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EFFECT OF UDP-GLUCURONYLTRANSFERASE INDUCTION ON ZEARALENONE METABOLISM

Camelia Bara, L. Bara , V. Bara , E. Jude , M. Osvat*

* University of Oradea, Faculty of Environmental Protection, 26 Gen. Magheru St., 410048 Oradea; Romania, e-mail: cameliabara@yahoo.com

Abstract

Experiments were conducted to determine the UDP-glucuronyltransferase (UDP-GT) isoenzyme which catalyzes zearalenone (Z) conjugation, and the effect of increased enzyme activity on Z metabolism. In competitive enzyme assays, the activity of rat liver UDP-GT towards Z was inhibited by 1-naphthol (NA), a GT_1 substrate, and 4-hydroxybiphenyl (HB), a GT_2 substrate. When enzyme activity was induced with either 3-methylcholanthrene (MC), a GT_1 inducer, or Phenobarbital (PB), a GT_2 inducer, increased UDP-GT activity towards Z, NA and HB was observed. UDP-GT induction by PB increased urinary excretion of conjugated α-zearalenol. These results indicate that UDP-GT isoenzymes have overlapping substrate specificities, and that Z detoxification may be enhanced by UDP-GT enzyme induction, resulting in increased urinary excretion of conjugated α-zearalenol.

Key words: UDP-glucuronyltransferase, zearalenone, mycotoxin

INTRODUCTION

Zearalenone (Z) is naturally occurring mycotoxin synthesized by *Fusarium* mold species endemic to temperate climates. Z and its metabolites α- and β-zearalenol belong to a rare class of natural products, the β-resorcylic acid-lactones that mimic estrogenic actions in animals by binding to estrogen receptors. Dietary Z acts systematically producing an "estrus syndrome", and toxicity occurs when contaminated food/feed is ingested by man, livestock or experimental animals. The effect of Z toxicosis has been recently reviewed.

Z is a hydrophobic xenobiotic which is metabolized by two different reactions, a reduction to an alcohol and a conjugation with glucuronic acid. Reduction and conjugation of Z increase its solubility and may promote its excretion in the urine. Conjugation of Z increases its molecular weight which may act to increase biliary excretion. It has been suggested that nutritional regimen affects Z metabolism, excretion and toxicity: i.e. diet can affect the total amount of Z and its metabolites excreted as glucoronide conjugates in the urine, which acts to limits enterohepatic recirculation and reduces the toxic expression of this xenobiotic.

UDP-glucuronyltransferase activity (UDP-GT) toward various xenobiotics is mediated by a family of isoenzymes. The model of differential induction, based upon the selective induction of enzyme activity

towards specific substrates by 3-methylcholanthrene (3-MC) or phenobarbital (PB), has been used to classify these isoenzymes. For example, UDP-GT enzyme activity towards GT_1 substrates is induced by 3-MC and activity towards GT_2 substrates is induced by PB.

Dietary Z has been shown to induce UDP-GT activity; however, the specific isoenzyme responsible for Z conjugation has not been identified.

The purpose of this study was to determine the UDP-GT isoenzyme which catalyzes Z conjugation, and examine the effect of this conjugation on Z metabolism and excretion.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats were used when required for the three separate experimental studies. All animals were housed in individual cages. The room temperature was maintained at 21±1 C with a relative humidity of 50% and a 14-10h light/dark cycle. Rats were fed a commercial chow diet throughout the study. Animals were randomly assigned to the various experimental groups.

Competitive enzyme assay

The rats were killed by decapitation; their livers were excised, chilled on ice and immediately assayed for UDP-GT enzyme activity. About 2g of fresh liver was homogenized for 1 min at 0˚ C in 15 ml of 0.154 M KCl. The homogenate was centrifuged at 2000 x g for 20 min at 4 C and the supernatant was extracted and kept on ice. The protein content was determined according to Lowry *et al*., and the protein concentration of the supernatant was adjusted to 0.7 – 0.9 mg/ml by dilution with 0.1 % Triton X-100 (W/V) in 0.154 M KCl. The spectrophotometric method of Mulder and Van Doorn was used to determine the UDP-GT enzyme activity. Validation of this method was carried out, and a minor modification to the method adopted resulting in the final concentration of the acceptor substrates of 0.15mM. Six sets of acceptor substrates were used to initiate the reaction in this assay: 1.) 1-naphtol (NA, GT1-specific substrate), 2.) 4-hydroxybiphenyl (HB, GT_2-specific substrate), 3.) Z, 4.) NA and Z and NA and HB. Assays were performed in triplicate. The activity of UDP-GT was measured as the change in optical density at 340 nm and was expressed as nmol of NADH oxidized per h per mg protein.

The spectrophotometric assays of UDP-GT enzyme activity were confirmed by HPLC analysis. Modifications were made to the method of Mulder and Van Doorn for the direct determination of glucuronidation. The homogenate was prepared as previously described, and the protein

concentration of the supernatant was adjusted to 2 mg/ml by dilution with 0.1 % Triton X-100 (W/V) in 0.154 M KCl. The assay medium contained 33.5 mg of UDP glucuronic acid, 7.62 mg of D-saccharic acid, 1,4-lactone, and 1ml of $MgCl_2$ solution (33.8 mg $MgCl_2$ per ml H_2O) and was diluted to a final volume of 25 ml in a 0.105 M Tris buffer (pH 7.3). Six hundred µl of assay medium was pipetted into a 5 ml test tube and placed in a 37 C shaking water bath. After 5 min, 30 µl of 4mM acceptor substrate and 200 µl of homogenate were added to the reaction mixture, which was then vortexed and returned to the shaking water bath. After 10, 20 and 50 min, 100 µl aliquots were removed from the test tube, transferred to an 8 ml screw top test tube containing 2 ml of 10% 2-propanol in ether and mixed thoroughly. To extract the sample and prepare it for HPLC analysis, 2 ml of distilled water was added, vortexed thoroughly, and centrifuged for 2 min at 1500xg. The ether layer was transferred to an 8 ml screw-top test tube. The aqueous layer was washed with 2 ml of 10% 2-propanol in ether; the extracted ether layers were combined and evaporated to dryness under nitrogen. Samples were taken up in a known amount of solvent and quantified by HPLC as previously described. Retention time for NA, HB and Z was 5.1, 6.1 and 8.5 min, respectively. Assays were performed in duplicate and the activity of UDP-GT was expressed as nmol acceptor conjugated per h per mg protein.

Enzyme induction study

The rats received intraperitoneal treatment with PB, 3-MC, or the appropriate control vehicle, as follows: PB an initial dose of 30 mg/kg body wt., in saline, was given on day 1, and 60 mg/kg wt. on days 2 and 3; controls received saline on all 3 days. 3-MC: a dose of 30 mg/kg body wt. in corn oil was given for 3 days; controls received corn oil. Animals were killed 4 days after the initial treatment; their livers were excised and immediately assayed for UDP-GT enzyme activity.

Metabolism study

Animals were pretreated with PB i.p. at the following doses: 30 mg/kg body weight on day 1 and 60 mg/kg body weight on days 2 and 3, while controls received saline on all 3 days. On day 4, rats were dosed with Z, 5 mg/kg body weight, by stomach intubation. Animals were transferred to polycarbonate metabolic cages, urine was collected every 24 h for 4 days and was kept frozen at -20 C until analyzed. Z and its metabolites were extracted and analyzed by HPLC.

Statistical analysis

For the competitive inhibition study, means were calculated on the treatment groups and compared using a paired t-test. For the induction and the metabolism studies, significant differences were estimated by Student's t-test.

RESULTS AND DISCUSSION

UDP-GT enzyme activity towards Z was significantly affected by the presence of either NA or HB in the reaction medium. The theoretical sum of UDP-GT enzyme activity towards Z and NA, or Z and HB was significantly more than the observed enzyme activity when the two substrates were present in the reaction medium. If two acceptors are conjugated by different UDP-GT isoenzymes, the enzyme activity observed when these two acceptor substrates are present in the assay medium should be additive. However, when two substrates which are conjugated by the same GT form are present in the medium, the observed enzyme activity will not be additive. NA has been reported to be a GT_1 substrate, while HB has been shown to be a specific GT_2 substrate. Since both substrates affect UDP-GT enzyme activity towards Z, it appears that Z is conjugated by both UDP-GT forms. However, it must be noted that NA and HB, the GT_1 and GT_2 acceptor substrates, enzyme activities were not additive. This suggests that both substrates are conjugated by the UDP-GT enzyme forms that have overlapping substrate specificities.

The confirmatory analysis, a direct measurement of substrate conjugation by HPLC, supported these observations, with both NA and HB affecting UDP-GT enzyme activity towards Z. Conjugation of Z was 251, 181 and 160 nmol h^{-1} mg^{-1} protein when alone with NA and with HB, respectively, in the reaction medium. It should be noted that HB conjugation was 244 and 217 nmol h^{-1} mg^{-1} protein when alone and with NA in the reaction medium, a significant decrease in HB conjugation. This result suggests that there is a competition between these substrates for active sites on the UDP-GT enzyme.

Glucuronidation reactions have been reported to be differentially inducible by two prototype inducing agents, MC and PB. NA has been reported as a typical GT_1 acceptor substrate and GT_1 enzyme activity is preferentially induced by MC, while HB is thought to be a GT_2 substrate and GT_2 enzyme activity is preferentially induced by PB. In the enzyme induction study, pretreatment with MC resulted in significantly greater conjugation of NA, HB and Z, an increase of 290, 43 and 53%, respectively. Pretreatment with PB resulted in increases in UDP-GT enzyme activity towards NA, HB and Z of 41, 52 and 40% respectively. This observation

was confirmed by a direct measurement of substrate conjugation with both MC and PB affecting UDP-GT enzyme activity.

These observations are in general agreement with results reported in the literature. For example, Koster *et al.* observed that MC pretreatment resulted in a 330% increase in NA conjugation and a 50% increase in HB glucuronidation.

Pretreatment with PB increased UDP-GT enzyme activity towards NA and HB by 50 and 230%, respectively: i.e. while pretreatment with an inducing agent increased UDP-GT activity towards specific classes of substrates, there were overlapping substrate specificities and cross-induction is observed.

The pretreatment of rats with either MC or PB resulted in similar increases in hepatic UDP-GT enzyme activity towards Z. Since both inducers affect UDP-GT enzyme activity towards Z, it appears that Z is conjugated by both UDP-GT isoenzyme forms. Olsen *et al*. reported that there was no correlation between the rates of Z and NA glucuronide conjugation in sow intestinal mucosa, and suggested that these substrates are metabolized by different UDP-GT isoenzymes. However, our results, from both the competitive enzyme assays and the induction studies, indicate that Z is an acceptable substrate for both UDP-GT forms. Overlapping substrate specificity within the UDP-GT isoenzymes has been previously reported.

Conjugation is a deactivation reaction; the newly formed conjugates have decreased membrane permeability and increased water solubility, thereby facilitating renal clearance of the substrate. The molecular weight of a compound affects its metabolism and excretion and there appears to be a urinary threshold for excretion of 350±50 in the rat. Since the molecular weight of Z is 318 and the molecular weight of glucuronic acid is 194, conjugation could theoretically promote billiary excretion. We have suggested that the conjugation of Z and its metabolites acts to reduce the toxic expression of Z by increasing urinary excretion, thus limiting enterohepatic recirculation. The induction of UDP-GT activity by PB pretreatment in the metabolism study resulted in a significant increase in the urinary excretion of conjugated α-zearalenol from 5.4 to 9.9 μg. Since α-zearalenol is 10 times more potent as an estrogen than Z, this observation is biologically significant. An increase in free Z excretion from 143 to 153.9 μg was observed. While the amount of conjugated Z increased 22% from 38.7 to 47.4 μg, the response was too variable to demonstrate significance. These observations are consistent with our previous report that increased in UDP-GT activity result in increases in both free and bound metabolites in the urine.

These results indicate that UDP-GT isoenzymes have overlapping substrate specificities, and that Z detoxification may be enhanced by UDP-

GT enzyme induction, resulting in increased urinary excretion of Z and its metabolites.

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