Biochemical Pharmacology, Vol. 43, No. 4, pp. 902–904, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

Stereospecific glutathione conjugation of (*R*)- and (*S*)-2-bromoisovalerylurea in freshly isolated rat kidney proximal tubular cells

(*Received* 23 *August* 1991; *accepted* 4 *November* 1991)

Abstract—The glutathione conjugation of 2-bromoisovalerylurea (BIU) was studied in isolated proximal tubular kidney cells of the rat. Racemic (*R*,*S*)-BIU was incubated with the cell suspension, and the incubation medium was analysed for the diastereomeric glutathione (GSH) conjugates, cysteine conjugates and mercapturates that can be formed from (*R*)- and (*S*)-BIU. Only the mercapturate formed from (*R*)-BIU was found, as well as its cysteine precursor. No GSH conjugates were detected. These results indicate that these cells conjugate only the (*R*)-BIU enantiomer, and that the GSH conjugate is immediately further metabolized to its cysteine conjugate and mercapturate.

The kidneys are an extrahepatic site of drug metabolism. Although they represent less than 1% of the total body mass in mammals, they receive approximately 25% of the cardiac output. Therefore, they are extensively exposed to xenobiotics in the blood, especially the highly perfused proximal tubular cells (PTC*) in the cortex. Drug metabolism is quite active in the kidney [1–3]; PTC in particular contain many drug metabolizing enzymes at high activity.

The kidneys play a major role in the metabolism of GSH and GSH conjugates; all the enzymes required for the synthesis as well as for the degradation of GSH and GSH conjugates are present. Renal GSH and GSH-*S*-transferases (GSTs) are located mainly in the PTC [4]. The enzyme responsible for the rapid degradation of GSH or its conjugates is γ-GT. The kidney has the highest γ-GT activity of all the organs, mainly located in the brush border of the S3-segment [5]. After degradation by γ-GT a GSH conjugate is further hydrolysed by peptidases to the corresponding cysteine-*S*-conjugate; the latter can be N-acetylated to form the mercapturic acid, which may be actively excreted by the kidneys [6].

Whereas many studies have been performed with purified enzyme preparations or subcellular fractions, little information is available on GSH conjugation in intact kidney PTC. The aim of this work was to study GSH conjugation in these cells, using BIU as substrate. Since BIU has a chiral center, two diastereomeric GSH conjugates can be formed after conjugation of its (*R*) and (*S*) enantiomers. These can be further metabolized to two diastereomeric mercapturates that are excreted in the urine. Because GSH conjugation involves complete inversion of conformation, (*R*)-BIU gives rise to the (*S*)-conjugate, (*S*)-IU-S-G, and the (*S*)-mercapturate, (*S*)-IU-S-MA. Similarly, (*S*)-BIU results in (*R*)-IU-S-G and (*R*)-IU-MA. *In vivo* experiments in anesthetized rats and *in vitro* experiments with liver perfusions and isolated hepatocytes consistently showed a large difference in the formation rates of the diastereomeric BIU metabolites: the conjugation rate of (*R*)-BIU was much faster than that of (*S*)-BIU [7–9]. The stereoselectivity of GSH conjugation in isolated PTC was also studied.

Materials and Methods

Chemicals. The materials used to isolate PTC from rat kidney are the same as described in Ref. 10. BIU (*N*-aminocarbonyl-2-bromo-3-methylbutamide) was purchased from Brocacef B.V. (Maarsen, The Netherlands). All solvents and reagents were of analytical grade [11]. Throughout the study deionized water was used (Milli Q Purification System, Millipore, Bedford, MA, U.S.A.). The cysteine conjugates, GSH conjugates and mercapturates of BIU were synthesized as described in Ref. 11.

Animals. Male Wistar rats (200–250 g body wt) of the Sylvius Laboratories, University of Leiden, were used. The rats were housed in Macrolon cages on standard hard wood bedding. The animals had free access to tap water and standard lab chow (SRM-A, Hope Farms, Woerden, The Netherlands). Lights were on from 6 a.m. to 6 p.m.

Cell isolation. The isolation procedure of PTC is described in Ref. 10. Briefly, rats were anesthetized and the abdomen was opened. The coelic and upper mesenteric arteries and the lower vena cava were ligated, the aorta was subsequently cannulated and the renal veins were opened. The kidneys were removed and subsequently perfused with calcium-free Hanks'–HEPES buffer containing 0.5 mM ethyleneglycolbis(aminoethylether)tetraacetate, and Hanks'–HEPES buffer containing 4 mM $CaCl_2$ and 0.08% (w/v) collagenase. After 18 min the cortical tissue was gently dispersed in ice-cold Hanks'–HEPES buffer supplemented with 2.5% (w/v) BSA and filtered through a nylon gauze (pore size 60 μm). The cell suspension was washed three times with ice-cold incubation medium and viable PTC were separated from other cell types, cell debris and tubular fragments by isopycnic centrifugation on a discontinuous Nycodenz gradient.

The cells were incubated in Hanks' buffer (pH 7.4) supplemented with 25 mM HEPES and 2.5% (w/v) BSA, at 37° under 95% O_2/5% CO_2 on a rotary shaker (160 cycles/min). Cells were preincubated for 30 min with 1 mM L-methionine to stimulate GSH synthesis [9].

Racemic (*R*,*S*)-BIU was dissolved in Hanks'–HEPES buffer supplemented with 2.5% (w/v) BSA while heating with hot water, and the solution was vortexed and sonicated until total dissolution.

After the preincubation, 200 μL of the racemic BIU solution was added to 1.8 mL of cell suspension; the final concentration of BIU was 0.25 mM. To samples of 100 μL (cells plus medium), 200 μL of ice-cold methanol was added after various incubation times to stop the incubation; the mixture was placed on ice. After centrifugation, the deproteinized samples were subjected to HPLC analysis.

Analytical procedures. The chromatographic apparatus consisted of a Spectroflow model 400 pump (Kratos Analytical, Ramsey, NJ, U.S.A.) equipped with a membrane-type liquid pulse damper, model 12-01125, a WISP autoinjector (Millipore) and a Spherisorb ODS2 column (5-μm particles, 3.9 × 150 mm). Detection was based on the on-line generation of bromine, subsequent reaction of bromine with eluted thioethers and electro-

* Abbreviations: PTC, proximal tubular cells; GSH, glutathione; γ-GT, γ-glutamyltranspeptidase; BIU, 2-bromoisovalerylurea; BSA, bovine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; GST, GSH-*S*-transferase.

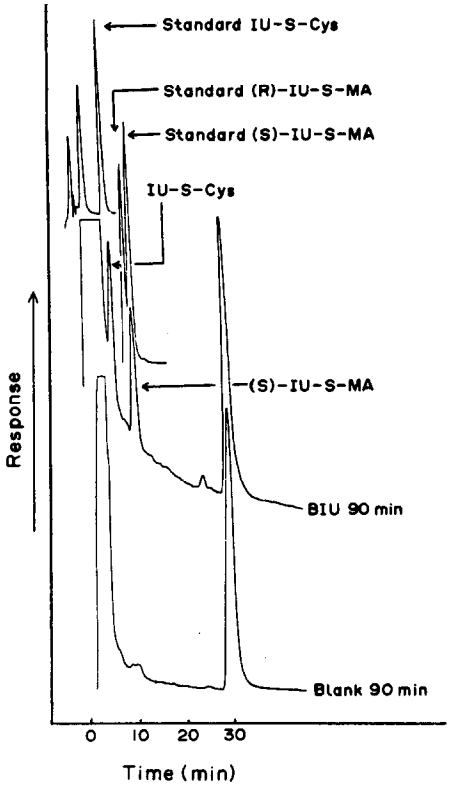

Fig. 1. HPLC chromatograms of BIU metabolites formed by isolated PTC. Samples of incubation media without (blank) or with racemic (*R*,*S*)-BIU obtained after 90 min incubation were analysed by HPLC and electrochemical detection. The position of the injected standards is also indicated.

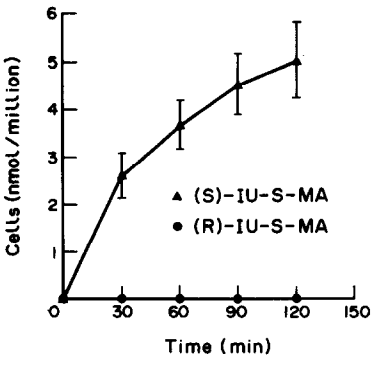

Fig. 2. Time-course of the formation of mercapturates from racemic BIU by PTC.

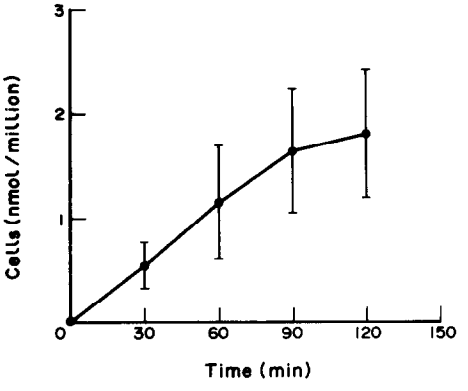

Fig. 3. Time-course of the formation of the cysteine conjugate from racemic BIU by PTC.

chemical measurement of the remaining amount of bromine [11]. Under appropriate conditions the Cys-conjugate and the diastereomers of IU-S-MA could be separated in one run with an isocratic chromatographic system using decanesulfonate as ion-pair forming agent and a flow rate of 0.45 mL/min. The eluent consisted of an aqueous buffer [0.1 M sodium nitrate, 0.01 M potassium bromide and 0.01 M citric acid buffer (pH 2.5)] and methanol in the presence of sodium decanesulfonate [11].

Results

Following incubation of freshly isolated kidney PTC with racemic BIU (0.25 mM) samples were analysed by HPLC in order to detect the formation of GSH conjugates or their breakdown products. Only two peaks were found with retention times of approximately 7 and 11 min (Fig. 1) which were identified as the cysteine conjugate and (*S*)-IU-MA formed from (*R*)-BIU, by co-chromatography of the chemically synthesized standards [11]. No BIU glutathione conjugates and only one of the two possible BIU mercapturates, (*S*)-IU-MA, were present.

The time-course showed a rapid formation of both IU-Cys and (*S*)-IU-MA in the first 30 min of incubation and a moderate increase afterwards. The accumulation of (*S*)-IU-MA was higher than that of IU-Cys (Figs 2 and 3).

Discussion

PTC formed only two metabolites from racemic BIU: IU-Cys and (*S*)-IU-MA. We assume that the IU-Cys represents the (*S*)-IU-Cys diastereomer since the only mercapture detected was (*S*)-IU-MA; our HPLC system does not separate the IU-Cys diastereomers. These results indicate that BIU conjugation with GSH was stereospecific: only one of the two enantiomers, (*R*)- BIU, was converted.

The GSH conjugate itself could not be detected. This must have been due to a very efficient breakdown of the GSH conjugate to the cysteine conjugate. A similar finding was reported by Moldéus *et al.* [12] for paracetamol in their kidney cell suspension.

When similar experiments were carried out with isolated rat hepatocytes, only the two diastereomeric BIU glutathione conjugates were formed but no Cys conjugates or mercapturates were detected [7] indicating that γ-GT is not very active in this system. The isolated hepatocytes showed only stereoselectivity of BIU conjugation: an approximately 5-fold higher rate of GSH conjugation of the (*R*)-enantiomer as compared to that of the (*S*)-BIU. In contrast, renal GSTs are stereospecific with respect to (*R*)-BIU because only the metabolites of this enantiomer could be detected.

Isoenzymes of the GST μ multigene family (namely subunits 3 and 4) preferentially conjugated (*R*)-BIU, while α multigene family isoenzymes (subunit 2, but also the less active subunit 1) favored (*S*)-BIU [13]. Further studies with another chiral substrate, 2-bromoisovaleric acid, revealed that subunits 1 and 2 were stereospecific for the (*S*) enantiomer. Recently, we found that isoenzyme 12-12 was also stereospecific [Polhuijs *et al.*, unpublished data]. Further studies are needed to identify the GST isoenzymes in kidney cells involved in BIU conjugation.

Acknowledgements—Monica Mestre I Viver was supported by a grant from the Ministerio de Asuntos Exteriores, Dirección General de Relaciones Culturales y Cientificas, Madrid, Spain.

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Biochemical Pharmacology, Vol. 43, No. 4, pp. 904–907, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

Kinetic and substrate binding characterization of hepatic mixed function oxidase system in monkeys with primaquine and (N^1-3-acetyl-4-5-dihydro-2-furanyl)-N^4-(methoxy-8-quinolinyl) 1,4-peptane-diamine

(*Received* 20 *June* 1991; *accepted* 1 *November* 1991)

Recently, our Institute developed a schizontocide named CDRI compound 80/53 [(N^1-3-acetyl-4-5-dihydro-2-furanyl)-N^4-(methoxy-8-quinolinyl) 1,4-peptane-diamine] (80/53*) (Fig. 1). This is an enamine analogue of the well known tissue schizontocidal PQ. Although PQ is the first

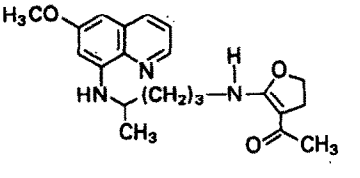

Fig. 1. Compound 80/53.

* Abbreviations: 80/53, CDRI Compound 80/53, N^1-3-acetyl-4,5-dihydro-2-furanyl)-N^4-(methoxy-8-quinolinyl)1,4-peptane-diamine; PQ, primaquine; AH, aniline hydroxylase; AND, aminopyrine-*N*-demethylase.