Loss of thyroid hormone receptor activity in primary cultured rat hepatocytes is reversed by 2-mercaptoethanol

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In primary cultures of rat hepatocytes, specific thyroid-hormone-binding activity diminished with time and was hardly detectable at 24 h. In accordance with the loss of 3,5,3'-tri-iodothyronine (T_3) binding, responses to the hormone disappeared, as indicated by low induction of the thyroid-hormone-responsive gene S14. In contrast, thyroid hormone receptor proteins were present, as determined by immunostaining with a specific antibody against the receptor. Thus the loss of T_3 binding was due to receptor inactivation. After various attempts to restore the T_3 -binding activity, we found that 2-mercaptoethanol, a reducing agent, when added to the culture medium restored the hormone binding activity in a dose- and time-dependent manner. The observed kinetics and experiments using cycloheximide suggested that mercaptoethanol prevented inactivation of the newly synthesized receptors. Oxidoreductive conditions within cells may have a role in determining the level of activity of thyroid hormone receptors.

INTRODUCTION

Thyroid hormone regulates the gene expression of various enzymes. Some of these are inducible enzymes participating in lipogenesis, including malic enzyme, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase [1]. S14 is also a thyroid-hormone-responsive gene, the product of which may be involved in lipid metabolism; the level of its hepatic nuclear transcript increases as early as 10 min after thyroid hormone administration to rats [1,2].

Adult rat hepatocytes in primary culture are used in studies of hormonal regulation. At 24 h after plating the hepatocytes show liver-specific functions and respond to various hormones that are present *in vivo* [3]. We found that the cultured hepatocytes rapidly lose the response of the S14 gene to 3,5,3'-tri-iodothyronine (T_3). We report here that, regardless of the expression of the thyroid hormone receptor (TR), nuclear T_3 -binding activity rapidly decreased in cultured hepatocytes, but this loss was reversed when 2-mercaptoethanol was present in the media.

MATERIALS AND METHODS

Materials

3,5,3'-Tri-[125 I]iodo-L-thyronine ($[^{125}\text{I}]\text{T}_3$, 44.4 MBq/ μg), [α - ^{32}P]dCTP (111 TBq/mmol) and Genescreen nylon filter were purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). Unlabelled T_3 and insulin were obtained from Sigma (St. Louis, MO, U.S.A.); cycloheximide was from P-L Biochemicals Inc. (Milwaukee, WI, U.S.A.); dexamethasone, collagenase (1000 units/mg) and 2-mercaptoethanol were from Wako Pure Chemical Industries (Osaka, Japan); and fetal calf serum and Williams' medium E were from Flow Laboratories (Irvine, Scotland, U.K.). Kanamycin was purchased from Meiji Seika (Tokyo, Japan), collagen from Koken (Tokyo, Japan) and Vectastain ABC immunostaining kit from Vector Laboratories

Inc. (Burlingame, CA, U.S.A.). β -Actin DNA was obtained from Onco (Gaithersburg, MD, U.S.A.).

Primary culture of hepatocytes

Hepatocytes were isolated from male Wistar rats (180–250 g) using Seglen's *in situ* collagenase perfusion method [4] (originally developed by Berry & Friend [5]), and incubated in Williams' medium E [0.05 mg of glutathione/litre (0.16 μM), pH 7.4] supplemented with 5% fetal calf serum, 1 nM-dexamethasone, 1 nM-insulin and 30 μg of kanamycin/ml at a density of 10^5 cells/cm² on collagen-coated culture dishes. The viability of isolated cells exceeded 90%. The incubation was maintained at 37 °C in 5% CO₂/20% O₂/75% air.

Measurement of T_3 binding to hepatocyte nuclei

T_3 binding in cultured cells was determined as described elsewhere [6]. At the indicated times, the hepatocytes were incubated with [125 I] T_3 for 1 h in the presence or the absence of a 10^4 -fold molar excess of unlabelled T_3 , and specific T_3 binding to the nuclei was determined.

T_3 binding to the nuclear extract

TR was extracted from the nuclei of rat liver and of 24 h primary hepatocytes, and the specific [125 I] T_3 binding was determined by the filter-binding assay or by the AG 1X8 resin method [7,8]. Each assay was performed in duplicate. Values are presented as means with range.

Labelling of DNA probes

cDNA inserts of the pS14-cl clone (a gift from Dr. H. C. Towle, Department of Biochemistry, University of Minnesota, Minneapolis, MN, U.S.A.) [9] and β -actin [10] were labelled with [α - ^{32}P]dCTP by the random primer extension method [11].

RNA preparation and Northern blotting

Total RNA was prepared from hepatocytes and liver according to the method of Chomczynski & Sacchi [12], with slight

Abbreviations used: T_3 , 3,5,3'-tri-iodothyronine; TR, thyroid hormone receptor; DAB, diaminobenzidine tetrahydrochloride; DTT, dithiothreitol.

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modification: the extraction step using acid/guanidinium thiocyanate, phenol and chloroform followed by propan-2-ol precipitation was repeated. Total RNA (30 μ g) was electrophoresed in 1% agarose gels containing formaldehyde, then blotted to nylon filters according to the manufacturer's instruction. The filters were prehybridized in $5 \times$ SSC (0.75 M-NaCl/75 mM-sodium citrate)/ $5 \times$ Denhardt's solution/50 mM-sodium phosphate (pH 6.5)/0.1% SDS/1 mM-EDTA/50% formamide/heat-denatured salmon sperm DNA (300 μ g/ml) at 42 °C for 1 h, and hybridized in fresh solution containing labelled DNA probe at 42 °C for 24 h. Filters were washed in $2 \times$ SSC for 10 min at room temperature, $2 \times$ SSC/1% SDS at 65 °C for 1 h and $0.1 \times$ SSC at room temperature for 30 min, and then autoradiographed on Kodak X-OMAT films at -70 °C with an intensifying screen.

Immunostaining of TR

The antibody 4B II, which recognizes TR α_1 and TR β , but not TR variant α_2 [13–16], was used for immunostaining of primary cultured hepatocytes. Cells were fixed with 4% formaldehyde for 4 min and with 70% ethanol for 1 min, then permeabilized with 0.1% Triton X-100 for 20 min. Endogenous peroxidase was inactivated with 0.33% H_2O_2 in methanol for 1 min, and the non-specific reaction was blocked with 20-fold-diluted normal goat serum for 15 min. The primary antibody (4B II or control rabbit serum) was allowed to react overnight at 4 °C. The specimens were washed with phosphate-buffered saline (0.14 M-NaCl, 2.7 mM-KCl, 8.1 mM- Na_2HPO_4 , 1.5 mM- KH_2PO_4 , pH 7.4), treated for 1 h each with the secondary antibody [biotinylated goat anti-(rabbit antibody)], and avidin-biotinylated-peroxidase complex, and finally stained with diaminobenzidine tetrahydrochloride (DAB) in the presence of H_2O_2 .

RESULTS

Nuclear T_3 binding in cultured hepatocytes

Adult rat hepatocytes in primary culture were found to drastically diminish their nuclear T_3 -binding activity. Hepatocytes cultured for various times were further incubated with [125 I] T_3 for 1 h, and specific T_3 binding to the cell nuclei was determined. The specific binding, which was significant in the early periods after plating, decreased to less than 10% of the initial activity by 24 h (Fig. 1). Further culture up to 72 h did not restore the lost T_3 -binding activity. T_3 (0.1 μ M) added from the initiation of cell culture did not improve the nuclear T_3 -binding activity (results not shown).

T_3 -binding activity in the nuclear extract of primary hepatocytes

We searched for an explanation for the loss of T_3 binding to the nuclear sites. Nuclear receptors were extracted with 0.4 M-NaCl from the purified nuclei of rat liver and 24 h-cultured hepatocytes, and incubated with [125 I] T_3 . The specific binding activity of the extract from cultured hepatocytes was less than 5% of that of the rat liver extract containing the same amount of protein (results not shown).

Saturation curves are shown in Fig. 2. Saturable binding sites were present in the liver extract. From Scatchard plots, a maximum capacity of binding sites of 2.9×10^{-13} mol/mg of protein and a K_d of 1.4×10^{-11} M were obtained by computer-assisted analysis. No meaningful plots were obtained for the extract from 24 h-cultured hepatocytes (Fig. 2b).

Decreased response of the S14 gene

The S14 gene, which responds dramatically *in vivo* within 20 min after T_3 administration [2], was examined for induction in cultured hepatocytes. The level of S14 transcript in 28 h-cultured

Fig. 1. Time course of T_3 binding to hepatocyte nuclei

At the indicated times after plating, hepatocytes were further incubated for 1 h at 37 °C with [125 I] T_3 (0.2 nM) with or without a large excess of unlabelled T_3 . [125 I] T_3 bound to the nuclei was determined and corrected for non-specific binding, as described in [6]. The Figure is representative of five similar experiments having different time points and cell densities, giving essentially the same results. Values are the means with ranges of duplicate determinations.

Fig. 2. T_3 -binding activity of extracted nuclear receptors

T_3 receptors were extracted with 0.4 M-NaCl from the nuclei of rat liver and the 24 h-cultured hepatocytes, and then incubated with [125 I] T_3 at various concentrations (0.1–4 nM) for 20 h at 0 °C in the presence or the absence of a large excess of unlabelled T_3 . ○, Receptors from 24 h-cultured hepatocytes (108 μ g of protein/tube); ●, receptors from rat liver (135 μ g of protein/tube). Values are the means with range of duplicate determinations. (a) Dose-dependent saturation curves; (b) Scatchard plots.

Fig. 3. Northern analysis of S14 expression in rat liver and cultured hepatocytes

Total RNA (30 μ g) was electrophoresed and blotted. Lane 1, RNA from rat liver; lanes 2 and 4, RNA from hepatocytes cultured for 28 h; lanes 3 and 5, RNA from hepatocytes cultured for 24 h and further incubated with T_3 (50 nM) for 4 h. The filter was hybridized with labelled S14 cDNA (lanes 1–3) or β -actin cDNA (lanes 4 and 5).

hepatocytes had decreased drastically to about 5 % of that in the liver cells in euthyroid rats. There was little hormonal response even when 50 nM- T_3 was added to the medium (Fig. 3). The β -actin 2 kb transcript was, however, detected in the cultured hepatocytes, and its signal became 10 times stronger than that from rat liver, indicating that mRNAs were not destabilized in the cultured hepatocytes.

TR molecules are present in nuclei of cultured hepatocytes

Despite the loss of specific T_3 binding, there were TR molecules in the hepatocytes. Hepatocytes were immunostained with an antibody, 4B II, which specifically reacts with TR α_1 and β , but not with TR α_2 [13–16]. At 1 h after plating, the round hepatocytes had attached to the plate, and within 24 h each hepatocyte had spread out and made contact, forming a monolayer structure similar to liver cell cords. As seen in Fig. 4, the anti-TR antibody stained the nuclei of hepatocytes at 24 h as well as at 1 h.

2-Mercaptoethanol restores the T_3 -binding activity of the hepatocytes

After various attempts to restore T_3 -binding activity by adding to the culture media various chemicals, including hormones such

Table 1. Effect of DTT on T_3 binding to rat liver nuclear extract

A nuclear extract of rat liver (50 μ l) was incubated at 0 °C for 8 h with [125 I] T_3 (0.2 nM) and DTT at various concentrations in the presence and the absence of unlabelled T_3 in Buffer T (50 mM-NaCl, 2 mM-EDTA, 20 mM-Tris/HCl, pH 8.2, 10 % glycerol). Specifically bound T_3 was determined as described. Values are means of duplicate determinations.

DTT (mM)	[125 I] T_3 bound	
	(fmol)	(fold)
0	1.67	1.0
0.1	14.12	8.4
0.2	16.10	9.6
1.0	16.08	9.6

Fig. 4. Immunostaining of TR in primary cultured hepatocytes

Hepatocytes, cultured for 1 h (a, b) and 24 h (c, d) were immunostained with 4B II (a, c) an antibody specific to TR α_1 and β , or with control normal serum (b, d). Note that the nuclei of hepatocytes cultured for 24 h were still stained with 4B II.

Table 2. Effect of air on the T_3 -binding activity of extracted nuclear TR

Air was delivered gently (6.5 ml/min) through a tubing into the rat nuclear extract (20 ml) at 0 °C, and then the extract (50 μ l/tube) was incubated with [125 I] T_3 (0.2 nM) as described in the Materials and methods section. Values are means of duplicate determinations.

Exposure time to air (h)	[125 I] T_3 bound	
	(fmol)	(%)
0	23.22	100.0
0.5	13.48	58.1
1	3.98	17.1
2	0.51	2.2

as glucocorticoids, insulin or T_3 , 2-mercaptoethanol was found to restore the T_3 -binding activity. In fact, as shown in Tables 1 and 2, the receptor required a reducing agent in the *in vitro* T_3 -binding assay (Table 1), and quickly lost its T_3 -binding activity upon exposure to the air (Table 2): the specific T_3 binding of the nuclear extract from rat liver was 9.6-fold higher with dithiothreitol (DTT) at 0.2 mM than without DTT (Table 1). The nuclear extract drastically decreased its hormone-binding activity (by 83 %) upon exposure to air for 1 h (Table 2).

Hepatocytes after 24 h of culture were incubated with various concentrations of 2-mercaptoethanol for a further 12 h. Without

Fig. 5. Effect of 2-mercaptoethanol on T₃ binding by hepatocyte nuclei

Hepatocytes were cultured with 3 mM-2-mercaptoethanol for the indicated times. The total incubation time from plating was 24 h. After the culture with 2-mercaptoethanol, the hepatocytes were assayed for specific T₃ binding by the nuclei. Values are the means of duplicate determinations.

Table 3. Effect of cycloheximide on the restoration of T₃ binding by 2-mercaptoethanol

Hepatocytes cultured for 24 h were further incubated for 4 h in the presence or the absence of 3 mM-2-mercaptoethanol (2ME) and 100 µM-cycloheximide (CHx). Values are the means with range of duplicate determinations.

Additions		[¹²⁵ I]T ₃ bound (fmol/mg of DNA)
2ME (mM)	CHx (µM)	
0	0	0.0 ± 0.1
0	100	0.0 ± 0.1
3	0	6.7 ± 0.1
3	100	2.6 ± 0.6

2-mercaptoethanol, binding was low, but with 0.1 mM-2-mercaptoethanol there was 4-fold higher specific binding, and at 3 mM an increase in specific binding exceeding 20-fold was detected (results not shown). The time course of the 2-mercaptoethanol effect revealed that the specific binding activity increased in a linear fashion with time (Fig. 5).

Cycloheximide blocks the 2-mercaptoethanol effect

Cycloheximide, an inhibitor of protein synthesis, blocked the effect of 2-mercaptoethanol. Hepatocytes after 24 h of culture were incubated with 3 mM-mercaptoethanol in the presence or the absence of 0.1 mM-cycloheximide for 4 h. The T₃-binding activity restoration by 2-mercaptoethanol was suppressed by cycloheximide (Table 3).

DISCUSSION

Hepatocytes in culture show specific functions similar to those seen *in vivo*, and respond well to various hormones and amino acids [3]. However, when we examined T₃ induction of the S14 gene, the transcription of which is immediately enhanced by the hormone in the liver *in vivo*, no significant response was detected in the primary cultured rat hepatocytes.

When HTC Morris hepatoma cells were incubated with [¹²⁵I]T₃, specific T₃ binding to the cell nuclei was observed [6]. Using the

same procedure, we assayed the T₃-binding activity of primary cultured hepatocytes. Hepatocytes in early periods after plating showed significant binding activities. Mariash *et al.* [17] also reported that freshly isolated hepatocytes have nuclear T₃-binding characteristics identical to those of liver *in vivo*. Our study demonstrated that the activity thereafter drastically decreased.

If the T₃ degradation rate in hepatocytes is accelerated or the cellular translocation system of the hormone is hindered during culture, nuclear T₃ binding would decrease, while extracts of nuclear receptors would retain T₃-binding activity. However, the nuclear extracts from cultured hepatocytes again showed little T₃-binding activity. Thus the decrease in T₃ binding in hepatocytes was attributed to a decrease in the nuclear T₃-binding activity.

Unexpectedly, the synthesis of TR did not appreciably diminish in the hepatocytes. Since the known natural inhibitors, non-esterified fatty acids and fatty acyl-CoAs [6,8] were not the cause of the loss of T₃-binding activity (results not shown), we thought it likely to be due to denaturation of the receptor. Whereas the specific T₃-binding drastically diminished in the hepatocytes, the non-specific binding activity increased reciprocally within the cells in a well-correlated fashion (results not shown).

The liver is an anaerobic organ. In contrast, in cultures under conditions of 20% O₂ and 5% CO₂, hepatocytes are exposed to much more aerobic circumstances, and receptors in the hepatocytes may be influenced. Glucocorticoid and oestrogen receptors are members of the steroid/thyroid receptor superfamily. It has been shown that in their ligand-binding domains they have critical SH groups that are essential for steroid binding [18,19]. Grippo *et al.* showed that thioredoxin, an endogenous reductant, maintains the glucocorticoid receptor in a steroid-binding form in the liver cytosol [20,21]. Similarly, Takagi *et al.* reported that thioredoxin and glutaredoxin enhanced T₃ binding by its nuclear receptor *in vitro* [22]. In T₃-binding assays *in vitro*, inclusion of reductants such as DTT or 2-mercaptoethanol is important to obtain a high T₃-binding activity. We also observed that the receptor extracted from rat liver quickly lost its hormone-binding activity when air (20% O₂) was delivered into the extract. Hence we concluded that under the aerobic conditions of hepatocyte culture the T₃-binding activity of the receptor was decreased by oxidative inactivation. We thus examined the effects of various reducing agents on the hepatocytes in culture, and observed that 2-mercaptoethanol increased specific T₃ binding by hepatocytes most effectively and in a dose-dependent manner.

The T₃-binding activity was restored gradually and linearly up to 12 h (Fig. 5). This may mean that in the recovery of T₃ binding the inactivated TRs were not reactivated (if this is the case, the recovery would occur around the same time, for a relatively short period of time). In addition, cycloheximide blocked the effect of 2-mercaptoethanol. This supports the notion that the reducing agent exerted its effect not on the inactivated receptor but on the newly synthesized receptor. 2-Mercaptoethanol could act directly on the receptor or by increasing the levels of endogenous reductants such as thioredoxin and NADPH.

In contrast to the present results, induction of malic enzyme by T₃ was noted in hepatocytes prepared from chick embryos and from neonatal chicks [23]. The different biological cellular contexts of adult liver and embryonal or neonatal liver may explain the difference in the T₃-binding activities in cultured hepatocytes. Thus fetal hepatocytes in culture may be able to adapt to these more aerobic conditions to maintain T₃-binding activity.

In the cultured hepatocytes incubated with 3 mM-2-mercaptoethanol for 4 h, S14 mRNA synthesis increased 2.2-fold in the

presence of T_3 as compared with the situation without T_3 (results not shown). In rats, however, the induction is usually 4–7 times larger than this. There may be other transcriptional factor(s) necessary for full S14 gene expression which have become deficient in the hepatocytes, or the chromatin structure of the S14 gene may have changed in the culture.

Zilz *et al.* [24] reported that the S14–CAT fusion gene transfected to primary cultured hepatocytes was induced by T_3 only when a vector containing TR cDNA was co-transfected, thereby suggesting that endogenous TRs are either not expressed or do not function in primary cultured hepatocytes. It is not clear why TR expressed from a vector was not inactivated along with the endogenously encoded TR.

In trying to restore T_3 -binding activity, we added to the medium various chemicals including hormones, or changed the medium. Waymouth's medium had some (not significant) restoring activity. It is noteworthy that Waymouth's medium contains 300 times (15 mg/litre) more glutathione than does Williams' medium E.

In conclusion, we found that TRs lost T_3 -binding activity in cultured hepatocytes, that the loss was attributable primarily to receptor inactivation, probably due to aerobic circumstances, and that the loss could be counteracted by adding 2-mercaptoethanol to the medium. These observations suggest that oxidative circumstances are important in determining the levels of activity of intracellular receptors.

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