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Selective Inhibition of Glucuronidation by 2,2,2-Triphenylethyl-UDP in Isolated Rat Hepatocytes: Conjugation of Harmol, 3,3′,5-Triiodothyronine, and *N*-Hydroxy-2-acetylaminofluorene

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SUMMARY

2,2,2-Triphenylethyl-UDP (TPEU) was synthesized as an analogue of the transition state of the glucuronidation reaction catalyzed by UDP-glucuronosyltransferase; it contains both a uridine and an acceptor substrate moiety. It inhibits rat liver microsomal UDP-glucuronosyltransferase [*Eur. J. Biochem.* **188**:309–312 (1990)]. In the present work, TPEU was tested as an inhibitor of glucuronidation in intact rat hepatocytes. Two phenols (harmol and 3,3′,5-triiodothyronine) and a hydroxamic acid (*N*-hydroxy-2-acetylaminofluorene) were used as substrates for glucuronidation. The glucuronidation of these substrates was strongly decreased by TPEU at 0.3–5 mM. Up to 5 mM TPEU did not kill the cells, as shown by unimpaired trypan blue exclusion at the end of the incubation. When glucuronidation was inhibited, the sulfation of harmol increased, as did the production of reactive species generated from *N*-hydroxy-2-acetylaminofluorene that bind to cellular macromolecules. This indicates that a decreased substrate consumption by loss of glucuronidation leads to increased conversion by competing pathways. The results show, therefore, that TPEU is an effective inhibitor of glucuronidation in this cellular system *in vitro*.

Glucuronidation is an important conjugation reaction in the biotransformation of both endogenous and exogenous compounds (1, 2). It is catalyzed by UDP-glucuronosyltransferases, a family of (iso)enzymes that are located in the endoplasmic reticulum membrane. The highest activity is found in the liver. Glucuronidation plays a major role in the detoxification of many xenobiotics but is also involved in the metabolism of many endogenous compounds.

Recently, we started a program directed towards the development of selective inhibitors of UDP-glucuronosyltransferases. In a preceeding paper, we reported that several potential transition state analogues of the glucuronidation reaction were inhibitory towards UDP-glucuronosyltransferase activity in the microsomal fraction from rat liver (3). If such inhibitors are also active *in vivo*, they could be useful tools in studies on the role of glucuronidation in the toxification and detoxification of xenobiotics (4).

In the present paper, we report on the effect of the most active inhibitor, TPEU (Fig. 1), on glucuronidation in an intact cellular system *in vitro*, i.e., isolated rat hepatocytes. Three very different compounds, which have in common the fact that sulfation of the same functional group in the substrate is a competing biotransformation, have been used as substrates. Harmol (5) and the thyroid hormones T_4 and T_3 (6), which are phenols (T_4 and T_3 being endogenous compounds), have been used as substrates for glucuronidation. *N*-OH-AAF is a hydroxamic acid that is detoxified by glucuronidation of the hydroxyl group but becomes more toxic upon sulfation of this group (4). The results show that TPEU selectively inhibits glucuronidation, while competing reactions are increased, indicating that TPEU is effective in an intact cell system.

Experimental Procedures

Materials. TPEU was synthesized as described elsewhere (7). [^{3}H] Harmol and harmol were obtained from sources reported before (5). [^{3}H]*N*-OH-AAF was obtained as described earlier (8). T_3 and T_4 were obtained from Henning Berlin GmBH (Berlin, Germany). β-Glucuronidase (from *Escherichia coli* K12) was purchased from Boehringer (Mannheim, Germany). TLC analysis was performed on silica gel 60 F254 plates (Merck, Darmstadt, Germany). Plasmasol was obtained

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ABBREVIATIONS: TPEU, 2,2,2-triphenylethyl-UDP; T_3, 3,3′,5-triiodothyronine; T_4, 3,3′,5,5′-tetraiodothyronine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); BES, *N*,*N*′-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; *N*-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; T_3G, 3,3′,5-triiodothyronine glucuronide; T_3S, 3,3′,5-triiodothyronine sulfate; TLC, thin layer chromatography.

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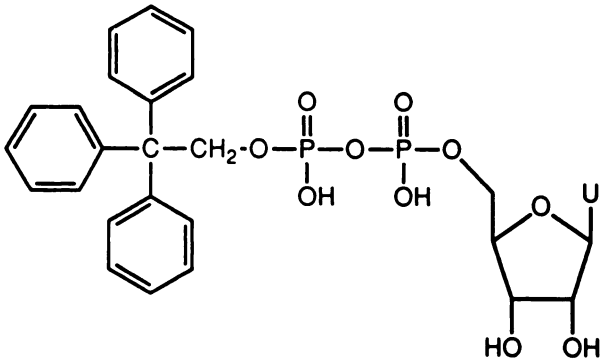

Fig. 1. Chemical structure of TPEU. *U*, uracil.

from Packard (Downers Grove, IL). Media and chemicals used for the hepatocyte primary cultures were from sources reported before (9).

Assay of UDP-glucuronosyltransferase. UDP-glucuronosyltransferase activities for T_3 and T_4 were assayed essentially as previously described, by reaction of 1 μM radioactive T_3 or T_4 for 1 hr at 37° with 1 mg of microsomal protein/ml, in the presence of 5 mM UDPGA (6). Bry 56 (0.05%) was included for complete "activation" of the enzyme. The glucuronide formed was quantified by Sephadex LH-20 analysis.

Isolation of hepatocytes. For the isolation of hepatocytes, male Wistar rats (body weight, approximately 200 g) from the Sylvius Laboratory (University of Leiden, Leiden, The Netherlands) were used; they had free access to food and water. Hepatocytes were isolated according to a collagenase perfusion method, originally described by Seglen (10) and modified as described elsewhere (11). Hepatocyte suspensions were made in Hanks'-HEPES buffer (pH 7.6) containing bovine serum albumin (1.5%, w/v), at a concentration of 3×10^6 cells/ml (harmol experiments) or 6×10^6 cells/ml (*N*-OH-AAF experiments). Viability was >95%, as determined by trypan blue exclusion.

For studies of T_3 metabolism, rat hepatocytes were isolated using a similar procedure, but they were allowed to form primary monolayer cultures. For this purpose, 10^6 liver cells were suspended/ml of culture medium (Ham's F-10 medium supplemented with 10.6 mM PIPES, 11.2 mM BES, 8.9 mM HEPES, 1.8 mM $CaCl_2$, 1 mM glutamine, 12 milliunits/ml insulin, 50 nM dexamethasone, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10%, v/v, fe l bovine serum) (9). Two-milliliter aliquots of this suspension were plated into uncoated 3.5-cm wells of plastic six-well dishes. The plates were kept for 4 hr at 37° under air, in an incubator. Cell viability, as tested by trypan blue exclusion, exceeded 85%.

Incubation procedure for harmol and analysis of its conjugates. In plastic tubes, 300 μl of hepatocyte suspension (3×10^6 cells/ml) in Hanks'-HEPES buffer (pH 7.6) and 25 μl of a solution of TPEU in the same Hanks'-HEPES buffer, or solvent only, were preincubated for 15 min on a rotary shaker at 190 rpm and 37°. Oxygenation of the incubation mixtures was performed with 95% O_2/5% CO_2 (11). The reaction was started by addition of 25 μl of a solution of [^{3}H]harmol (5.4 μCi/ml) in Hanks'-HEPES buffer (final concentration in the incubation mixture, 200 μM) to the incubation mixture. After 30 and 120 min, a 125-μl sample was taken, which was rapidly vortexed with 125 μl of ice-cold methanol and kept on ice for 30 min, followed by centrifugation at 1200 × *g* for 15 min. A sample of the methanol extract was applied to a TLC plate. Methanol/chloroform/isopropanol/ammonia (10:90:95:5, v/v/v/v) was used as eluent (12). The fluorescent spots of harmol glucuronide, harmol sulfate, and unconjugated harmol were scraped off and put in counting vials, together with water (0.5 ml). Then, Plasmasol (8 ml) was added, followed by thorough mixing of the vial on a Vortex mixer. Before liquid scintillation counting, the silica gel was allowed to settle for 24 hr in the dark.

Incubation procedure for *N*-OH-AAF and determination of its glucuronidation and covalent binding to macromolecules. *N*-OH-AAF can be conjugated to a stable glucuronide, but it can also be metabolized to extremely reactive products that become covalently bound to macromolecules, mainly to protein.

In plastic tubes, 4200 μl of a hepatocyte suspension (6×10^6 cells/ml) in Hanks'-HEPES buffer (pH 7.6) and 350 μl of a solution of TPEU in Hanks'-HEPES buffer (final concentration in the incubation mixture, 5 mM), or its solvent, were preincubated for 15 min on a rotary shaker at 190 rpm and 37°. Oxygenation of the incubation mixtures was performed with 95% O_2/5% CO_2 (11). Then, 350 μl of a solution of freshly dissolved [^{3}H]*N*-OH-AAF in Hanks'-HEPES buffer (final concentration in the incubation mixture, 100 μM; 0.065 μCi/μmol) were added to the incubation mixture. After 30 and 120 min, 2-ml samples were taken, which were rapidly vortexed with 4 ml of ice-cold methanol, followed by centrifugation at 300 × *g* for 10 min. The supernatant was used to determine the amount of the glucuronide formed, whereas the pellet was used to assay for covalent binding to macromolecules.

In order to determine the formation of the glucuronide conjugate, the water of the supernatant was removed in a SpeedVac apparatus, and the residue was taken up in 1 ml of 0.2 M sodium acetate buffer (pH 6.0), followed by extraction with 1 ml of diethyl ether (three times). The aqueous layer (300 μl) and 5 μl of the undiluted β-glucuronidase solution were incubated at 37° for 90 min. Then, each mixture was extracted twice with an equal volume of diethyl ether. The organic layers were collected and evaporated to dryness under a stream of nitrogen. The residues were dissolved in ethanol (0.5 ml). *N*-OH-AAF was determined by TLC; a 25-μl sample was applied to a TLC plate (aluminium foil sprayed with a solution of 0.5 M Na_4EDTA (5–10 ml/30 cm^2). After chromatography (eluent, *n*-butanol/ethyl acetate/ammonia, 70:30:4, v/v/v), the TLC plates were cut in small pieces and taken up in Plasmasol (8 ml). Radioactivity was determined by liquid scintillation counting after 2 hr (13).

In order to determine the amount of [^{3}H]*N*-OH-AAF covalently bound to the precipitated protein, the pellet was extensively washed with methanol and ethanol. The pellets were washed twice with 5 ml of methanol/water (67:33, v/v), followed by washing with ethanol (two times). The pellet was dried overnight *in vacuo*. The weight of the dried material was determined, and the dry material was dissolved in 1 N NaOH (24 hr) to obtain a solution of 20 mg of dry material/ml. For liquid scintillation counting, 50 μl were taken up in Ultima Gold (Packard, Downers Grove, IL). Protein contents were determined according to the method of Lowry *et al.* (14). Covalent binding of [^{3}H]*N*-OH-AAF to protein was expressed as pmol/mg of protein.

Incubation of T_3 with hepatocytes and analysis of products. After aspiration of culture medium, the monolayers of 2×10^6 hepatocytes were incubated for 3 hr at 37° under air with 10 nM T_3 and 0.1 μCi of [3′-^{125}I]T_3, in 2 ml of incubation medium (Ham's F-10 medium supplemented with PIPES, BES, HEPES, calcium, chloride glutamine, insulin, dexamethasone, and 0.1% bovine serum albumin). Medium samples (200 μl) were obtained at 1-hr intervals and analyzed on Sephadex LH-20, as previously described (9). For this purpose, samples were brought to 1 ml with 0.1 M HCl and applied to small Sephadex LH-20 columns. Using a stepwise elution scheme, separation was achieved between iodide, T_3G, T_3S, and remaining T_3. Products were quantified on the basis of the radioactivity in the different fractions, corrected for the low blank values observed after incubation without hepatocytes (9).

Results and Discussion

TPEU was previously shown to be inhibitory towards glucuronidation by the rat liver microsomal fraction, of a number of substrates. Approximately 80% inhibition was observed for several substrates at 0.5 mM TPEU. Now TPEU has been tested on the UDP-glucuronosyltransferase activity of rat liver microsomes for T_4 and T_3. It has previously been demonstrated that multiple UDP-glucuronosyltransferases are involved in the conjugation of thyroid hormones (6). Hepatic glucuronidation of T_4 may be catalyzed by 3-methylcholanthrene-inducible

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phenol UDP-glucuronosyltransferase as well as clofibrate-inducible bilirubin UDP-glucuronosyltransferase, whereas T_3 glucuronidation shows a genetic linkage with androsterone UDP-glucuronosyltransferase (6). Fig. 2 shows that TPEU produced a concentration-dependent inhibition of the glucuronidation of T_4 and T_3, amounting to about 50% at 0.1 mM and 90% at 1 mM inhibitor. Similar effects were observed with both substrates; little or no inhibition was noted at 0.01 mM TPEU (data not shown).

In order to find out whether TPEU was also inhibitory towards glucuronidation in hepatocytes, a number of substrates were used that can be both glucuronidated and sulfated at the same acceptor group in the molecule.

Effects of TPEU on glucuronidation and sulfation of harmol. Harmol [7-hydroxy-1-methyl-9*H*-pyrido-(3,4*b*)-indole] is a phenolic compound that is a substrate for both UDP-glucuronosyltransferase and sulfotransferase. Sulfation is catalyzed by sulfotransferases located in the cytosol. The fraction of harmol that is sulfated decreases as the concentration of harmol increases, whereas glucuronidation becomes more important at higher concentrations of harmol. This is due to the fact that the K_m values for the phenol sulfotransferases are much lower than those for UDP-glucuronosyltransferases (15). Earlier we reported that sulfation of harmol could be inhibited by 2,6-dichloro-4-nitrophenol and pentachlorophenol; enhanced glucuronidation compensated for the loss of sulfation (16).

In order to determine whether TPEU can also inhibit glucuronidation in intact cells, isolated hepatocytes were incubated with different concentrations of TPEU and [^{3}H]harmol for different periods of time, followed by analysis of the conjugates. The results of this experiment (Fig. 3) demonstrate that glucuronidation of harmol is decreased in the presence of TPEU and that sulfation is somewhat increased. The fact that after 30 min of incubation there was hardly any increase in sulfation

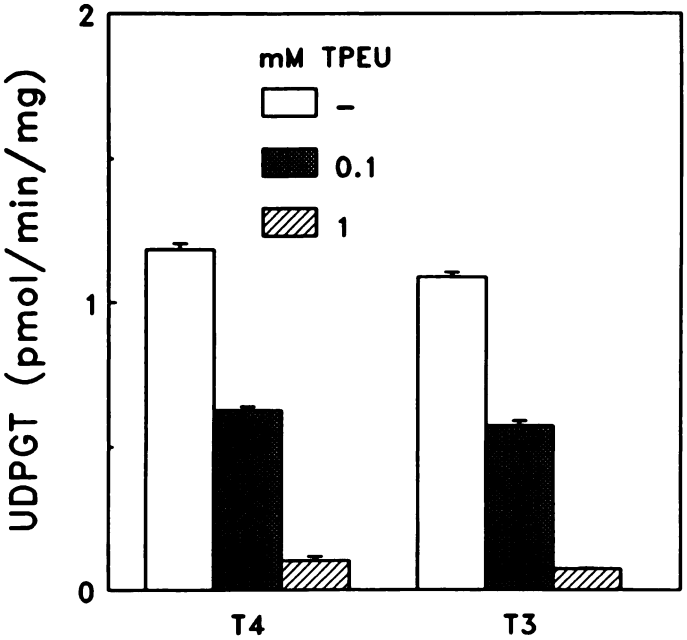

Fig. 2. Inhibition by TPEU of the glucuronidation of T_3 and T_4 in rat liver microsomes. The results are the means ± standard deviations of triplicate determinations in a representative experiment. TPEU was used at 0.1 and 1.0 mM. *UDPGT*, UDP-glucuronosyltransferase.

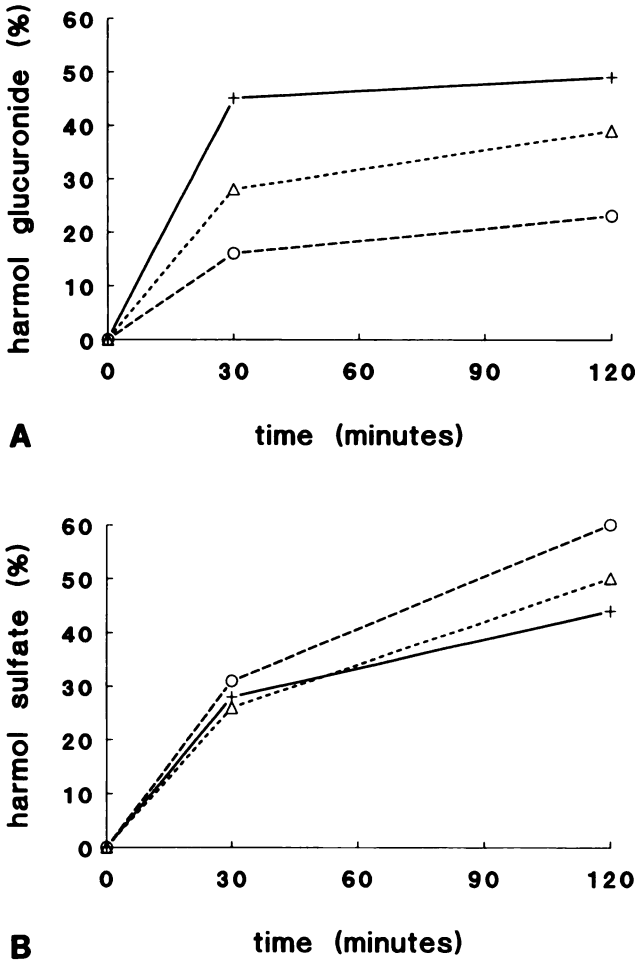

Fig. 3. Time course of glucuronidation (A) and sulfation (B) of harmol in the presence of TPEU. Rat hepatocytes were incubated with [^{3}H]harmol and various concentrations of TPEU. After the incubation, the harmol conjugates and unconjugated harmol were separated and isolated by TLC and quantified by liquid scintillation counting. ○, [TPEU] = 5.0 mM; △, [TPEU] = 0.3 mM; +, [TPEU] = 0.0 mM.

of harmol is probably due to saturation of the sulfotransferases during that period (15). After 2 hr, harmol was almost completely metabolized. At that time, the percentage converted to harmol glucuronide was 49 ± 4% in controls and 23 ± 4% with 5 mM TPEU. For harmol sulfate, this was 44 ± 8% and 60 ± 9%, respectively (mean ± standard error, three experiments).

These results indicate that TPEU is not very hepatotoxic, because sulfation is unimpaired. This was supported by the outcome of viability tests on the isolated hepatocytes; exclusion of trypan blue was not affected by incubation with TPEU.

Effect of TPEU on glucuronidation and covalent binding of *N*-OH-AAF. The carcinogen *N*-OH-AAF can be metabolized by several routes. Some metabolites, such as the *N*-sulfate conjugate, may generate reactive species that can bind to macromolecules like nucleic acids and proteins (4). Meerman *et al.* (13) demonstrated that covalent binding of *N*-OH-AAF depended largely on sulfation. Because the sulfate ester of *N*-OH-AAF is extremely unstable and highly reactive towards nucleophilic groups present in macromolecules (17), its formation by sulfotransferases can only be demonstrated indirectly, by determination of the covalent binding of *N*-OH-AAF to macromolecules in the isolated hepatocytes (13). Isolated he-

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patocytes were incubated with TPEU and [^{3}H]*N*-OH-AAF for various periods of time, and the amounts of *N*-OH-AAF glucuronide present in the various incubation mixtures were determined. Glucuronidation was significantly lower in the incubation mixtures with TPEU, compared with controls; 14% of the added dose was converted to the glucuronide in the presence of TPEU, compared with 51% in controls. However, the amount of unchanged *N*-OH-AAF after incubation with TPEU (10%) was approximately identical to the amount of *N*-OH-AAF found in the control incubation (7%), indicating that other pathways had "compensated" for the loss of glucuronidation.

The covalent binding of *N*-OH-AAF to macromolecules was dramatically enhanced by the presence of TPEU (Fig. 4). This suggests that sulfation or other reactions leading to covalent binding of *N*-OH-AAF are strongly increased in the presence of TPEU.

Effects of TPEU on metabolism of T_3. Because in normal rat hepatocytes T_4 is only slowly glucuronidated (18, 19), the effects of TPEU on the metabolism of iodothyronines in liver cells were only tested using T_3 as the substrate. The hepatic metabolism of T_3 proceeds via three pathways, i.e., glucuronidation, sulfation, and deiodination (9). Whereas T_3G is a stable conjugate, T_3S is rapidly degraded by enzymatic deiodination. Under normal conditions, therefore, incubation of hepatocytes with [3′-^{125}I]T_3 yields predominantly labeled T_3G and iodide as products. Fig. 5 clearly demonstrates that TPEU is a selective inhibitor of T_3 glucuronidation in cells. Addition of 0.5 mM TPEU decreased T_3G formation by more than 80%, whereas iodide production was diminished by less than 20%. Because iodide is produced by successive sulfation and deiodination, our results suggest that TPEU has little effect on the enzymes that catalyze these reactions. At the end of the incubation, 53 ± 2% and 39 ± 1% of the T_3 had been converted in the absence and presence of TPEU, respectively. The percentage present as T_3S was 6.5 ± 0.4% in both cases.

Conclusions

The findings described above indicate that TPEU is a selective and relatively nontoxic inhibitor of glucuronidation in intact liver cells. TPEU was previously shown to be a competitive inhibitor of microsomal UDP-glucuronosyltransferases (3). The fact that it inhibits glucuronidation of three different substrates in isolated hepatocytes shows that it is also effective in intact cells and that its effect can be observed with several, structurally very different substrates. This offers a new approach to selective inhibition of glucuronidation, for which, as yet, no selective inhibitors are available. The fact that the production of metabolites other than glucuronides was not (or hardly) affected indicates that the effect of TPEU shows some selectivity. Because TPEU was devised as a transition state analogue, we hope that its specificity for UDP-glucuronosyltransferases will be high, but this requires further investigations.

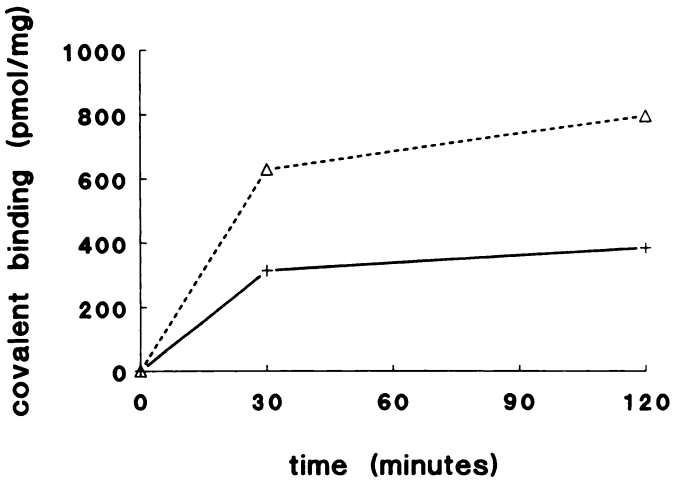

Fig. 4. Time course of covalent binding of *N*-OH-AAF to protein in the presence of TPEU. Rat hepatocytes were incubated with [^{3}H]*N*-OH-AAF and TPEU. After the incubation was stopped, the precipitated cells were extensively washed with methanol and ethanol to remove unbound radioactive material. After the pellets were dried, protein contents were determined and covalent binding of [^{3}H]*N*-OH-AAF was quantified with liquid scintillation counting. △, [TPEU] = 5.0 mM; +, [TPEU] = 0.0 mM.

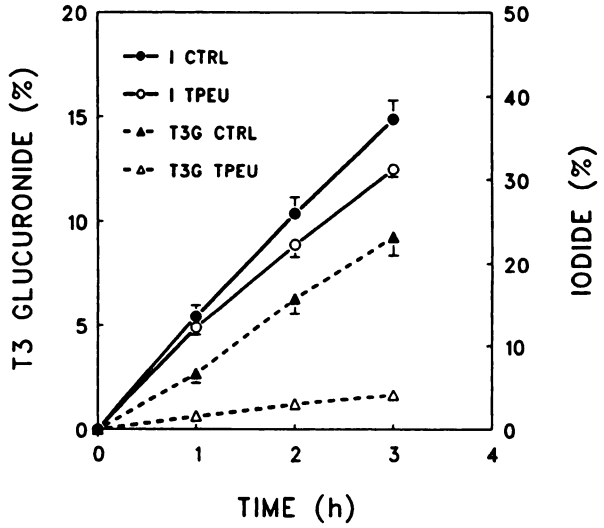

Fig. 5. Effects of TPEU on the production of radioactive iodide and T_3G from T_3 in hepatocyte primary culture. The TPEU concentration was 0.5 mM, whereas 10 nM (≈0.1 μCi) [3′-^{125}I]T_3 was used as substrate, with 2 × 10^6 hepatocytes/incubation. The data are expressed as percentage of medium radioactivity and are displayed as the means ± standard deviations of triplicate observations in a representative experiment. *I*, radioactive [^{125}I]iodide.

Acknowledgments

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