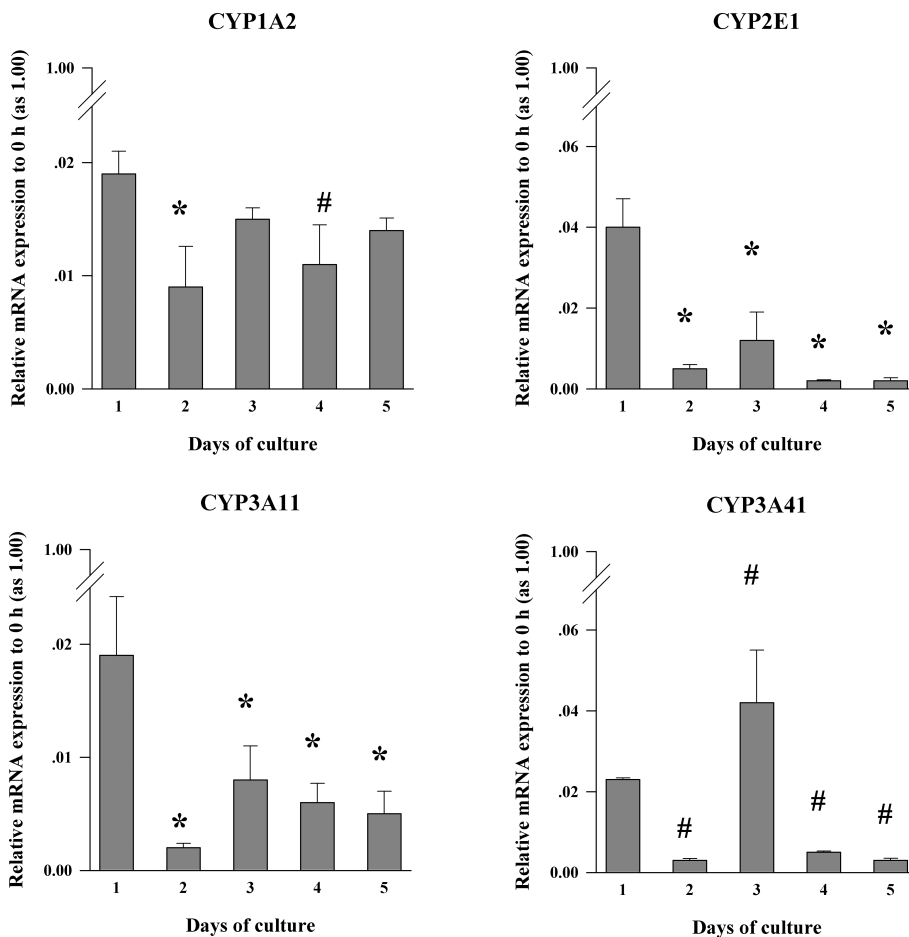


Fig. 3. Expression of mRNA of P450 Enzymes Involved in APAP Metabolism

Total RNA was prepared from mouse hepatocytes in spheroid cultures at the indicated times from days 1–5. Expression was evaluated by quantitative real-time RT-PCR. Values were normalized to that of GAPDH and calculated relative to that on the day of perfusion (0 hr), which was taken as 1.00. Each column represents the mean \pm S.D. ($n = 6$). Significance was calculated using ANOVA with the Student-Newmann-Keuls, post-hoc testing method between day 1 and other days. #, $p < 0.05$, *, $p < 0.01$.

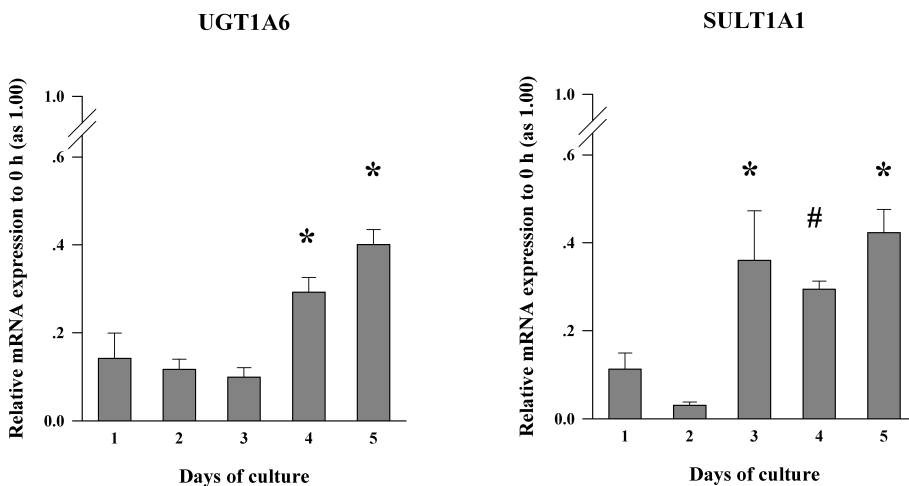


Fig. 4. Expression of mRNA of Phase II Enzymes Involved in APAP Metabolism

Total RNA was prepared from mouse hepatocytes in spheroid cultures at the indicated times from days 1–5. The expression of UGT1A6 and SULT1A1 mRNAs was determined by quantitative real-time RT-PCR. Values were normalized to that of GAPDH and calculated relative to that on the day of perfusion (0 hr), which was taken as 1.00. Each column represents the mean \pm S.D. ($n = 6$). Significance was calculated using ANOVA with the Student-Newmann-Keuls, post-hoc testing method between day 1 and other days. #, $p < 0.05$, *, $p < 0.01$.

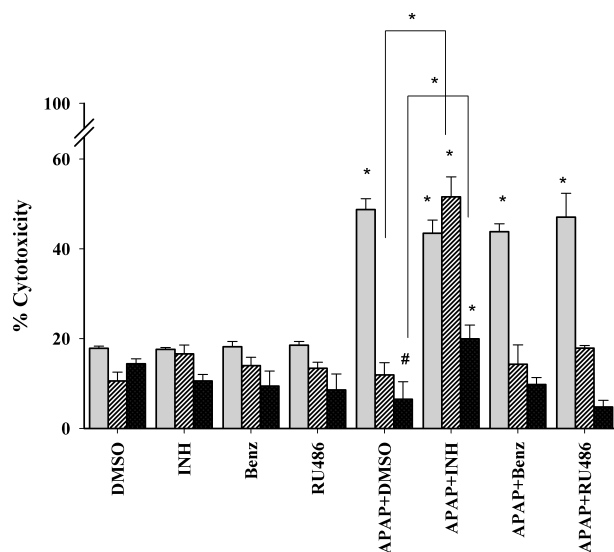


Fig. 5. Modification of APAP-induced Cytotoxicity by P450 Inducers

Mouse hepatocytes in spheroid cultures were treated with APAP and either P450 inducer. Treatment with 25-mM APAP and either 1-mM INH, 0.01-mM RU486, 13- μ M Benz, or vehicle (0.5% DMSO) was started from 24 hr (opened bars), 48 hr (dashed bars), or 72 hr (closed bars). After incubation for a further 24 hr, the level of activity of LDH released from hepatocytes into the medium was determined. LDH activity after the treatment of hepatocytes with 0.3% Tween80 was taken as 100% toxicity. Values are expressed as the mean \pm S.D. ($n = 9$). The significance was calculated using ANOVA with the Student-Newmann-Keuls, post-hoc testing method between hepatocytes treated with APAP and/or either inducer in the same period. #, $p < 0.05$, *, $p < 0.01$.

groups treated for 48–72 and 72–96 hr ($p < 0.01$), whereas RU486 and Benz had no significant effect. These observations confirmed the importance of CYP2E1 in the cytotoxicity of APAP^{12, 13} under the present culture conditions.

Effect of APAP on mRNA Expression of Enzymes Involved in Its Activation and Detoxification

Figure 6 shows that treatment with APAP for 24–48 hr at non-toxic concentrations significantly enhanced the expression of CYP2E1, 3A11, and 3A41 mRNAs. The observation of enhanced expression of CYP1A2 mRNA was delayed after treatment for 48–72 hr. However, treatment for 48–72 hr decreased the expression of CYP3A11 and 3A41 mRNAs. The mRNA expression of SULT1A1 was not increased, but rather decreased.

DISCUSSION

The cytotoxicity of APAP using a primary culture of mouse hepatocytes was evaluated. The re-

sults indicated that apparent cytotoxicity was observed at different concentrations of APAP, depending on treatment points after the start of cultivation, and reciprocal values between MTT and LDH assays at the same concentrations of APAP were not clearly observed. Furthermore, although the mRNA expression of metabolizing enzymes involved in the activation or detoxification of APAP varied markedly, their altered expression might in some aspects correlate with the altered cytotoxicity against APAP. As new evidence, APAP by itself at non-toxic concentrations was found to enhance the expression of some P450s, which are involved in APAP activation.

It was reported that at least 0.1 mM of APAP in plasma was required for antipyretic activity.²⁵ However, a patient suffering from APAP-induced liver toxicity reportedly showed a plasma concentration of 1–2 mM,²⁶ at which level significant cytotoxicity was observed in primary cultures of human and rat hepatocytes in previous studies.^{27, 28} Moreover, APAP at similar concentrations exerted clear toxicity within a few hours of incubation after the isolation of mouse hepatocytes.^{3, 29} In the present experiments, we determined the cytotoxicity after 24 hr, and if the treatment was started just after the cells attached to the dish, there was a clear cytotoxic effect at 3.125 mM of APAP (Fig. 1). However, we observed cytotoxicity at higher concentrations of APAP in other experimental protocols.

After treatment with 25-mM APAP, a relationship between cytotoxicities and treatment points was suggested; namely the higher the values, the longer the culture period. However, this entirely changed to become inverse after treatment at higher concentrations of APAP in the LDH assay. As shown in Figs. 1 and 2, the LDH values after treatment with 50-mM APAP may lead to the conclusion that the treatment vitalized cells, although the MTT values indicated strong cytotoxicity at this concentration. It is generally accepted that hepatocytes show increased resistance to APAP exposure after longer culture period, however, this applied the experiments after treatment with limited concentrations of APAP, if we refer to the results shown in Fig. 2A and 2B. Therefore, it is necessary to clarify the reason why the measured values using the LDH assay draw a convex curve against APAP concentrations.

Enzymes exerting the hepatotoxicity of APAP have been indicated to be CYP1A2, CYP2E1, and CYP3A in humans and rodents.^{9–11} These activi-

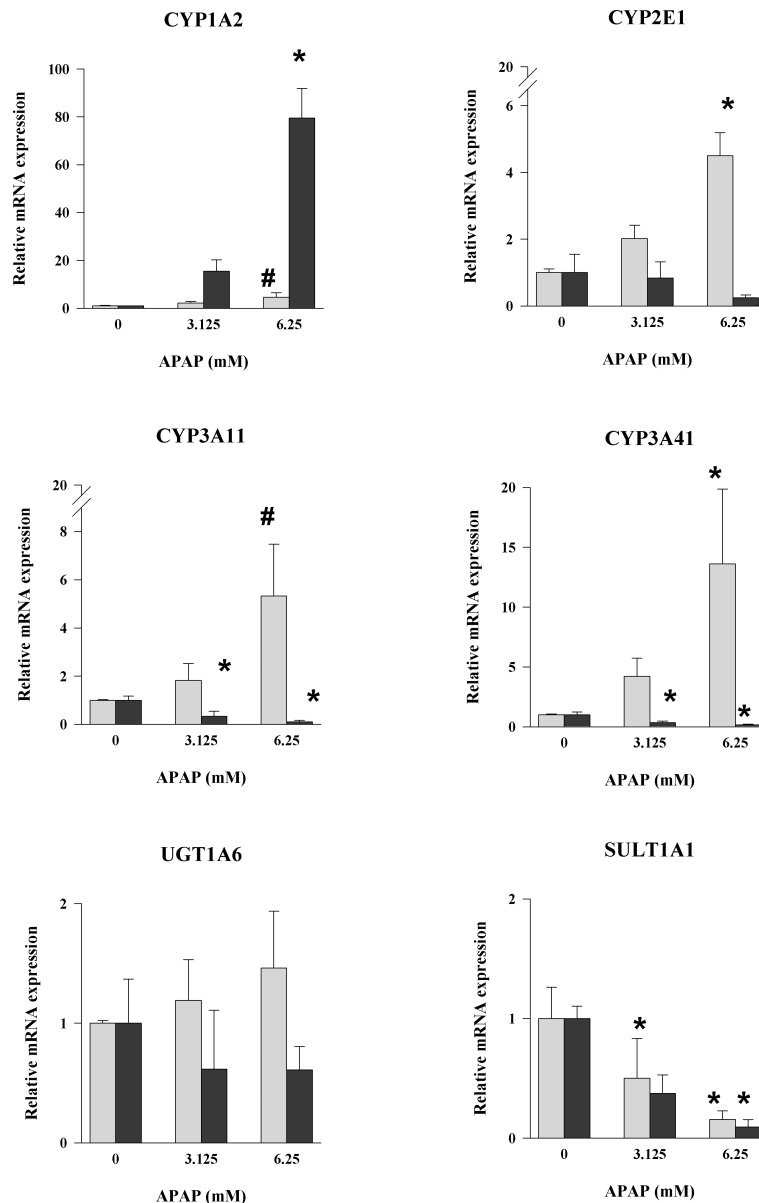


Fig. 6. Expression of mRNA of P450 and Detoxification Enzymes after Treatment with APAP

Mouse hepatocytes in spheroid cultures were treated with APAP at 0, 3.125, and 6.25 mM from 24 to 48 hr (opened bars) or from 48 to 72 hr (closed bars) of culture. The mRNA expression after treatment was evaluated by quantitative real-time RT-PCR. Values were normalized to that of GAPDH and calculated relative to that in untreated cells during the same culture period (taken as 1.00). The expression of GAPDH mRNA remained unchanged after either treatment. Each column represents the mean \pm S.D. ($n = 6$). Significance was calculated using ANOVA with the Student-Newmann-Keuls, post-hoc testing method between control and APAP-treated groups on the same day. #, $p < 0.05$, *, $p < 0.01$.

ties are usually markedly decreased after the start of a primary culture of hepatocytes.^{30,31} The present investigation quantitatively confirmed a rapid decline in the mRNA expression of these P450s. In contrast, the expression of the mRNA of UGT1A6 and SULT1A1 recovered to approximately 40% of that *in vivo* with an increasingly longer culture period. This evidence indicates a deficiency on the primary culture of hepatocytes, which does not precisely reflect the situation *in vivo*. The altered cytotoxicity of APAP during the observation period,

namely the toxicity found at higher concentrations later in the culture, in accordance with the reports on human, rat, and mouse hepatocytes in primary culture,³² might be in some sense due to a partial recovery of the expression of detoxification enzymes during that period. Furthermore, the observation of the elevated cytotoxicity of APAP after treatment with INH, conversely, suggests that the present culture system would be useful for the screening of chemopreventive compounds, which could modify the expression of enzymes involved in APAP

metabolism.

APAP at non-toxic concentrations significantly enhanced the expression of CYP1A2, CYP2E1, CYP3A11, and CYP3A41, although this enhancement was observed during a limited culture period (Fig. 6). There have been a few reports on the effects of APAP alone on hepatic P450 expression in cultured cells. For example, APAP increased mRNA levels of CYP1A2 and CYP3A11 2 hr after administration,³³⁾ with a return to basal levels by 7 hr³⁴⁾ in mice. Additionally, the expression of both proteins was increased, although not significantly.³³⁾ Therefore, the possibility that APAP by itself is able to alter P450 expression cannot be excluded.

In the present investigation, the concentrations of APAP at which the expression of some P450 isoforms was induced were revealed to be non-toxic for hepatocytes, and, indeed, were rather hepatoprotective; namely lower levels of LDH activity and higher MTT values were observed in treated cells than in the controls. This phenomenon is difficult to explain, but was consistently observed during the experiments. Considering the metabolism of APAP, it is difficult to identify a metabolite which would exert a hepatoprotective effect. In toxicology and radiology, hormesis after exposure to toxic compounds or radiation at very low levels is recognized as beneficial to the body, but the mechanism thereof has not yet been clearly resolved. Although there is no evidence that the present finding whereby APAP-treated cells showed lower LDH or higher MTT activity than control cells is analogous to hormesis, the primary culture system using mouse hepatocytes would be useful for investigating the mechanism of various actions of toxic compounds.

In conclusion, since the physiological condition of mouse hepatocytes in primary culture might change markedly during short-term culture for less than a week, the cytotoxic response was revealed to be diverse even after treatment with the same toxic compound employing an identical protocol. It is recommended for the determination of cytotoxicity to employ multiple assay methods, perform tests on multiple days, and apply the sample compound at a wide range of concentrations. Furthermore, it would be necessary to reveal the mechanism for the assay results indicating a more vitalized state after treatment with the compound at low concentrations.

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