Thyroid hormone metabolism in primary cultured rat hepatocytes. Effects of glucose, glucagon, and insulin.

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Research Article

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Thyroid Hormone Metabolism in Primary Cultured Rat Hepatocytes

EFFECTS OF GLUCOSE, GLUCAGON, AND INSULIN

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ABSTRACT Primary cultured adult rat hepatocytes were used to study the regulation of thyroid hormone deiodination. Control studies showed that these cells survived for at least 4 d, during which time they actively deiodinated both the phenolic (5'-) and nonphenolic (5-) rings of L-thyroxine (T_4),3,5,3'-triiodo-L-thyronine, and 3,3',5'-triiodothyronine. Increasing the substrate concentration caused a decrease in fractional iodide release and a corresponding increase in conjugation with sulfate and glucuronide. Propylthiouracil strongly inhibited the 5'-deiodinase activity and caused only a slight decrease in 5-deiodinase activity. Thus, these monolayer-cultured cells preserved many of the properties of normal hepatocytes. Incubation with combinations of insulin, glucagon, and/or glucose for 5 h showed that insulin stimulated T_4 5'-deiodination, whereas glucagon inhibited the insulin stimulation but had no effect in the absence of insulin. Glucose had no effect and did not alter the effect of the hormones. The insulin-enhanced deiodination increased between 1 and 5 h, which suggests that the previous inability to demonstrate an insulin effect was due to the short survival of the in vitro liver systems used in those studies. The present data suggest that the inhibition of T_4 5'-deiodination observed during fasting, and its restoration by refeeding, may be related to the effects of feeding on insulin and glucagon release rather than on glucose per se.

INTRODUCTION

The mammalian organism has an apparent adaptation mechanism for thyroid hormone metabolism by which the conversion of L-thyroxine (T_4)[1] to biologically active 3,5,3'-triiodo-L-thyronine (T_3) is decreased in fasting and increased by refeeding, especially of carbohydrate (1–5). This regulatory system may operate in starvation to conserve limited energy sources for essential biochemical processes. Although, in the starved rat, thyroid gland secretion is also diminished (6, 7), a number of studies have demonstrated decreased production of T_3 from T_4 in the liver. Various hypotheses have been proposed to explain this altered production of T_3; namely, decreased cofactor activity in the cytosol (8–11), such as nonprotein sulfhydryl compounds (mainly glutathione), decreased NADPH concentration and decreased glutathione transhydrogenase (12), a decreased amount of deiodinases (13), or decreased cellular uptake of T_4 (14). Most of these studies, however, were performed using crude liver homogenate under quite unphysiological conditions. Although a few studies used liver slices (15) and perfused liver (14), their limited viability, several hours at most, make them unsuitable in an investigation of the regulatory mechanism of the starvation effect, which takes place in days rather than hours (1–5). This disadvantage can be resolved by using primary cultured adult rat hepatocytes (16), which also offer the advantage of cellular homogeneity, the opportunity to conduct experiments with homologous cells from a single rat, and especially the ability to study the effect of various nutrients or hormones without the secondary hormonal responses from other organs, as can occur in whole animal studies.

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[1] *Abbreviations used in this paper*: Arg(−)DV medium, arginine-free Dulbecco-Vogt medium; complete Arg(−)DV medium, Arg(−)medium supplemented with bovine insulin and hydrocortisone hemisuccinate; EE medium, Eagle's minimal essential medium with Earle's balanced salt solution; complete EE medium, EE medium supplemented with insulin and hydrocortisone hemisuccinate; PTU, 6-*n*-propyl-2-thiouracil; rT_3, 3,3',5'-triiodo-L-thyronine; T_3, 3,5,3'-triiodo-L-thyronine; T_4, L-thyroxine.

Previously, we reported (17) that primary cultured adult rat hepatocytes maintained 5'- and 5-deiodinating activities for at least 4 d. In contrast to poorly differentiated monkey hepatocarcinoma cells (NCLP-6E), these well-differentiated rat hepatocytes preserved enzymes of the urea cycle (conversion of [^{3}H]ornithine to [^{3}H]arginine), the transsulfuration pathway (glutathione synthesis from methionine), and sulfo- and glucurono-conjugation (17), and thus are considered to be a more suitable model for studying thyroid hormone metabolism. By culturing the hepatocytes in cystine and methionine-deficient medium, thereby decreasing the intracellular glutathione concentration to <10% of the control (17), we also demonstrated that thyroid hormone deiodination is not modulated by nonprotein sulfhydryl levels in the liver.

Using these well-differentiated adult rat hepatocytes, we investigated the effects of glucose, glucagon, and insulin. We found that thyroid hormone metabolism was not affected by glucose alone, but rather by insulin, and that the stimulatory effect of insulin was abolished by glucagon. Furthermore, the insulin effect was evident at 10 ng/ml, a concentration found in the portal vein (18). This suggests that the fasting- and feeding-induced alteration of thyroid hormone metabolism may be regulated by these hormones.

METHODS

[3',5'-^{125}I]T_4 (~1 mCi/μg) and [3'-^{125}I]T_3 (~1 mCi/μg) were purchased from New England Nuclear, Boston, Mass. [3,5-^{125}I]T_4 (~2 mCi/μg) and [3,5-^{125}I]T_3 were synthesized according to Cahnmann's methods (19, 20). All radioactive tracers stored >10 d (in ethanol at 4°C) were purified by ion-exchange column chromatography (0.9 × 10 cm, Dowex 50W-X4, 30–35 μm, Bio-Rad Laboratories, Richmond, Calif.), followed by passing through a short column (0.9 × 1.0 cm) of Dowex 50W-X4 to remove $^{125}I^-$ as described previously (17). Other materials were 6-*n*-propyl-2-thiouracil (PTU) (Sigma Chemical Co., St. Louis, Mo.), bovine insulin (Sigma), bovine glucagon (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), and hydrocortisone hemisuccinate (Sigma).

Primary culture of adult rat hepatocytes. Culture medium routinely used was arginine-free Dulbecco-Vogt medium containing 10 mM Hepes, 15% dialyzed fetal bovine serum, streptomycin sulfate (100 μg/ml), penicillin (100 U/ml), and ornithine (0.4 mM) [Arg(−)DV medium], which was supplemented with bovine insulin (10 μg/ml) and hydrocortisone hemisuccinate (10 μg/ml) [complete Arg(−)DV medium]. In this complete Arg(−)DV medium, only cells containing enzymes for the urea cycle (hepatocytes) can grow (21).

In some cases, Eagle's minimal essential medium with Earle's balanced salt solution containing 10% undialyzed fetal bovine serum, 10 mM Hepes, 2 mM glutamine, 1% nonessential amino acids, streptomycin sulfate (100 μg/ml), and penicillin (100 U/ml) (EE medium) was used and was supplemented with insulin (10 μg/ml) and hydrocortisone hemisuccinate (10 μg/ml) (complete EE medium).

Primary culture of adult rat hepatocytes was performed by the method of Leffert et al. (16), with slight modifications as described previously (17). In brief, collagenase-dispersed hepatocytes from a single rat (200–300 g) were suspended in complete Arg(−)DV medium (cell density, 0.5–0.6 × 10^6/ml). Aliquots (5-ml) of the cell suspension were added to 25 cm^2 Falcon plastic flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.), and kept at 37°C under 5% CO_2 and 95% air; at this optimal cell density the recovery efficiency (ratio of attached cells to total cells 24 h after plating) was 20–30%. In the presence of supraphysiological concentrations of insulin and hydrocortisone, and without changing the medium, the number of cells increased after 3 d to ~1.5 × 10^6/flask. The hepatocytes became flattened, polyhedral in shape, and formed trabeculae which reached confluence in some places. After 4 d, the cells began to degenerate, becoming round and granular, and losing adherence to the plastic. By day 7 almost all cells became detached.

When hepatocytes were grown in complete EE medium, there was better recovery efficiency (>30%) at 24 h, and a greater number of cells survived after 3 d (~2 × 10^6/flask); but hepatocytes grown in complete EE medium started to degenerate at the beginning of the 4th day after plating, and nonparenchymal or nonarginine-synthesizing cells could also grow. Therefore, arginine-free, ornithine-supplemented Dulbecco-Vogt medium was usually used.

Metabolism of various concentrations of T_4, T_3, and 3,3',5'-triiodithyronine (rT_3) added to the medium. Cells grown in complete EE medium for 2 d were washed three times with 5 ml of Dulbecco buffer, and 3 ml of serum-free EE medium was added. After a 1-h preincubation at 37°C under 5% CO_2 and 95% air, T_4, T_3, or rT_3 dissolved in 30 μl of 5 mM NaOH was added to a final concentration of 1 nM–10 μM, followed by the addition of ~300,000 cpm of [3',5'-^{125}I]T_4, [3'-^{125}I]-T_3, or [3',5'-^{125}I]rT_3, respectively. In some experiments, [3,5-^{125}I]T_4 or [3,5-^{125}I]T_3 was used. After an additional 3 and 15 h incubation, 0.5 ml of medium was taken and 10 μg of cold T_4, T_3, or rT_3 dissolved in 10 μl of alkalinized *n*-butanol was added as carrier. After lyophilization of the samples, ^{125}I-labeled iodothyronine metabolites were analyzed by thin-layer chromatography (TLC) with the solvent system of *n*-butanol saturated with 2 N NH_4OH, as described before (22).

Effect of PTU on T_4 and T_3 metabolites in the medium. 2 d after growing the hepatocytes in complete Arg(−)DV medium, spent medium was aspirated, and the cell monolayer was washed three times with 5 ml of Dulbecco-Vogt buffer (pH 7.4). Then, 3 ml of serum-free Arg(−)DV medium containing various concentrations of PTU (0.001–0.1 mM) was added. After a 1-h preincubation at 37°C under 5% CO_2 and 95% air, [3',5'-^{125}I]T_4 [3,5-^{125}I]T_4, [3'-^{125}I]T_3 or [3,5-^{125}I]T_3 dissolved in 50 μl of saline was added to a final concentration of ~0.1 nM. After an additional 3-h incubation, 0.5 ml of medium was taken and [^{125}I]iodothyronine metabolites released into the medium were analyzed by TLC as above.

T_4 metabolites in the cells. In some experiments, intracellular T_4 metabolites at 3 h incubation were analyzed after [^{125}I]T_4 metabolites were extracted with *n*-butanol by a modification of Flock's method (23). After an aliquot of medium was taken for analysis, the rest of the medium was aspirated. Cell monolayers were washed with 5 ml of ice-cold Dulbecco buffer (pH 7.4). Then 1 ml of 0.1% sodium dodecyl sulfate solution containing 1 mM PTU, 5 mM diamide, 1 mM *N*-ethylmaleimide, and 10 μM T_4 was added and incubated at 37°C for 1 h. Cell lysates were acidified with 0.1 ml 4 N HCl, and the acidified samples were extracted twice with 2 ml of *n*-butanol. Then 4 ml of chloroform was added to the combined *n*-butanol extract and mixed well. The turbid mixture was extracted with 2 and 1 ml of 2 N NH_4OH, successively. The combined extracts were lyophilized and T_4 metabolites were analyzed by TLC with 5 μg of T_4, T_3, and rT_3 as carriers and markers. The recovery of ^{125}I was ~80%. When 1 ml of [^{125}I]T_4-

containing medium was treated in the same way, ~1% of ^{125}I comigrated with T_3, 1% with rT_3, and 94% with [^{125}I]T_4.

Effect of glucose, glucagon, and insulin. Hepatocytes were grown for 2 d in complete Arg(−)DV medium. Monolayer cells were washed three times with 5 ml of Dulbecco buffer, then incubated for 15 h with 3 ml of serum-free Arg(−)DV medium. (In this serum-free, hormone-free Arg(−)-DV medium, hepatocytes usually survived for up to 48 h, but sometimes started to degenerate after 36 h. All the experiments were therefore performed within 3 d after plating.) Then 30 μl of insulin or glucagon dissolved in 10 mM HCl was added to a final concentration of 10 ng/ml to 10 μg/ml. For control cells, 30 μl of 10 mM HCl was added. After a 5-h preincubation at 37°C under 5% CO_2 and 95% air, [3′,5′-^{125}I]-T_4 dissolved in 30 μl of 0.9% NaCl was added, and after an additional 3-h incubation, [^{125}I]T_4 metabolites in the lyophilized medium were analyzed by TLC as above.

To investigate the fasting and feeding effects on thyroid hormone metabolism, the effects of glucose, glucagon, and insulin in various combinations were studied after the hepatocytes were grown for 2 d in complete Arg(−)DV medium. Washed monolayer cells were then incubated with 3 ml of serum-free, glucose-free Arg(−)DV medium for 15 h. Insulin, and/or glucagon were added to a final concentration of 5 μg/ml for each. For control cells, 30 μl of 10 mM HCl was added. In some flasks, 30 μl of glucose was added to a final concentration of 250 mg/dl. After a 5-h incubation, [3′,5′-^{125}I]T_4 dissolved in 30 μl of saline was added to a final concentration of ~0.1 nM, and, after an additional 3-h incubation, [^{125}I]T_4 metabolites secreted in the medium were analyzed by TLC. In this serum-free, glucose-free medium, cells appeared intact for >24 h, but some degeneration started by 40 h. Therefore, all the experiments were completed within 24 h after changing the medium, or within 72 h after plating.

After the medium was taken for analysis of [^{125}I]T_4 metabolites, washed monolayer cells were treated with 1 ml of 0.05% trypsin dissolved in Ca^{++}, Mg^{++}-free Dulbecco buffer containing 0.5 mM EDTA. After 2–3 min incubation at room temperature, 3 ml of EE medium was added and the number of cells was counted in a hemocytometer. Since there was no significant difference in cell numbers in control, glucagon, and insulin (~1.5×10^6 flask), the results were expressed as percentage of total ^{125}I.

To investigate a more prolonged period of T_4 metabolism approaching the steady-stage condition, [^{125}I]T_4 metabolism was investigated in the presence of serum in a few experiments. After the cells were grown in complete Arg(−)DV medium for 24 h, medium was replaced with 3 ml of Arg(−)-DV medium containing 10% dialyzed serum, streptomycin (100 μg/ml), penicillin (100 U/ml), 10 mM Hepes, various concentrations of glucose (0, 1, and 8 mg/ml), and ~500,000 cpm of [3′,5′-^{125}I]T_4. The T_4 concentration in the dialyzed fetal bovine serum was 9 μg/dl. As a cell-free control, [^{125}I]T_4 was incubated in the same medium in a cell-free flask. After a 48-h incubation, 1 ml of medium was taken, and [^{125}I]T_4 metabolites were extracted according to Flock's (23) method, and analyzed by TLC. The recovery of radioiodine was >80%. Nonspecific deiodination of [^{125}I]T_4 was 1.3%, with <1% of T_3 and rT_3 contaminants.

RESULTS

Effect of T_4, T_3, and rT_3 concentration on thyroid hormone metabolism (Fig. 1). The major metabolite of [3′,5′-^{125}I]T_4 at low concentrations, as reported previously (17), was $^{125}I^-$, whereas T_4-conjugates were found with little iodide release at higher T_4 concentration 1–10 μM. The same metabolites were obtained when [3,5-^{125}I]T_4 was used as a tracer (data not shown). The ratio of $^{125}I^-$ released from [3,5-^{125}I]T_4 to that from [3′,5′-^{125}I]T_4 was ~1 at all substrate concentrations. Since little iodide was released from either ring of T_4 at the higher concentrations, the bands designated as C_1 (R_f 0.04) and C_2 (R_f 0.14) were nondeiodinated T_4 derivatives. The treatment of isolated C_1 and C_2 bands with β-glucocuronidase or sulfatase revealed that these were composed mainly of T_4 glucuronide and T_4 sulfate, respectively.

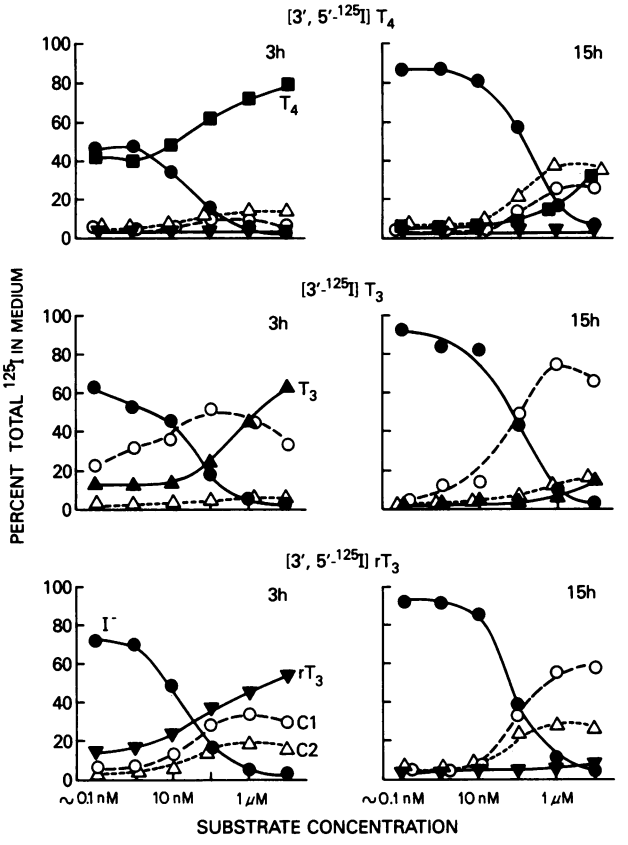

FIGURE 1 Thyroid hormone metabolism at various substrate concentrations. Hepatocytes were grown for 2 d in complete EE medium. Monolayer cells were incubated with 3 ml of serum-free EE medium. After a 1-h preincubation at 37°C, T_4, T_3, or rT_3 dissolved in 30 μl of 5 mM NaOH was added to a final concentration of 1 nM–10 μM followed by the addition of ~300,000 cpm of [3′,5′-^{125}I]T_4, [3′-^{125}I]T_3, or [3′,5′-^{125}I]rT_3. After an additional 3- and 15-h incubation, medium was taken for analysis. Note that major metabolites are $^{125}I^-$ (●) at the lower substrate concentration, but are the conjugates (dotted and broken lines) at the higher concentrations. Percentage of total ^{125}I is indicated as T_4 (■), T_3 (▲), rT_3 (▼), C_1 (glucuronoconjugates, △), and C_2 (sulfoconjugates, ○).

Similarly, the major metabolites of [3′-^{125}I]T_3 were iodide at low concentrations, but T_3 conjugates at the higher concentrations. When [3,5-^{125}I]T_3 was used, a

qualitatively similar chromatogram pattern was obtained with 4% $^{125}I^-$ release at 10 μM, which suggests that the C_1 and C_2 bands were T_3 conjugates. The ratio of $^{125}I^-$ released from [3,5-^{125}I]T_3 to that from [3′-^{125}I]T_3 at 15 h was 1.9, 3.3,and 3.1, at 0.1, 1, and 10 μM, respectively, which suggests that the major deiodination pathway of T_3 was to 3,3′-diiodo-L-thyronine (3,3′-T_2), which was identified in the autoradiogram exposed >3 d (data not shown). The treatment of C_1 and C_2 bands with β-glucuronidase or sulfatase revealed that these bands were mainly T_3 glucuronide, and T_3 sulfate (with small amounts of 3,3′-T_2-sulfate), respectively. In contrast to the T_4 conjugates, much more T_3 sulfate was formed, compared with the glucuronoconjugate.

[3′,5′-^{125}I]rT_3 metabolism also proceeded rapidly through outer ring deiodination at the lower concentrations, but, at the higher concentrations, it was sulfo- and glucurono-conjugated.

It can be seen that thyroid hormone 5′-deiodination proceeds rapidly in the order of $rT_3 > T_3 > T_4$ (Fig. 1 at 3 h), and that sulfoconjugation takes place in the order of $T_3 > rT_3 > T_4$, with reciprocally decreased formation of glucuronoconjugates ($T_3 < rT_3 < T_4$) (Fig. 1 at 15 h). Using T_3, the best substrate for phenol sulfotransferase, [^{125}I]T_3 metabolism was studied in the absence of exogenous sulfate (streptomycin sulfate) in the medium; no change in T_3 sulfate formation was detected. This indicates that primary cultured rat hepatocytes, in contrast to monkey hepatocarcinoma cells (17), can synthesize sulfate endogenously.

PTU effect on T_4 and T_3 metabolism. As shown in Table I and Fig. 2, PTU inhibited 5′-deiodination of [^{125}I]T_4 in a dose-dependent fashion. On the other hand, 5-deiodination of [^{125}I]T_4 was only slightly affected even at 0.1 mM PTU. The marked inhibitory effect on 5′-deiodination gave rise to an increased amount of rT_3. As expected, slightly more rT_3 was observed in metabolism of [3′,5′-^{125}I]T_4 than with that of [3,5-^{125}I]T_4. The inhibition of deiodination of T_4 was accompanied by an increase in T_4 conjugates. This was even more striking after further incubation for 5 h (Fig. 2A).

Analogous with its effect on [^{125}I]T_4 metabolism, PTU also markedly inhibited outer ring deiodination of [^{125}I]T_3 (Fig. 2B). Little inhibitory effect was observed on inner ring deiodination of T_3, but when the percentage of ^{125}I released from [3,5-^{125}I]T_3 was measured, a slight but significant inhibitory effect was observed as it was with T_4. The striking inhibition of phenolic ring deiodination and the slight inhibition of nonphenolic ring deiodination of T_3 gave rise to increased formation of conjugates, especially of sulfoconjugates (Table I and Fig. 2).

T_4 metabolites in the cells. Intracellular T_4 metabolites were analyzed after incubation for 3 h with ~0.1 nM [^{125}I]T_4. A slight but distinct amount of T_3 (2–3%) was detected. Radioactivity comigrated mostly with T_4

TABLE I
PTU Effect on [^{125}I]T_4 Metabolism

PTU	[^{125}I]T_4 metabolites					
	I^-	T_4	rT_3	C_2	C_1	Origin
mM	*% of total*					
[3′,5′-^{125}I]T_4						
0 (n = 4)	32.2±2.4	56.4±1.9	3.7±0.8	5.8±1.4	3.0±0.9	0.4±0.2
0.001 (n = 3)	9.3±2.7*	57.8±8.2	11.5±1.2‡	13.9±3.2	7.0±1.8	0.5±0.1
0.01 (n = 4)	4.9±0.7‡	60.9±8.8	14.4±2.6*	12.9±4.3	6.4±2.6	0.3±0.1
0.1 (n = 4)	3.5±0.4‡	62.6±8.5	13.8±2.5*	12.3±3.6	7.2±2.8	0.2±0.1
[3,5-^{125}I]T_4						
0 (n = 3)	27.7±1.3	58.2±2.4	2.0±0.7	8.6±1.7	2.8±0.4	0.7±0.1
0.001 (n = 2)	20.2	59.1	8.9	8.3	3.1	0.4
0.01 (n = 3)	19.5±1.0‡	61.0±0.3	8.2±0.3*	6.9±0.5	3.2±0.9	0.9±0.3
0.1 (n = 3)	16.6±2.2*	65.3±1.9*	7.7±0.1*	6.0±0.2	3.7±0.9	0.7±0.2

Rat hepatocytes were grown in complete Arg(−)DV medium for 2 d. Then medium was replaced with 3 ml of serum-free Arg(−)DV medium, and PTU was added. After a 1-h preincubation, [3′,5′-^{125}I]T_4 or [3,5-^{125}I]T_4 was added to a final concentration of 0.1–0.2 mM. After an additional 3-h incubation, 0.5 ml of the medium was taken for analysis of [^{125}I]T_4 metabolites released from the cells. No T_3 band was detected on the autoradiographs, and the T_3 area was not counted in these experiments. C_1 and C_2 represent glucurono- and sulfoconjugates, respectively. Data are mean±SEM. Statistical analyses were performed by paired t test.
* $P < 0.05$.
‡ $P < 0.01$.

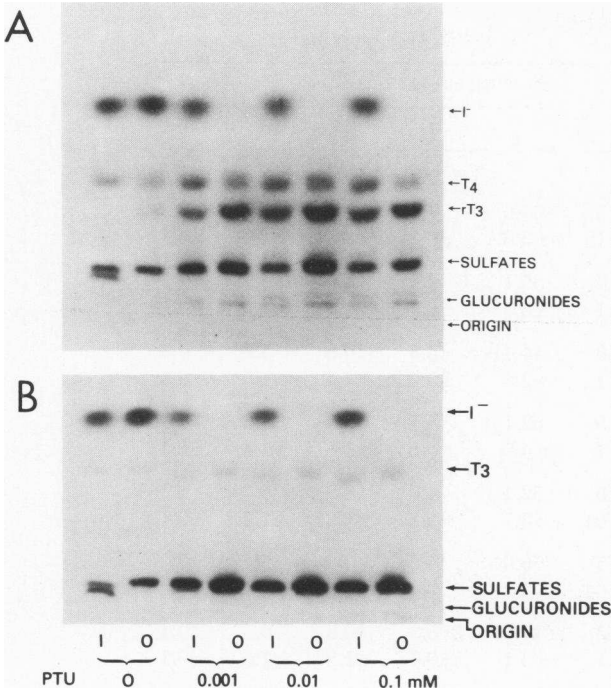

FIGURE 2 Effect of PTU on thyroid hormone metabolism. Hepatocytes were grown for 2 d in complete Arg(−)DV medium. Then monolayer cells were incubated with 3 ml of serum free Arg(−)DV medium containing various concentrations of PTU (0.001–0.1 mM). After a 1-h preincubation at 37°C, [3,5-^{125}I]T_4 (inner-ring labeled, I) or [3′,5′-^{125}I]T_4 (outer ring-labeled, O) (Fig. 2A) or [3,5-^{125}I]T_3 (I) or [3′-^{125}I]T_3 (O) (Fig. 2B) were added to a final concentration of ~0.1 nM. After an additional 5-h incubation for T_4 and 3 h for T_3, 0.5 ml of medium was taken and metabolites were analyzed by TLC. Note that PTU markedly inhibits outer ring deiodination of T_4 and T_3.

(~70%), accompanied by I^- (~4%), rT_3 (~10%), C_1-(~5%), and C_2(~4%).

Effect of glucose, glucagon, and insulin. As shown in Fig. 3, preincubation with insulin (10 ng/ml–10 μg/ml) for 5 h caused marked stimulation of 5′-deiodination of [3′,5′-^{125}I]T_4, whereas glucagon had no effect at any concentration. This stimulatory effect of insulin was less marked when [^{125}I]T_4 was added after only a 1-h incubation with insulin.

Next, the effects of glucose, glucagon, and insulin were studied in hepatocytes incubated in serum-free, glucose-free Arg(−)DV medium for 15 h. 5 h after the addition of glucose (2.5 mg/ml), glucagon (5 μg/ml), and/or insulin (5 μg/ml), [3′,5′-^{125}I]T_4 metabolites secreted in the medium during 3 h were analyzed by TLC. As shown in Table II, insulin significantly increased 5′-deiodination, and this stimulatory effect was inhibited by glucagon. This occurred either in the presence or absence of glucose. It should be pointed out that the stimulatory effect of insulin on T_4 metabolism is primarily ascribed to an increased rate of deiodination; conjugation was little affected. Glucagon alone had no effect on T_4 metabolism. In fact, in four of seven experiments, glucagon appeared to show a slight inhibitory effect on 5′-deiodination, but this was not statistically significant (Table II). There was also no effect on the amount of T_3 or rT_3 produced.

There was no difference in [3′,5′-^{125}I]T_4 metabolism between glucose-fed and glucose-deprived groups, which suggests that fasting-induced, and feeding-reversed alterations of thyroid hormone metabolism are modulated by glucoregulatory hormones but not by carbohydrate alone.

To investigate a prolonged effect of glucose-deprivation, cells were grown in aglycemic, normoglycemic (1 mg/ml), and hyperglycemic (8 mg/ml) medium for 48 h in the presence of 10% dialyzed serum containing [3′,5′-^{125}I]T_4. The cell number at 3 d was ~1.5×10^6/flask. Major [^{125}I]T_4 metabolites in the normoglycemic medium were $^{125}I^- = 22.8\%$, $rT_3 = 4.9\%$, $C_1 = 5.3\%$, and $C_2 = 3.5\%$, with little T_3 formation (<1%). Hyperglycemic medium caused no change. A slight decrease in $^{125}I^-$ release was observed in aglycemic medium (19.3%), but because the number of cells grown in glucose-free medium was ~10% less than that in the glucose-fed cells, the interpretation of this change is difficult.

DISCUSSION

Most of our experiments were carried out at a concentration of ~0.1 nM T_4 in the medium, since this is close to the free T_4 concentration in rat serum (24). In our system, ~50% of T_4 was metabolized in 3 h by 1.5×10^6 cells in 3 ml of serum-free medium containing 0.1–0.2 nM T_4 (0.1–0.2 ng of T_4/1.5×10^6 cells per 3 h

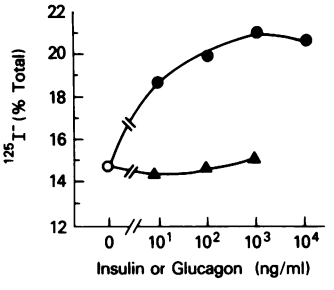

FIGURE 3 Effect of insulin and glucagon on 5′-deiodination of [3′,5′-^{125}I]T_4. Hepatocytes were grown for 2 d in complete Arg(−)DV medium. Monolayer cells were washed and incubated with 3 ml of serum-free, hormone-free Arg(−)DV medium for 15 h. Then 30 μl of insulin (●) or glucagon (△) dissolved in 10 mM HCl was added to a final concentration of 10 ng/ml–10 μg/ml. After a 5-h preincubation, [3′,5′-^{125}I]T_4 was added to a final concentration of ~0.1 nM, and after an additional 3-h incubation, [^{125}I]T_4 metabolites in the medium were analyzed by TLC. Data are means of duplicate determinations.

TABLE II

Effects of Glucose, Glucagon, and Insulin on [3′,5′-^{125}I]T_4 Metabolism

Glucose	Glucagon	Insulin	[^{125}I]T_4 metabolites						
			I^-	T_3	T_4	rT_3	C_2	C_1	Origin
			% of total						
(−)	(−)	(−)	25.5 ±2.4	0.5 ±0.1	54.0 ±3.6	4.8 ±0.8	9.4 ±1.3	5.2 ±1.3	0.3 ±0.1
(−)	(+)	(−)	24.1 ±2.8	0.5 ±0.1	55.1 ±5.3	4.9 ±0.6	9.4 ±1.6	5.7 ±1.6	0.3 ±0.1
(−)	(−)	(+)	32.8* ±1.7	0.6 ±0.1	44.4‡ ±2.8	5.3 ±0.4	10.8 ±1.4	5.7 ±1.5	0.3 ±0.1
(−)	(+)	(+)	26.5§ ±2.6	0.5 ±0.1	52.1§ ±4.7	5.1 ±0.6	9.8 ±1.6	5.5 ±1.4	0.4 ±0.1
(+)	(−)	(−)	26.7 ±1.8	0.5 ±0.04	52.1 ±3.7	4.7 ±0.5	9.7 ±1.3	5.7 ±1.7	0.3 ±0.1
(+)	(+)	(−)	24.0 ±2.5	0.5 ±0.04	56.0 ±5.2	4.2 ±0.4	9.3 ±1.6	5.4 ±1.7	0.3 ±0.2
(+)	(−)	(+)	32.6* ±2.1	0.5 ±0.1	44.8‡ ±3.1	5.3 ±0.4	10.8 ±1.3	5.7 ±1.6	0.4 ±0.1
(+)	(+)	(+)	26.5§ ±2.6	0.4 ±0.1	52.1§ ±4.9	5.1 ±1.0	9.8 ±1.6	5.8 ±1.8	0.3 ±0.1

Cells were grown for 2 d in complete Arg(−)DV medium. The medium was replaced with hormone-free, glucose-free, protein-free Arg(−)DV medium and incubated for 15 h. Then, glucose, glucagon, and/or insulin were added to 2.5 mg/ml, 5 μg/ml, and 5 μg/ml, respectively. After a 5-h incubation, [3′,5′-^{125}I]T_4 was added to a final concentration of ~0.1 nM. After an additional 3-h incubation, 0.5 ml of medium was taken, and [^{125}I]T_4 metabolites were analyzed by TLC. Data are means±SEM of seven experiments. C_1 and C_2 represent glucurono- and sulfoconjugates, respectively. Statistical analysis by paired *t* test are as indicated in footnotes.

* $P < 0.001$ when compared with the control.

‡ $P < 0.01$ when compared with the control.

§ $P < 0.05$ when glucagon(+)-insulin(+) groups are compared with glucagon(−) insulin(+) groups.

or 0.06–0.12 μg/10^8 cells per 24 h). This can be compared with the rate of T_4 metabolism in the liver in vivo. It is generally accepted that 2 μg of T_4/100 g of body weight is required to maintain a rat in a euthyroid state (25). On the assumption that 30% of T_4 is metabolized in the liver, which makes up ~4% of total body weight, and that 1 g of liver contains 1.3×10^8 hepatocytes (26), hepatocytes in vivo metabolize ~0.12 μg of T_4/10^8 cells per 24 h. Therefore, the present experimental condition is a suitable model to study the regulatory mechanism of thyroid hormone metabolism. At the lower concentration of T_4, the major metabolite was $^{125}I^-$; increasing the T_4 concentration to a supraphysiological level (1–10 μM), shifted the metabolism from the deiodinating pathway to the detoxification pathway involving sulfo- and glucurono-conjugation, as reported in vivo (27).

It is of interest to note the relative amounts of sulfo- and glucurono-conjugation for the three iodothyronines studied. For sulfation, these were $T_3 > rT_3 > T_4$. This phenomenon was recently elucidated by Sekura *et al.* (28, 29), who purified sulfotransferase from rat liver (28) and showed that the enzymes had an affinity for T_3, rT_3, and T_4, in this order (29). The poor activity of T_4 as a substrate for the phenolsulfotransferase probably causes it to take the alternate conjugating pathway. Nothing, however, is known about the reactivity of glucuronotransferase with T_4 analogues. In contrast to monkey hepatocarcinoma cells (17, 22), the hepatocytes were able to synthesize T_3 sulfates in the absence of exogenous sulfate, which indicates that the rat hepatocytes synthesize sulfate endogenously by the cysteine-oxidizing pathway (30). The greater percentage of T_3 sulfate found at 3 h compared with 15 h at 0.1–10 nM (Fig. 1) suggests that the sulfoconjugate is slowly hydrolyzed by an intracellular sulfatase (31).

As is well known from both in vivo and in vitro data (32, 33), PTU efficiently inhibits outer ring deiodination of T_4 and T_3. PTU also has a slight inhibitory ef-

fect on inner ring deiodination of T_4 and T_3 (34, 35), which results in decreased serum T_3 concentration with reciprocally increased rT_3 concentration (36). These inhibitory effects on T_4 metabolism increase T_4 metabolism through the conjugating pathways (37). These phenomena can be visualized in Fig. 2. It should be pointed out that the PTU concentration added in the medium was within the range achieved in human subjects taking 200 mg PTU per os (0.5–10 μg/ml or 0.003–0.06 mM) (38).

The most interesting findings in the present experiments are the effects of glucose, glucagon, and insulin on thyroid hormone metabolism. Our data clearly show that insulin increased 5′-deiodination of T_4 after several hours of incubation. The insulin effect was more evident after a 5-h incubation than after a 1-h incubation, and this effect was antagonized by glucagon. The insulin effect was observed at 10 ng/ml, a physiological concentration of insulin in the portal vein (18), which suggests that the hormone might regulate thyroid hormone metabolism at least in the liver in vivo. Since fasting is accompanied by hypoinsulinemia and hyperglucagonemia, and since refeeding, especially of carbohydrate, is accompanied by hyperinsulinemia and hypoglucagonemia (18), the present in vitro data support the hypothesis that thyroid hormone metabolism is modulated by hormones rather than by glucose alone. Recently, Balsam and Ingbar (15) reported that insulin administration to streptozotocin-induced diabetic rats normalized the diminished T_3 generation from T_4 in the liver. Also of interest is the low T_3 and high rT_3 serum concentration in patients with uncontrolled diabetes mellitus in spite of severe hyperglycemia (39). Glucagon is reported to cause low T_3 and high rT_3 levels in the rat (40), although our in vitro data showed no significant inhibitory effect on 5′-deiodination in the absence of insulin. It is of interest to note that the low insulin/glucagon ratio in catabolic states such as infection, traumatic and thermal injury, severe illness, and exercise (41) is also associated with decreased T_3 and increased rT_3 in serum (42, 43).

Insulin is a multipotent hormone that influences various metabolic functions of most tissues (44, 45). The effects may be rapid (within seconds to minutes, such as on membrane permeability), intermediate (within hours, such as on protein synthesis) or delayed (within days, such as on RNA and DNA synthesis). From this point of view, the greater stimulatory effect of insulin observed after a 5-h incubation than after a 1-h incubation suggests that the effect is an intracellular event, such as on *de novo* enzyme synthesis, on inhibition of enzyme degradation, or on activation and inactivation of enzymes. The enzymes modulated by insulin may be the deiodinases themselves, enzymes involved in carbohydrate metabolism, or both. It should be pointed out that the failure of Balsam and Ingbar (15) and of Jennings et al. (14) using liver slices and perfused liver, respectively, to obtain a positive insulin effect might be ascribed to the short incubation time inherent in their methodologies.

Generally, insulin and glucagon are considered to have antagonistic effects on hepatic metabolism. One of the few exceptions, however, is the regulation of liver cell growth; glucagon and insulin synergistically stimulate DNA synthesis and cell proliferation of neonatal or adult rat hepatocytes in primary culture (46, 47) and in vivo (48). Glucagon showed no effect on cell growth in our culture system, and our finding of its antagonistic effect suggests that insulin and glucagon are probably not acting through an effect on cell growth. Insulin is a very potent cell growth factor for hepatocytes, however, and this possibility should therefore be taken into consideration, even though the number of cells did not change significantly between control and insulin-treated cells within the time-course of the present experiments (8-h maximum).

As reported previously (17), 5′- and 5-deiodination proceeded always to the same extent in these primary cultured rat hepatocytes. Therefore, T_3 production should have occurred, and T_3 was in fact demonstrated intracellularly. A curious finding in the present series of experiments, however, was that we were able to detect rT_3 distinctly but could hardly detect T_3 in the culture medium (Tables I, II). We postulated previously (17) that a nonequilibrium condition between T_4 stored in the cell and [^{125}I]T_4 added exogenously might explain this failure, but this is unlikely because incubation with [^{125}I]T_4 in serum-containing medium for 48 h gave the same results. Another possibility is that the supraphysiological concentration of glucocorticoid in the culture medium, which is known to increase rT_3 and decrease T_3 concentration in humans (49), might be responsible. This is also unlikely since hepatocytes grown in hydrocortisone-free Arg(−)DV medium supplemented only with insulin for 1–3 d showed rT_3 but little T_3 either in the medium or in the cells (unpublished observations). The most likely explanation is that proliferating liver cells are no longer normal cells and metabolize T_3 more rapidly than normal. The present data using proliferating hepatocytes (50) are reminiscent of the "low T_3 syndrome" observed in the regenerating liver of partially hepatectomized rats (51). Furthermore, ontogenetic changes in thyroid hormone metabolism were recently reported in immature chicken liver in which T_4 deiodination mainly traverses the non-T_3-forming (probably rT_3-forming) pathway (52). Generally, various quantitative as well as qualitative alterations in enzymes occur during cell proliferation. In this culture method, 50% of cells in the logarithmic stage are in S phase (50), and we have demonstrated in synchronized monkey hepatocarcinoma cells that deiodinases are synthesized pe-

riodically with the highest activity in the late G_1 phase of the cell cycle (22). Our preliminary data suggest that rT_3 degrading enzymes are steadily decreasing in the cultured cells (unpublished observation), which accounts at least in part for the accumulation of rT_3 in the medium. In view of the fact that some enzymes undergo alteration from adult to fetal type in primary cultured hepatocytes (53, 54), phenotypic changes might also occur in the deiodinases in primary cultured rat hepatocytes as seen in monkey hepatocarcinoma cells (22). Although one of the greatest disadvantages of the primary culture of adult rat hepatocytes is the difficulty in obtaining nonproliferating hepatocytes for a number of days, methods have been reported extending the cell survival for up to 10–14 d (54). We are extending our research to learn whether this will provide a more suitable model to investigate the regulatory mechanism of thyroid hormone metabolism in the liver.

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