Biochemical Pharmacology, Vol. 43, No. 7, pp. 1649–1653, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

SHORT COMMUNICATION

Optimal detergent activation of rat liver microsomal UDP-glucuronosyl transferases toward morphine and 1-naphthol: contribution to induction and latency studies

(*Received* 2 *September* 1991; *accepted* 7 *December* 1991)

Abstract—The detergent-activation profiles of UDP-glucuronosyl transferases (UGTs, EC 2.4.1.17) toward 1-naphthol and toward morphine have been determined: three non-ionic detergents, Triton X-100, Brij 58 and Lubrol Px and one zwitterion detergent, 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonic acid (CHAPS) were studied. The results showed that marked inhibition of 1-naphthol-UGT and morphine UGT activities occurred with high concentrations of Triton X-100. Lubrol Px, at high concentrations, inhibited 1-naphthol-UGT but not morphine-UGT. It appeared that the detergent/protein ratio suitable for optimal activation of both isoenzymes was limited to 0.2 for these detergents. In contrast, Brij 58 and CHAPS displayed optimal activation of the two enzymes for a large range of detergent/microsomal protein ratios (respectively from 0.2 to 1 and from 0.4 to 1), making them the most suitable for induction and/or latency studies of both isoenzymes. The influence of maximal activation status on the effect of 3-methylcholanthrene and phenobarbital treatment on morphine-UGT and 1-naphthol-UGT activity has also been evaluated. The findings provided evidence that detergent-activation profiles and optimal detergent-activated versus "native" UGT activity determination give crucial informations about the characteristics of a given isoenzymic form of UGT, i.e. its sensitivity to specific alterations of the phospholipid environment, its latency and its inducibility.

Conjugation with glucuronic acid catalysed by microsomal UDP-glucuronosyl transferase (UGT,* EC 2.4.1.17) is quantitatively the most important phase 2 reaction of drug metabolism [1]. UGTs are inducible: between the eight groups of isoenzymes characterized in the rat liver [2], the 3-MC-inducible UGTs metabolize planar structures such as 1-naphthol while PB-type inducers stimulate glucuronidation of bulkier molecules like morphine. UGT activity is at least partially latent, maximal activity being expressed only after treatment with activators, generally detergents [3].

Determination of enzyme activity in both "native" and detergent-activated microsomes has been reported as a means to determine if UGT activity modifications are due to changes in protein level or to modifications in the enzyme membrane environment. It has thus been shown that cholesterol administration in the diet [4] induces an increase in UGT enzyme activity by decreasing membrane "fluidity". The isoenzyme-specific changes revealed in "native" liver microsomes from streptozotocin and alloxanic diabetic rats were abolished by maximal detergent activation *in vitro* suggesting an alteration of the membrane environment rather than a deficiency of isoenzyme proteins [5, 6]. Similarly, the lipophilic 1,4-benzodiazepines [6] and carbon tetrachloride [7] have been reported to alter UGT activity in "native" microsomes only, by changing the lipid environment of the enzyme through direct interaction with microsomal membranes. In contrast, the isoenzyme-specific influence of thyroid hormones on UGT was observed in both detergent-activated and "native" preparations suggesting changes in isoenzyme protein levels [8].

N-Nitrosodiethylamine, depending on the administered dose, induced and/or activated 4-nitrophenol and 2-aminophenol in the rat [9]. No apparent changes in enzyme activity in "native" microsomes may even mask a simultaneous process of induction and increase in the latency of the enzyme. Thus, a marked increase in the latency of 4-methylumbelliferone-UGT has been shown to accompany the synthesis of enzyme in the perinatal period, leading to an equivalent absolute amount of "native" activity from its fetal appearance until adulthood [10].

In this study, by using a simple and rapid HPLC method to evaluate glucuronide formation, we determined the detergent-activation profiles of 1-naphthol-UGT and morphine-UGT by three non-ionic detergents, Triton X-100, Brij 58 and Lubrol Px and by one zwitterion detergent, CHAPS. We also evaluated the influence of maximal activation status on the effect of 3-MC and PB treatment on morphine-UGT and 1-naphthol-UGT activity.

Materials and Methods

Animal treatment. Male Sprague–Dawley rats (Charles River) were used. For the induction studies, the rats were pretreated i.p. with either 3-MC (Sigma) 20 mg/kg in corn oil or PB (Rhône-Poulenc Rorer) 80 mg/kg in saline for 3 days before being killed. Respective control animals were administered the appropriate vehicle.

Microsomal preparation. Hepatic microsomes were prepared according to Amri *et al.* [11], aliquoted and frozen at −80° until analysis. For the preparation of "native" and detergent-activated microsomes, solutions containing 8 mg/mL microsomal proteins in 50 mM sodium phosphate buffer, 10 μM $MgCl_2$ pH 8, were preincubated at 37° for 10 min in the presence of a given detergent before being assayed for enzyme activity. Final detergent concentrations were chosen to vary from 0 ("native" microsomes) to 1 mg/mg microsomal proteins, according to the results of Dragacci *et al.* [12].

Enzyme analysis. UGT activity toward morphine (Merck Chemical Division) and 1-naphthol (Sigma) was assayed according to the method described by Liu and Franklin [13] with some modifications. Briefly, after the 10 min preincubation with a given detergent, microsomes (1.3 mg/mL) were incubated with either 1.2 mM 1-naphthol or 2.5 mM morphine in 50 mM sodium phosphate buffer, 10 mM $MgCl_2$ pH 8 for 5 min at 37°. Addition of 2 mM

* Abbreviations: UGT, UDP-glucuronosyl transferase; UDPGA, UDP-glucuronic acid; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonic acid; 3-MC, 3-methylcholanthrene; PB, phenobarbital.

UDPGA (Boehringer) initiated the reaction which was conducted at 37° for 0, 5, 15 and 30 min. The reaction was stopped by addition of 2% $HClO_4$ (w/v). After protein removal by centrifugation, the two supernatants were mixed and injected onto a 0.46 × 25 cm C18 HPLC column (SFCC). The mobile phase contained 2 mM sodium dodecyl sulfate and consisted of 60% of 20 mM sodium phosphate buffer pH 2.5 and 40% organic phase (2/3 acetonitrile: 1/3 methanol). The chromatograph was developed at a flow rate of 0.8 mL/min for 30 min. The eluate was continuously monitored at 220 nm (Kontron Instrument detector) for the detection of glucuronides and unconjugated substrates. For quantification of glucuronide formation, 20 μL of known concentrations of morphine 3-β-glucuronide (Francopia, 125 and 1389 μM) or naphthyl-β-glucuronide (Sigma, 10 and 250 μM) were also injected. By using the two calibration curves, the MT2 Kontron data system automatically determined the concentration of morphine- or 1-naphtholglucuronide in the samples. Glucuronidation of 1-naphthol was linear for at least 30 min in "native", Triton X-100 (Prolabo)-activated and CHAPS (Sigma)-activated microsomes, 15 min in Brij 58 (Sigma)-activated and 10 min in Lubrol Px (Sigma)-activated microsomes. Morphine glucuronide formation was linear for at least 30 min in all types of microsomes. Initial reaction rates were used to calculate enzyme activities, expressed as nmol 1-naphthol glucuronide or morphineglucuronide formed/min/mg microsomal proteins.

Statistical procedures. For statistical comparisons of data, the mean ± SD was calculated for each parameter and the treated groups were compared to the corresponding control group using either Student's *t*-test [14] when the variances were homogeneous according to Bartlett's test [15], or the non-parametric Wilcoxon signed rank test [16], when the variances were heterogenous. The statistical significance of any difference from the corresponding control value was reported.

Results and Discussion

The effect of the different detergents on UGT toward morphine and 1-naphthol are shown in Fig. 1. When the microsomal preparations were incubated with Triton X-100, an increase in both 1-naphthol and morphine glucuronidation occurred from a 0.1 detergent/protein ratio and maximal activation was obtained at a ratio of 0.2. Higher concentrations led to rapid inactivation of the isoenzymes. Similar activation/inactivation profiles by Triton X-100 of UGT towards various aglycones have been described in rat [17–19], mouse [19] and human [12] microsomes, and have been shown to be due to enzyme denaturation.

Lubrol Px displayed an activation pattern of microsomal 1-naphthol-UGT different from morphine-UGT. Both enzyme activities were increased in the presence of Lubrol Px from a detergent/protein ratio of 0.1 and maximal activation of the two enzymes occurred at a ratio of 0.2. However, at higher concentrations of Lubrol Px, 1-naphthol-UGT activity decreased while morphine-UGT activation plateaued until a detergent/protein ratio of 1. A gaussien type activation pattern of Lubrol Px similar to Triton X-100 has already been described for 4-nitrophenol-UGT [20]. Our results most probably reveal an increased sensitivity to denaturation by Lubrol Px of the 3-MC-inducible protein(s) involved in phenol glucuronoconjugation compared to the PB-inducible protein(s) and this could be explained by the well-documented differences in the interaction with microsomal membranes of the different isoenzymic forms of UGT [3].

The activation profiles of the two isoenzymes were in plateau with both Brij 58 and CHAPS: Brij 58 induced an increase of 1-naphthol-UGT and morphine-UGT activity from a detergent/protein ratio of 0.1 and maximal activation was achieved from 0.2 and maintained till 1 detergent/protein ratio. CHAPS activation occurred only from a

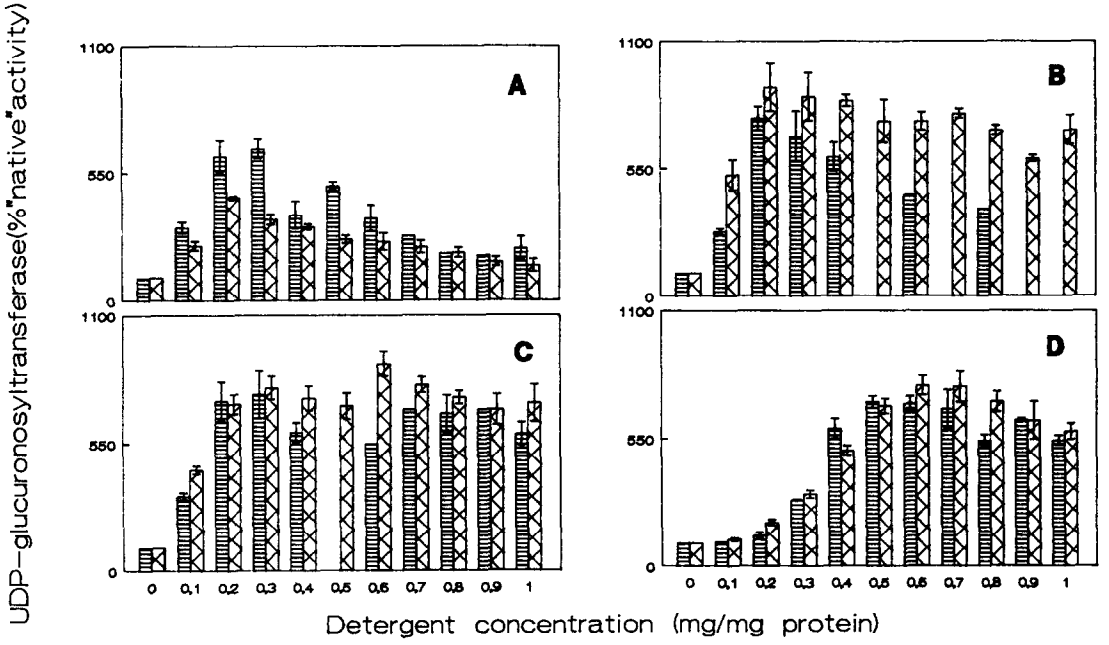

Fig. 1. Activation patterns of male rat hepatic microsomal UGT toward 1-naphthol (▤) and morphine (▨) by Triton X-100 (A), Lubrol Px (B), Brij 58 (C) and CHAPS (D). Enzyme activity in detergent-treated microsomes was expressed as percentage of "native" activity. Glucuronoconjugation rate (nmol/min/mg protein) in "native" microsomes was 3.7 ± 0.6 for 1-naphthol and 15 ± 2.6 for morphine. Values represent the mean ± SEM of 3–4 rats.

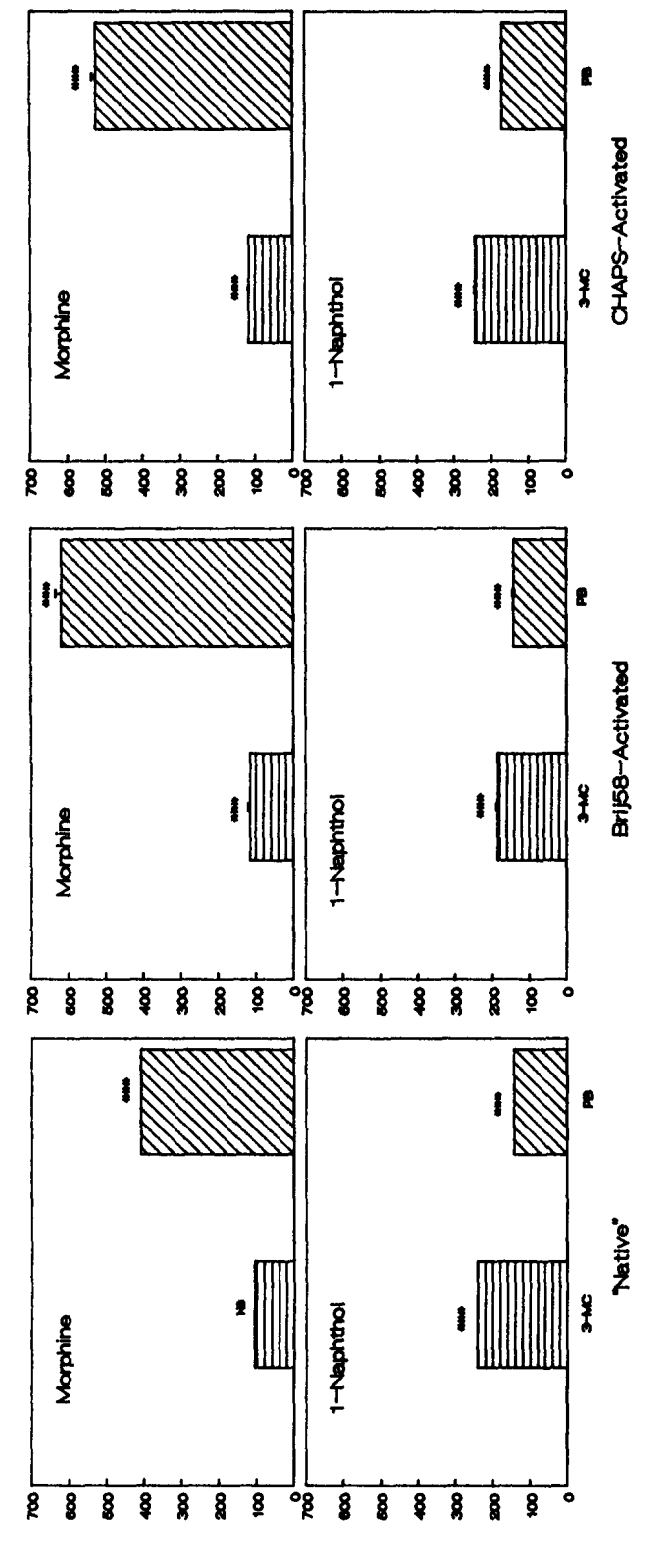

Fig. 2. UGT activities toward 1-naphthol and morphine were determined in hepatic microsomes from control rats and rats pretreated with 3-MC or PB. Enzyme activities were measured in both "native" and detergent-activated (0.3 mg/mg protein Brij 58 or 0.6 mg/mg protein CHAPS) microsomes and were expressed as percentage of respective controls. Glucuronoconjugation rate (nmol/min/mg protein) was 14.1 ± 4.2 for morphine and 4.3 ± 0.3 for 1-naphthol in control "native" microsomes; 62.2 ± 2.2 for morphine and 13.4 ± 1.7 for 1-naphthol in control CHAPS-activated microsomes; 62.1 ± 1.3 for morphine and 16.6 ± 0.2 for 1-naphthol in control Brij 58-activated microsomes. Values represent the mean ± SEM of 3 rats. NS, not significantly different from controls; ***significantly different from controls at the 1‰ ($P < 0.001$).

detergent/protein ratio of 0.3. Between 0.5 and 1 detergent/protein ratio of this detergent, activation of both enzymes was maximal. The findings with CHAPS support previous results [12, 17, 19, 21] demonstrating that it was poorly denaturating and that minimal concentration leading to maximal activation by this zwitterion detergent was higher than for non-ionic detergents. These results further indicate that Brij 58 does not inhibit 1-naphthol- and morphine-UGT activities at detergent/protein ratio up to 1.

The effect of the microsomal enzyme inducers 3-MC and PB on 1-naphthol and morphine UGTs, assayed in "native" and optimal detergent-activated microsomes are shown in Fig. 2. The glucuronidation of 1-naphthol was mainly increased by 3-MC treatment (2.5-fold in "native" and in CHAPS-activated and 2-fold in Brij 58-activated microsomes) but also by PB treatment (1.5-fold in both "native" and detergent-activated microsomes), this illustrating the overlapping substrate specificity of some UGTs: between the three UGT isoenzymes involved in the conjugation of 1-naphthol, one is strongly induced by 3-MC, the other one being induced by PB [2]. Enzyme activity toward morphine in PB-pretreated rats was increased 4-fold in "native", 5-fold in CHAPS-activated and 6-fold in Brij 58-activated microsomes, showing the well-described induction of morphine-UGT by PB, which has been confirmed by immunohistochemistry [2]. Morphine-UGT was also slightly (17%) but significantly increased in detergent-activated microsomes from 3-MC-treated rats, suggesting the induction by 3-MC of an isoenzyme slightly involved in morphine glucurono-conjugation. In addition, the results reveal that PB increases the latency of morphine-UGT. This supports earlier observations indicating (a) that PB and 3-MC cause alterations in the protein–lipid interactions of the microsomal membrane differently affecting UGT isoenzymes [22, 23] and consequently (b) that UGT enzyme activities determined in detergent-solubilized microsomes better approximate the amount of enzyme protein present.

In conclusion, the present findings show that the inhibitory effect of high concentrations of Triton X-100 (inhibition of 1-naphthol-UGT and morphine-UGT activities) and Lubrol Px (inhibition of 1-naphthol-UGT), are limited to a detergent/protein ratio of 0.2 for optimal activation of the two enzymes. In contrast, Brij 58 and CHAPS display optimal activation of the two enzymes over a large range of detergent/microsomal protein ratio, making them the most suitable for induction and/or latency studies of both isoenzymes. In addition, our results provide evidence that detergent-activation profiles and optimal detergent-activated versus "native" UGT activity determination give crucial information about the characteristics of a given isoenzymic form of UGT, i.e. its sensitivity to specific alterations of the phospholipid environment, its latency and its inducibility.

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