Alteration of Acetaminophen Metabolism by Sulfate and Steroids in Primary Monolayer Hepatocyte Cultures of Rats and Mice

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Sulfotransferase (ST) is considered to be generally not induced by xenobiotics. However, it has been reported that steroids such as dexamethasone (DEX) and pregnenolone-16α-carbonitrile (PCN) are effective ST inducers in rats, and sulfation of xenobiotics is quite different in rats and mice. The present study is primarily aimed at determining the effect of sulfate and steroids on the metabolism of acetaminophen (AA) in vitro using monolayer cultured hepatocytes of Sprague-Dawley rats and ICR mice. Hepatocytes of rats and mice were incubated with inorganic sulfate (0.25, 0.5, 1.0, 2.0, 4.0 mM) and AA in SO4-depleted media. AA sulfation rates increased as the sulfate concentration was raised to 1.0 mM in rats, whereas the addition of inorganic sulfate to the media had a lesser effect in mice hepatocytes. After pretreatment with DEX (0.1, 1.0, 10, 100 μM) and PCN (0.1, 1.0, 10 μM) for 3 d, hepatocytes were incubated with AA in media containing 4.0 mM SO4. Pretreatment of the hepatocytes with DEX caused an increase in the glucuronidation and sulfation of AA by 2–3 folds in rats, but to a lesser extent in mice. PCN significantly enhanced the formation of AA-glucuronide and AA-sulfate in mice, but had a minimal effect on rat hepatocytes. In summary, sulfate and DEX markedly enhanced the formation of AA-sulfate in rats hepatocytes, and DEX and PCN increased the sulfation of AA in mice hepatocytes. These results partially support the claim that DEX and PCN are effective ST and uridine diphosphate-glucuronosyltransferase inducers in vivo.

Key words sulfation; sulfate; steroid; rat hepatocyte; mouse hepatocyte

Overdoses of acetaminophen (AA) are known to produce acute liver injury in both laboratory animals and humans.1–4 However, AA-induced hepatotoxicity varies considerably among species.5,6 Hamsters and mice are most sensitive to the hepatotoxic properties of AA, whereas rats, rabbits and guinea pigs are relatively resistant to AA-induced liver injury. Species differences in the relative ratio of toxification/detoxication metabolic pathways appear to be the major factor causing the species variation in hepatotoxicity.7–9 Species differences in AA metabolism were also demonstrated in vitro, as reflected by very low sulfate conjugation in hepatocytes isolated from mice as compared to those isolated from the rats.10

The conjugation of hydroxylated drugs such as AA with inorganic sulfate or glucuronic acid involves competitive metabolic pathways in the liver. Sulfation often predominates over glucuronidation at low drug concentrations, due to the higher affinity of the sulfotransferases (STs) for the substrate.11 However, the sulfation pathway is easily saturated and becomes relatively less important than glucuronidation as the concentration of substrate is increased. At present, the factors responsible for this capacity-limited sulfation are not fully understood. Some reports have shown that the depletion of plasma sulfate levels reduced the rates of sulfation and this decrease in sulfation could be prevented by an infusion of inorganic sulfate in vivo.12,13 The results from these studies have suggested that the availability of inorganic sulfate may limit sulfation in rats. However, the effect of exogenous sulfate on sulfation in mice has been little reported so far.

Another important factor influencing sulfate conjugation, STs, are generally known to not be induced by xenobiotics. However, in recent publications, pregnenolone-16α-carbonitrile (PCN) and dexamethasone (DEX) have been reported to be effective ST inducers.14,15 Although PCN has been shown to be an effective ST inducer, Madhu and Klaassen have found that PCN did not affect AA sulfation, as indicated by there being no alteration of biliary and urinary excretion of AA sulfate in hamsters.16 Also, in mice it has been demonstrated that neither PCN nor DEX affect the biliary excretion of AA sulfate.17 However, because previous investigators have described not the formation but the excretion of AA sulfate after treatments of ST inducers, the direct effects of PCN or DEX on the formation of sulfate conjugate remain to be characterized.

The purpose of the present study is to explore species differences in the formation of sulfate metabolite after pretreatment with ST inducers or the addition of exogenous sulfate using rat and mice hepatocytes.

MATERIALS AND METHODS

Materials AA, PCN, DEX and collagenase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Williams medium E, Hanks balanced salt solution, and fetal bovine serum were obtained from Gibco (Grand Island, NY, U.S.A.). All other chemicals were of the highest purity available.

Cell Culture Rats hepatocytes were isolated from 6-week-old male Sprague-Dawley rats (180–200 g, KFDA, Seoul, Korea) using a modification of Williams’ methods.18 Briefly, livers were perfused for 5 min with Hanks calcium-magnesium-free balanced salt solution (HBSS) and then for 15 min with HBSS containing 100 U/ml collagenase type IV. The tissue was gently disrupted and the cell suspension was filtered through a 60 μm nylon mesh. Hepatocytes were cultured in Williams’ medium supplemented with 10% fetal bovine serum, 10 mM HEPES, 20 mM l-glutamine, insulin (0.4 mg/l), and 1% penicillin-streptomycin. Following incubation in a moist atmosphere of 5% CO2 in air at 37℃ for 4 h, the medium and non-attached cells were removed.

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Mice hepatocytes were isolated from adult male ICR mice (33—35 g, KFDA, Seoul, Korea) using a method reported by Klauning. Mice livers were perfused for 4 min with 100 ml of HBSS and then for 12 min with HBSS containing 100 U/ml collagenase type I. Subsequent manipulation of hepatocytes following perfusion was the same as described above for the primary culture of rats hepatocytes, except the attachment time was only 2 h. Only cells from isolations in which the hepatocyte viability was greater than 90% were used in the experiments.

Addition of Inorganic Sulfate to Hepatocytes 24 h after the attachment, inorganic sulfate (Na₂SO₄) was added to the hepatocyte to give final sulfate ion concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 mM. Ten minutes after the addition of sulfate ion, AA was added in a final concentration of 1.0 mM for rat hepatocytes. In the case of mice hepatocytes, AA was added to cells at 250 μM, because AA was cytotoxic in mice hepatocytes above 250 μM. Aliquots of the media were removed after 24 h of incubation for the measurement of AA metabolites. To eliminate the possible effect of sulfate in media, the hepatocytes were incubated in pH 7.4 Krebs-Henseleit buffer in which magnesium sulfate was replaced by magnesium chloride.

Pretreatment of Hepatocytes with Steroids After 24 h of attachment, rat and mice hepatocytes were pretreated daily with PCN (0.1, 1.0, 10 μM) or DEX (0.1, 1.0, 10, 100 μM) in dimethyl sulfoxide (DMSO) for 3 consecutive days and the control was given the DMSO. The day after the final steroid pretreatment, hepatocytes were incubated with inorganic sulfate (4.0 mM) and AA for 24 h in sulfate-depleted Krebs-Henseleit buffer. AA was added to rat and mice hepatocytes at 1.0 mM and 250 μM, respectively.

Analysis of AA and Its Metabolites After 24 h of incubation with AA, media were diluted 2-fold with methanol and centrifuged before analysis. AA and its metabolites in media were determined by isocratic reverse-phase HPLC with UV absorbance detection as described by Howie et al. AA and its metabolites were eluted with water—methanol—acetic acid (900:80:8) at a flow rate of 1.5 ml/min and monitored by absorbance at 254 nm. Quantification of AA and its metabolites was based on an integrated absorbance peak area. The concentrations of AA metabolites were calculated from AA standard curves as their molar extinction coefficients are the same that of AA. Protein concentrations were determined by the method of Lowry et al. with bovine serum albumin as a standard.

Statistics One way analysis of variance was used to assess the statistical significance of changes in all indices, followed by Duncan’s multiple range test with p<0.05 set at the minimum level of significance.

RESULTS

Effect of Sulfate Addition on the Formation of AA Metabolites The formation of AA glucuronide (AA-Glu) and AA sulfate (AA-SO₄) in rat or mice hepatocytes at various inorganic sulfate concentrations are shown in Figs. 1 and 2, respectively. The glutathione conjugate of AA was not detected in this condition. In rat hepatocytes, a large amount of AA-SO₄ was formed as the sodium sulfate concentration increased, and increased to 5-fold compared to the control at 4.0 mM inorganic sulfate. The rates of AA-SO₄ formation increased as the sulfate ion concentration was raised to 1.0 mM; however, raising the sulfate ion concentration above 1.0 mM did not produce further increases. Glucuronidation of AA was significantly suppressed at higher inorganic sulfate concentrations.

In mice hepatocytes, the formation of AA-SO₄ was enhanced about 1.5-fold by the addition of inorganic sulfate, but the increase in AA-SO₄ was not statistically significant. Compared to hepatocytes from rats, mice hepatocytes formed much fewer conjugates of AA-SO₄. The formation of AA-Glu in mice hepatocytes was not affected by sulfate addition.

Effect of DEX Pretreatment on the Formation of AA Metabolites After pretreatment with DEX, glucuronidation and sulfation of AA were significantly increased in rat hepatocytes (Fig. 3). The formation of AA-SO₄ in DEX-treated hepatocytes was approximately 2 times greater than that in the control. The sulfate conjugate of AA reached a maximum
Fig. 3. Effect of DEX on the Formation of AA Metabolites in Rat Hepatocytes

After pretreatment with DEX (0.1, 1.0, 10, 100 μM) for 3 consecutive days, hepatocytes were incubated with Na₂SO₄ (4.0 mM) and AA (1.0 mM) for 24 h in SO₄²⁻-depleted medium. Values are expressed as mean±S.D. of three separate experiments. An asterisk denotes a significant difference from the control at p<0.05.

Fig. 4. Effect of DEX on the Formation of AA Metabolites in Mice Hepatocytes

After pretreatment with DEX (0.1, 1.0, 10, 100 μM) for 3 consecutive days, hepatocytes were incubated with Na₂SO₄ (4.0 mM) and AA (250 μM) for 24 h in SO₄²⁻-depleted medium. Values are expressed as mean±S.D. of three separate experiments. An asterisk denotes a significant difference from the control at p<0.05.

Fig. 5. Effect of PCN on the Formation of AA Metabolites in Rat Hepatocytes

After pretreatment with PCN (0.1, 1.0, 10 μM) for 3 consecutive days, hepatocytes were incubated with Na₂SO₄ (4.0 mM) and AA (1.0 mM) for 24 h in SO₄²⁻-depleted medium. Values are expressed as mean±S.D. of three separate experiments. An asterisk denotes a significant difference from the control at p<0.05.

Fig. 6. Effect of PCN on the Formation of AA Metabolites in Mice Hepatocytes

After pretreatment with PCN (0.1, 1.0, 10 μM) for 3 consecutive days, hepatocytes were incubated with Na₂SO₄ (4.0 mM) and AA (250 μM) for 24 h in SO₄²⁻-depleted medium. Values are expressed as mean±S.D. of three separate experiments. An asterisk denotes a significant difference from the control at p<0.05.

duced at 1.0 μM of DEX and maintained a plateau up to 100 μM of DEX. The conjugation of AA-Glu was also markedly enhanced 2-fold with DEX pretreatment in a dose-dependent manner from 0.1 to 10 μM, as shown in Fig. 3. Pretreatment of rat hepatocytes with 10 μM of DEX caused a peak of AA-Glu formation, whereas a higher concentration (100 μM) of DEX had a lesser stimulatory effect.

As in rat hepatocytes, the sulfate conjugates of AA in mice hepatocytes were significantly increased, by 1.5—2-fold at 0.1 and 1.0 μM of DEX-treated groups. Although DEX also tended to enhance the formation of AA-Glu, only 100 μM of DEX caused a statistically significant increase.

**Effect of PCN Pretreatment on the Formation of AA Metabolites** Figures 5 and 6 illustrate the effect of PCN pretreatment on the metabolism of AA in rat and mice hepatocytes. In rat hepatocytes, PCN pretreatment had no statistically significant effect on either AA-sulfation or AA-glu-}

curonidation. In contrast, the formation of AA-SO₄ was significantly enhanced in all of the PCN-treated mice hepatocytes, exhibiting a 2.7-fold increase at 10 μM, compared with the control. AA-Glu was increased following PCN pretreatment, but the increase was not statistically significant.

**DISCUSSION**

Several studies have explored the relationships between sulfate ion concentrations and rates of sulfation in rat hepatocytes. However, no information is available on the relationships between sulfate concentration or ST activity and the capacity for sulfation in the primary monolayer hepatocyte of rats or mice. The present study was undertaken to determine whether the sulfate concentrations are related to the rates of sulfation in rat and mice hepatocytes. The results from rat hepatocytes demonstrate that at sulfate ion concentrations below 1.0 mM, the rates of AA sulfation were highly
dependent on the availability of sulfate ions. This finding is consistent with results conducted with a suspension of rats hepatocytes by Sweeny and Reineke and by Koike et al.\textsuperscript{23,25} It is important to note that the normal serum concentration of inorganic sulfate in the rat is about 0.9 mm,\textsuperscript{23} so that the conjugation of drugs with sulfate is likely to be limited by 3'-phosphoadenosine 5'-phosphosulfate (PAPS) availability under normal physiological conditions. Because PAPS levels are strongly dependent on sulfate ion concentrations,\textsuperscript{23} a reduction in plasma sulfate below physiological levels, as seen after high doses of AA,\textsuperscript{20} can be expected to result in lower rates of sulfation. On the other hand, increasing plasma sulfate ion concentrations will result in increased hepatic PAPS levels, but may have little effect on the rates of AA sulfation because the activity of the ST appears to be a limiting factor above 1.0 mm of sulfate ion. The effect of sodium sulfate on the AA glucuronidation was opposite in rat hepatocytes. At the AA dosage used in this study, a large proportion of the drug entering the cells is consumed preferentially by ST in the cytosol due to a higher affinity of ST to the substrate, and the effective AA concentration for microsomal uridine diphosphate-glucuronosyltransferase (UDP-GT) became low. Consequently, the AA glucuronidation was significantly suppressed by the addition of inorganic sulfate. There were two major differences between rat and mice hepatocytes concerning the sulfation of AA. First, sulfate conjugates in mice hepatocytes were only about 10 percent of that in rat hepatocytes. Second, sulfation was not affected by the addition of sulfate in mice hepatocytes. This result suggests that the activities of ST rather than sulfate concentration may play an important role in the sulfate conjugation reactions of mice hepatocytes.

In the present study, pretreatment with DEX for 3 d significantly increased the sulfation of AA, by 2-fold in both rat and mice hepatocytes, implying that DEX can induce ST in vitro. This result also supports the claim that sulfation capacity can be elevated by the induction of ST. DEX pretreatment markedly enhanced AA glucuronidation in rat hepatocytes, but failed to increase the formation of AA-Glu in mice hepatocytes. There are several reports that pretreatment with DEX markedly induced UDP-GT in rats,\textsuperscript{27–32} but had no effect in mice.\textsuperscript{37} Therefore, the enhancement of AA-Glu formation in DEX-treated rat hepatocytes could be due to the induction of UDP-GT, and the lack of effect of DEX on glucuronidation in mice hepatocytes could be explained by species differences in the induction of UDP-GT.

PCN significantly increased the AA-SO\textsubscript{4} in mice hepatocytes, supporting the claim that PCN induces the ST. In contrast to the mice hepatocytes, PCN treatment had no effect on sulfation or glucuronidation of AA in rat hepatocytes. This finding is not in agreement with the results of previous studies in which activities of ST and UDP-GT were increased in PCN-pretreated rats in vivo,\textsuperscript{16,27–32} Further studies will be needed to determine if a difference in the induction of enzymes does exist between in vivo and in vitro.

In summary, the results of the present study indicate there are marked differences in the formation of AA metabolites and in the properties of ST and UDP-GT induction in rat and mice hepatocytes. These results also support in part that DEX and PCN are effective inducers of ST and UDP-GT in vivo.

REFERENCES