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Uptake and Metabolism of 3,5,3′-Triiodothyronine and 3,3′,5′-Triiodothyronine by Human Liver-Derived Cells: HepG2 Cells as a Model for Thyroid Hormone Handling by Human Liver*

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ABSTRACT

The uptake and metabolism of T_3 and rT_3 was studied in human liver-derived HepG2 cells. The results showed a saturable, time-dependent, and ouabain-sensitive increase in nuclear bound T_3. The effects of ouabain (0.5 mmol/L) and unlabeled T_3 (10 nmol/L and 10 μmol/L) were much more pronounced at the nuclear level, suggesting the presence of a nonspecific component in total cellular binding. Nuclear binding of rT_3 remained below the detection limit in all experiments. Comparison of rT_3 metabolism in HepG2 cells and primary cultures of rat hepatocytes showed an approximately 10-fold lower iodide production in HepG2 cells. Iodide production was decreased in the presence of ouabain and almost absent in the presence of propylthiouracil (100 μmol/L). Our data confirmed the presence of a carrier-mediated uptake system for both T_3 and rT_3. Metabolism data indicated functional type I deiodinase activity in HepG2 cells, the presence of glucuronidating enzymes, and the absence of thyroid hormone sulfotransferase activity. Based on these data, we propose that HepG2 cells provide an appropriate model for thyroid hormone handling by human liver. In addition, we suggest that in human liver sulfation of thyroid hormone, and therefore deiodination of T_3 is of only minor importance. (*J Clin Endocrinol Metab* **81:** 244–248, 1996)

IN BOTH MAN and rat, the conversion of T_4, the main secretory product of the thyroid gland, to the biologically active T_3 takes place in the liver. After entrance of T_4 into the cellular compartment, it can either be activated by deiodination to T_3 or inactivated by deiodination to rT_3 (1–3). In the liver, both reactions are mediated via type I deiodinase, which has recently been cloned (4). Alternatively, the iodothyronines can be conjugated by glucuronyltransferases, with subsequent excretion into the bile, or sulfotransferases (1).

Many studies on the hepatic entry of thyroid hormone have been performed using primary cultures of rat hepatocytes (5–9), rat liver-derived cell lines (10, 11), and rat liver *in vivo* (12). It has been shown that the uptake process is carrier mediated, being saturable, stereospecific, and energy and temperature dependent. This process has also been shown to be operative in other types of cells of several species (13–20) and is not only essential for the intracellular activation of T_4, but also for the biological effects of circulating T_3, which is mediated via specific nuclear receptors.

Recently, we reported data on thyroid hormone transport in primary cultures of human hepatocytes (21). Furthermore, transport into human fibroblasts (22), neuroblasts (23), and glioma cells (24) has been described. Because of difficulties in routinely obtaining viable human hepatocytes we decided to study the human liver-derived cell line HepG2, in which only uptake and nuclear binding of T_3 have been studied (23, 25).

HepG2 cells have been shown to retain many of the liver-specific metabolic enzymes and are thought to be a suitable model for human liver cells (26). In this report we studied T_3 and rT_3 uptake and deiodination in human HepG2 cells and compared the results with those obtained in the previously used rat and human hepatocyte model. As we have shown that there is no deiodination of T_3 in HepG2 cells due to deficient T_3 sulfation (27), we have used nuclear binding of T_3 to study internalization of this hormone.

Materials and Methods

Materials

L-[3′-^{125}I]T_3 and L-[3′-5′-^{125}I]rT_3 (SA of both, >1200 μCi/μg) were obtained from Amersham International (Aylesbury, UK). Unlabeled T_3 and rT_3 were obtained from Hennig (Berlin, Germany). Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden). All tissue culture chemicals and plastics were obtained from Life Technologies (European Division, Breda, The Netherlands). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

Cell culture. HepG2 cells were obtained from Dr. Knowles, Wistar Institute (Philadelphia, PA). Cells were grown in a 5% CO_2, 37 C, humidified incubator in DMEM-Ham's F-12 nutrient mixture (1:1, vol/vol)

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supplemented with 10% FCS, penicillin (100 IU/mL), streptomycin (100 μg/mL), and glutamine (2.4 mmol/L). Cells were subcultured at confluence using trypsinization (0.5 g/L trypsin, 0.02 g/L collagenase, and 0.5 mmol/L ethylenediamine tetraacetate).

Whole cell incubations. Subconfluent cultures of HepG2 cells were harvested using trypsinization and transferred to six-wells plates. After reaching confluence, medium was replaced with 2 mL incubation medium/well [culture medium without FCS, with 5 g/L BSA, with or without 0.5 mmol/L ouabain, or 100 μmol/L propylthiouracil (PTU)] for 30 min. Incubation was started by replacing medium with 1 mL fresh incubation medium containing the same additions and 90,000 cpm/mL ^{125}I-labeled iodothyronine (total T_3 concentration, 24 pmol/L; total rT_3 concentration, 60 pmol/L). Cells were incubated for indicated periods of time in a dark humidified 5% CO_2 incubator at 37 C. Parallel incubations without cells were performed to correct for spontaneous deiodination. After incubation, medium was removed for further analysis. Cells were washed with 2 mL ice-cold PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, and 1.5 mmol/L KH_2PO_4, pH 7.4) and lysed in 0.1 mol/L NaOH. Total cellular binding (TCB) was determined by measuring cell lysate radioactivity. The media were analyzed for iodide, conjugates, and remaining iodothyronines using Sephadex LH-20 chromatography, as described previously (28). All tracers used were always LH-20 purified on the day of the experiment.

For comparison, studies with primary cultures of rat hepatocytes were performed as described previously (8).

Nuclear binding. Experiments were performed essentially the same as whole cell incubations with the following modifications, as described previously (25). After reaching confluence, cells were preincubated, as described above, in the presence and absence of 0.5 mmol/L ouabain and 10 nmol/L or 10 μmol/L unlabeled T_3, followed by incubation with 1 mL incubation medium containing the same additions and 150,000 cpm/mL 3′-L-[^{125}I]T_3 (total T_3 concentration, 40 pmol/L) for the indicated periods of time. After incubation, the medium was discarded, and the cells were washed twice with 2 mL ice-cold PBS. Cells were scraped from the wells using a rubber policeman and resuspended in 2 mL ice-cold PBS. All of the following procedures were performed on ice. The radioactivity of cells was measured to determine TCB. The cells were pelleted by centrifugation (300 × *g*, 4 C, 7 min), and subsequently solubilized in 1 mL PBS-0.5% Triton X-100. After 2 min of continuous vortexing, nuclei were spun down (900 × *g*, 4 C, 5 min) and washed twice in PBS-0.5% Triton X-100. After the final wash, the radioactivity of the nuclear pellet was measured to determine nuclear binding.

General. All metabolism experiments were standardized for protein contents, determined in the individual cell lysates using a commercially available kit based on the method of Bradford (Bio-Rad, Veenendaal, The Netherlands). Nuclear binding experiments were standardized for cell density. To monitor the quality of cells after incubation, ATP was determined in perchloric acid cell extracts using the luciferin/luciferase system (Lumac Adenylate Energy Charge Kit Lumac, 3 M bv, Landgraaf, The Netherlands). For several experiments, the protein/DNA ratio was determined to verify whether protein contents indeed reflects cell number. The DNA concentration was determined using Hoechst dye, as described previously (29). Data are expressed as the mean ± SD for incubations assayed in triplicate, with each experiment performed at least three times. Statistical analysis of data was performed using Student's *t* test for unpaired observations.

Results

All metabolism incubations were standardized for protein contents, as these values can routinely be obtained for each individual sample without the need for parallel incubations. For control incubations, a protein/DNA ratio of 1.44 ± 0.24 mg protein/μg DNA was found, without significant changes under any of the incubation conditions. Therefore, we believe that this is a very useful standardization technique. Nuclear binding experiments were standardized for cell density, as in these experiments the cells are used for the isolation of nuclei, excluding the possibility of lysing cells in NaOH for protein determination.

Figure 1 shows the time-dependent increase in total cellular binding of T_3. The presence of 10 μmol/L unlabeled T_3 significantly reduced T_3 uptake during the entire time course ($P < 0.001$). Incubations in the presence of 0.5 mmol/L ouabain start to differ significantly from controls after 120 min or longer ($P < 0.001$), similar to incubations in the presence of 10 nmol/L unlabeled T_3 ($P < 0.01$ at 2 h and $P < 0.001$ at 17 h). After 17 h, the total cellular binding is decreased by 39.7 ± 4.6% in the presence of 0.5 mmol/L ouabain and by 28.5 ± 1.8% in the presence of 10 nmol/L unlabeled T_3.

Figure 2 shows the time-dependent increase in nuclear bound T_3. Uptake of T_3 into HepG2 cells was studied using nuclear binding of radiolabeled T_3 as proof that T_3 had entered the cells. Nuclear binding increased as a percentage of the total cellular binding from 4.1 ± 1.0% at 15 min to 19.6 ± 0.71% at 17 h. In the presence of 10 μmol/L unlabeled T_3, nuclear binding did not increase above background during the entire time course. Incubations in the presence of 10 nmol/L unlabeled T_3 differed significantly from control incubations after 15 min or longer ($P < 0.01$ and $P < 0.001$, respectively). Incubations in the presence of 0.5 mmol/L ouabain differed significantly from control incubations after 120 min or longer ($P < 0.005$ and $P < 0.001$ respectively). After 17 h, nuclear binding was decreased by 71.5 ± 4.5% in the presence of ouabain and by 92.6 ± 0.6% in the presence of 10 nmol/L unlabeled T_3.

The metabolism of rT_3 by HepG2 cells was compared with incubations of primary cultures of rat hepatocytes. Figure 3 shows the iodide and conjugate production from rT_3 during a 17-h incubation with HepG2 cells. Iodide production was 15.90 ± 0.80 fmol/mg protein and decreased to 13.56 ± 0.27 fmol/mg protein in the presence of 0.5 mmol/L ouabain

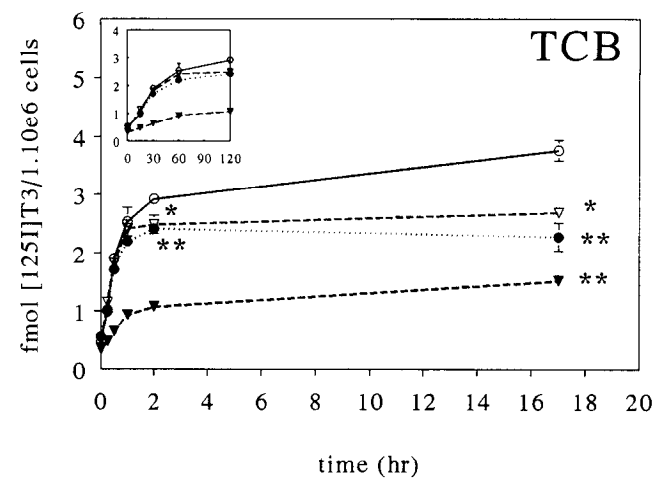

FIG. 1. TCB of [^{125}I]T3 (total T_3 concentration, 40 pmol/L in culture medium containing 0.5% BSA) by HepG2 cells at 37 C. The *inset* shows the initial uptake for 0–120 min. Total cellular binding is decreased in the presence of 0.5 mmol/L ouabain (●) and in the presence of 10 nmol/L (▽) and 10 μmol/L (▼) unlabeled T_3. Ouabain and 10 nmol/L unlabeled T_3 incubations start to differ significantly from control incubations (○) after 120 min ($P < 0.001$ and $P < 0.01$, respectively), whereas incubations in the presence of 10 μmol/L unlabeled T_3 are significantly lower during the entire time course ($P < 0.001$). **, $P < 0.001$; *, $P < 0.01$.

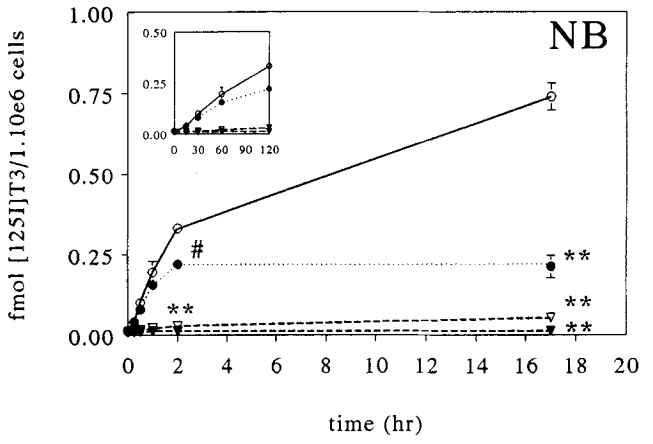

FIG. 2. Nuclear binding of [^{125}I]T_3 (total T_3 concentration, 40 pmol/L in culture medium containing 0.5% BSA) by HepG2 cells at 37 C. After incubation, nuclei were isolated by repeated washing of cells with PBS-0.5% Triton X-100. The *inset* shows the initial uptake for 0–120 min. There is a time-dependent increase in nuclear bound T_3 in control incubations (○), which is decreased in the presence of 0.5 mmol/L ouabain (●; $P < 0.005$ for 120 min or longer). The decrease in the presence of 10 nmol/L (▽) unlabeled T_3 is significant at 15 min ($P < 0.01$) or longer ($P < 0.001$), whereas in the presence of 10 μmol/L (▼) unlabeled T_3, all data are significantly less than those from control incubations ($P < 0.001$). **, $P < 0.001$; #, $P < 0.005$.

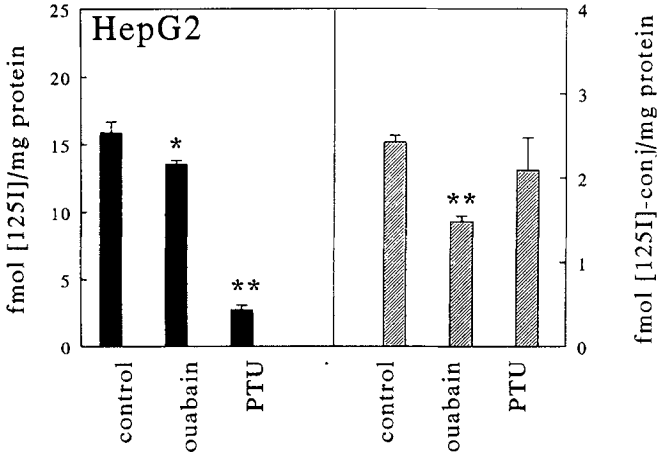

FIG. 3. Metabolism of [^{125}I]rT_3 (total rT_3 concentration, 60 pmol/L in culture medium containing 0.5% BSA) by HepG2 cells during a 17-h incubation at 37 C. Iodide production (*left axis*) decreases in the presence of 0.5 mmol/L ouabain ($P < 0.01$) and 100 μmol/L PTU ($P < 0.001$). Conjugate production (*right axis*) decreases in the presence of 0.5 mmol/L ouabain ($P < 0.001$), but is not significantly different from control incubations in the presence of PTU. **, $P < 0.001$; *, $P < 0.01$.

($P < 0.01$) and to 2.75 ± 0.34 fmol/mg protein in the presence of 100 μmol/L PTU ($P < 0.001$). Conjugate production in these incubations decreased from 2.43 ± 0.08 to 1.48 ± 0.07 fmol/mg protein in the presence of ouabain ($P < 0.001$), without changes in the presence of PTU (2.09 ± 0.39 fmol/mg protein; not significant).

Iodide and conjugate production from rT_3 during a 90-min incubation with rat hepatocytes is shown in Fig. 4. Iodide production decreased from 17.63 ± 1.14 to 1.99 ± 0.04 fmol/mg protein in the presence of PTU ($P < 0.001$), without significant changes in the presence of ouabain (17.60 ± 1.47 fmol/mg protein). Conjugate production increased from

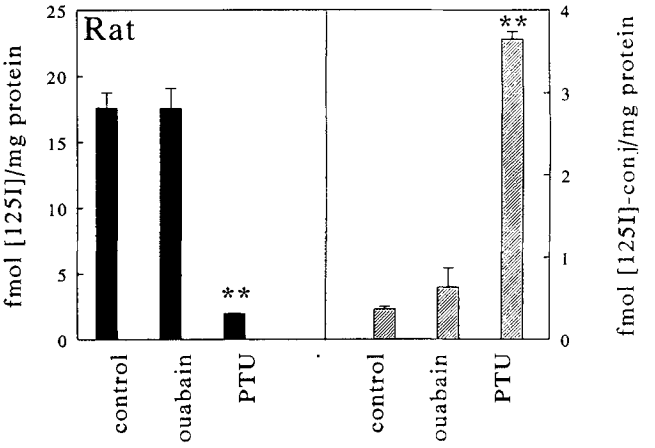

FIG. 4. Metabolism of [^{125}I]rT_3 (total rT_3 concentration, 60 pmol/L in culture medium containing 0.5% BSA) by primary cultures of rat hepatocytes during a 90-min incubation at 37 C. Iodide production (*left axis*) decreases in the presence of 100 μmol/L PTU ($P < 0.001$), but shows no significant changes in the presence of 0.5 mmol/L ouabain. Conjugate production (*right axis*) shows no significant changes in the presence of 0.5 mmol/L ouabain and an almost 10-fold increase in the presence of PTU ($P < 0.001$). **, $P < 0.001$.

0.37 ± 0.03 to 3.65 ± 0.09 fmol/mg protein in the presence of PTU ($P < 0.001$), but showed no significant change in the presence of ouabain.

Discussion

In this study the uptake and metabolism of T_3 and rT_3 in human liver-derived HepG2 cells were investigated. We show, by measuring nuclear bound hormone, that T_3 enters the cellular compartment. There is a time-dependent increase in total cellular binding of T_3. Initial uptake is linear up to 30 min and falls off subsequently. Suppression of linear initial uptake by the high unlabeled T_3 concentration represents saturation of the plasma membrane transport process. As ouabain does not interfere with intracellular binding of thyroid hormone (30), its suppressive effect on T_3 uptake indicates sodium dependency of the plasma membrane transport process. In the same experiments, nuclear bound radioactivity was determined. There is a clear time-dependent increase in the percentage of hormone bound to nuclear sites, suggesting movement of T_3 from extranuclear sites (plasma membrane and cytoplasma) to the nucleus. The effects of the low concentration of unlabeled hormone (10 nmol/L T_3) and ouabain are much more pronounced at the nuclear level, suggesting partial nonspecific and ouabain-insensitive binding in the total cellular binding data. This is explained by the fact that there is a substantial amount of T_3 aspecifically bound to the plasma membrane that does not enter the cells (5). The observation that nuclear bound T_3 is decreased by almost 75% in the presence of ouabain suggests that at least 75% of nuclear T_3 must have entered the cell by ouabain-sensitive transport systems, as this concentration of ouabain (0.5 mmol/L) has no influence on cell viability, as monitored by ATP and DNA measurements (data not shown). Our data show that although plasma membrane transport inhibitors have low to moderate effects on total cellular binding, the effects on the actual transport process, and thus, the avail-

ability of intracellular thyroid hormone may be substantial. At the nuclear level this has its consequences for occupancy of hormone receptors and, consequently, for initiation of biological effects.

As there is very little nuclear binding of rT_3, which is also reflected by its very low total cellular binding (data not shown), we have used the intracellular deiodination and conjugation of this hormone to study cellular entry in human liver-derived HepG2 cells. Analogous to previous studies on T_3 metabolism in HepG2 cells (27), we have performed parallel incubations with primary cultures of rat hepatocytes for comparison.

Taking the different incubation times into consideration, there is an approximately 10-fold higher rate of deiodination and conjugation in rat hepatocytes *vs.* HepG2 cells. This is observed not only for rT_3, but also for T_4 metabolism. For this reason, we were not able to study T_4 metabolism, which would require long incubation times such that cell growth would be compromised.

In this study we present data from 90-min rat hepatocytes incubations compared to those from 17-h human HepG2 incubations. There is substantial rT_3 deiodination in HepG2 cells, comparable to that in rat hepatocytes, in contrast to the previously reported absence of T_3 deiodination (27). This confirms the presence of functional type I deiodinase activity in HepG2 cells and is in concert with our previous conclusion that the lack of T_3 deiodination is due to deficient T_3 sulfation. There is a small but significant decrease in iodide production in the presence of ouabain ($P < 0.01$) in HepG2 cells, which is not found in rat hepatocytes. This could be explained by the fact that in the present studies rat hepatocytes were cultured in HepG2 medium. This medium differs slightly from the normal rat hepatocyte medium. Under optimal culture conditions, we always find ouabain inhibition of thyroid hormone transport in rat hepatocytes (5–8). In the presence of PTU, however, iodide production by both cell types is reduced dramatically by inactivation of type I deiodinase.

Comparison of conjugate production in HepG2 cells and rat hepatocytes shows a relatively high conjugate production in HepG2 cells in control incubations (2.43 ± 0.08 *vs.* 0.37 ± 0.03 fmol/mg protein, HepG2 *vs.* rat). Conjugates of HepG2 cells contained only glucuronides, whereas conjugates produced by rat hepatocytes contained glucuronides but little sulfate. There is a decrease in conjugate production in HepG2 cells in the presence of ouabain, explained by decreased cellular entry of the hormone, which is not found in rat hepatocyte incubations due to suboptimal incubation conditions (see above). The increase in rT_3 conjugates by PTU in rat hepatocytes is explained by the decreased deiodination of rT_3 by type I deiodinase and, consequently, the increased availability of rT_3 for conjugation. There is no increase in conjugate production by HepG2 cells in the presence of PTU. One theoretical explanation for this phenomenon could be that in control incubations, the glucuronyl transferases in HepG2 cells are already saturated using this rT_3 concentration. This is supported by the observation that in HepG2 control incubations, there is a relatively high glucuronidation of rT_3 compared to rat hepatocytes. A more likely explanation is a shift from 3,3′-reverse diiodothyronine glucuronides in control incubations to rT_3 glucuronides in PTU incubations. Both compounds accumulate in the same fractions after LH-20 chromatography.

It has previously been reported that amino acids interfere with the uptake of thyroid hormone. Studies using rat erythrocytes suggest inhibition of T_3 uptake by tryptophan (17, 19), whereas studies using brain-derived cells suggest involvement of the amino acid uptake system L (14, 31). All experiments described in this paper were performed in cell culture medium, but all amino acid concentrations remained well below the reported K_i values for T_3 uptake.

In conclusion, we have shown that there is a time-dependent ouabain-sensitive increase in nuclear binding of T_3 in human liver-derived HepG2 cells. These cells do not deiodinate T_3, due to deficient sulfation (27), but do deiodinate rT_3, which enters the cells through an ouabain-sensitive transport system. There is substantial glucuronidation, but no detectable sulfation of rT_3 and no increase in glucuronidation in the presence of PTU.

Combining all data that we have obtained in our laboratory using the human liver-derived cell line HepG2 and our previous data concerning the uptake and metabolism of iodothyronines in primary cultures of human hepatocytes (21), we propose that HepG2 cells are a suitable model for the human liver. Both human hepatocytes as well as HepG2 cells show an approximately 10-fold lower rate of metabolism as rat hepatocytes. In addition, both human hepatocytes and HepG2 cells show, in contrast to rat hepatocytes, no iodide production from T_3 and no increase in T_3 conjugates in the presence of PTU. In HepG2 cells we have shown that this is caused by deficient sulfation of T_3 (27) despite sulfation of other compounds in these cells (26). Based on these data we propose that in the human liver, deiodination of T_3 is only of minor importance due to deficient sulfation of this hormone. These observations caution against uncritical use of the rat liver as a model for hepatic thyroid hormone metabolism in man.

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