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Peroxisome proliferators and T3 operate by way of distinct receptors

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Peroxisome proliferators and thyroid hormones have a number of common metabolic effects. The possibility that the signal transduction pathways of both groups of effectors converge at the receptor level was investigated. It was shown that T3, specifically bound to the rat thyroid β-receptor, was not displaced to a significant extent by ciprofibrate or bezafibrate. No specific binding of T3 to the mouse peroxisome proliferator activated receptor could be demonstrated. In transactivation experiments peroxisome proliferators were unable to activate the thyroid receptor and T3 did not activate a chimeric receptor containing the ligand binding domain of the peroxisome proliferator activated receptor. It is concluded that peroxisome proliferators and thyroid hormone do not cross-react at the level of their nuclear receptors.

Thyroid hormone; Peroxisome proliferator activated receptor; Signal transduction

1. INTRODUCTION

A number of reports have appeared in recent literature indicating that peroxisome proliferators and thyroid hormones have overlapping metabolic effects: both are hypolipidemic and calorigenic and both, in vitro as well as in vivo, induce enzymes that are classically considered to be thyroid hormone dependent, e.g. malic enzyme, glycerol-3-phosphate dehydrogenase and S14 (see [1] and references therein). The inductive effects of peroxisome proliferators are independent of the presence of thyroid hormones and are, at least in some cases, the result of increases in the corresponding mRNAs due to transcriptional activation of the respective genes [2,3]. On the other hand, evidence for the peroxisome proliferative effect of thyroid hormones is conflicting [3,4]. The capacity of peroxisome proliferators to induce peroxisomal enzymes is not always correlated with their thyromimetic or hypolipidemic effects, implying distinct mechanisms [3]. Moreover, peroxisome proliferators and thyroid hormones have different tissue specificities, suggesting that both groups of effectors use different transduction pathways [1,3]. In some cases peroxisome proliferators have a higher inductive capacity than thyroid hormones [3] and their effects can be additive [2], corroborating the thesis of independent mechanisms.

Thyroid hormone action is mediated by specific nuclear receptors interacting with cognate nucleotide sequences (response elements) in the promoter of affected genes [5,6]. Peroxisome proliferators appear to operate by a similar but less well-characterized mechanism: 'peroxisome proliferator activated receptors' (PPARs) were recently isolated and found to be structurally related to thyroid hormone receptors [7,8]. Respons elements for PPARs have been described [9,10] but the endogenous ligand of this receptor remains elusive.

In this paper we present evidence that peroxisome proliferators and thyroid hormones do not cross-react at the receptor level.

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Abbreviations: PPAR, peroxisome proliferator activated receptor; TR, thyroid β-receptor; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethanesulphonylfluoride; CAT, chloramphenicol acetyltransferase; TRE, thyroid hormone response element.

2. EXPERIMENTAL

2.1. *Plasmids*

The cDNA for the mouse PPAR was obtained by PCR from mouse liver RNA and subcloned in the CDM expression vector. The rat thyroid β-receptor (TR) expression vector was provided by D.D. Moore (Boston, USA). Reporter plasmids were constructed in the pUTKAT vector [11]. The TR-PPAR chimera was generated using PCR with primers flanking the ABC domains of the TR and the DEF domains of the PPAR. At the N-terminus of the D domain of the chimeric construct a serine residue was replaced by an arginine an a histidine by an alanine.

2.2. *Other materials*

All other materials were of the highest purity available and were obtained from Boehringer-Mannheim Belgium (Brussels, Belgium) or Sigma Chemical Co. (St. Louis, MO, USA). [^{125}I]T3 was purchased from DuPont-New England Nuclear (Brussels, Belgium). Ciprofibrate, bezafibrate and methylclofenapate were generous gifts from, respectively, Winthrop Laboratories (Brussels, Belgium), Boehringer-

Mannheim Belgium (Brussels, Belgium) and Imperial Chemical Industries (Macclesfield, UK).

2.3. *Overexpression of TR and PPAR*

10^6 COS-M6 cells (American Type Culture Collection, Rockville, MD, USA) were seeded into 10 cm dishes in DMEM+10% (v/v) newborn calf serum one day prior to transfection. The medium was changed to DMEM+10% (v/v) NuSerum one hour before transfection. Each dish was transfected with 5 μg receptor expression vector using the DEAE-dextran method [12]. Chloroquine (100 μM) was added to increase transfection efficiency. After 3.5 h of incubation, cells were shocked with 10% (v/v) DMSO and grown for 2 days in DMEM supplemented with 10% (v/v) charcoal-stripped calf serum.

2.4. *Preparation of a COS-M6 nuclear extract*

Cells were scraped in PBS, pelleted and swollen for 5 min on ice in 25 mM KCl, 2 mM Mg-acetate, 1 mM DTT, 10 mM Tris-HCl, pH 7.5. The suspension was triturated through a 25-gauge needle to lyse the cells. The nuclei were pelleted and resuspended in a hypertonic buffer (0.4 M KCl, 2 mM Mg-acetate, 1 mM DTT, 0.5 mM PMSF, 10 μg/μl leupeptin, 10 mM Tris-HCl, pH 7.5). After 10 min on ice, the nuclei were homogenised and left to stand for another 10 min on ice. The crude nuclear extract was cleared by centrifugation for 10 min at $16,000 \times g$ and diluted with one volume of 0.1 M KCl, 20% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF, 90 mM Tris-HCl, pH 7.5, to create optimal binding conditions.

2.5. *Binding assay*

The nuclear extract containing TR or PPAR was incubated overnight at 4°C with 3×10^{-9} M [^{125}I]T3 (2200 Ci/mmol) in 0.25 M KCl, 10% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF, 5 μg/μl leupeptin, 50 mM Tris-HCl, pH 7.5 (binding buffer), in the presence or absence of an excess of cold competitor (T3, ciprofibrate or bezafibrate). Free [^{125}I]T3 was separated from protein-bound [^{125}I]T3 by adsorption to 1.25% (w/v) dextran-coated charcoal, suspended in binding buffer. Radioactive T3 bound to the receptor was measured by γ-counting of the supernatant. The displacement of [^{125}I]T3 from the receptor by the cold competitor was used as a measure for specific binding.

2.6. *Cell culture and transfections*

JEG-3 cells (American Type Culture Collection, Rockville, MD, USA) were seeded into 6 cm dishes in DMEM supplemented with 10% (v/v) charcoal-stripped fetal bovine serum one day prior to transfection. Transfections were carried out using calcium phosphate precipitation with 6 μg of reporter plasmid, 3 μg of expression plasmid and 5 μg of pSV2APAP (coding for alkaline phosphatase) for normalization purposes. After 18–24 h, cells were shocked with 20% (v/v) DMSO and refed with DMEM containing charcoal-stripped serum and either vehicle (DMSO), T3, ciprofibrate or methylclofenapate. After 2 days, cell extracts were prepared and CAT activities were analyzed as described by Brent et al. [12]. Alkaline phosphatase activity was measured by incubating 2 μl of cell extract with 1 ml of 5 mM *p*-nitrophenylphosphate in 1 M diethanolamine buffer, pH 9.8, 0.28 M NaCl, 0.5 mM $MgCl_2$ for 1 h at 30°C and reading absorbance at 405 nm.

3. RESULTS AND DISCUSSION

We first wanted to investigate possible displacement by peroxisome proliferators of T3 bound to the TR and possible binding of T3 to the PPAR.

COS-M6 cells were transiently transfected with TR and nuclear extracts were prepared. The extracts were incubated with 3×10^{-9} M [^{125}I]T3, in the absence or presence of cold 'competitor', and bound labeled T3 was measured. Table I shows the results of a typical experiment. In the absence of competitor, approx. 115,000 dpm of label bound to the nuclear extract. Cold T3 (100-fold excess) displaced approx. 63,000 dpm, clearly attesting to specific binding of T3. It can be seen that ciprofibrate and bezafibrate, up to a 10,000-fold excess, displaced very little specifically bound T3. This indicates that both compounds, which are very potent peroxisome proliferators, do not bind to the TR, at least under the given conditions. This in agreement with the results of Hertz et al. [3] who could not show displacement of T3 by bezafibrate in a nuclear extract from rat liver.

Table I

Displacement of [^{125}I]T3 (3×10^{-9} M) from the TR in a nuclear extract prepared from COS-M6 cells transiently transfected with TR

Competitor		Displacement of [^{125}I]T3 from the TR
T3	3×10^{-7} M	100
Ciprofibrate	3×10^{-7} M	8
	3×10^{-6} M	8
	3×10^{-5} M	15
Bezafibrate	3×10^{-7} M	0
	3×10^{-6} M	0
	3×10^{-5} M	12

The radioactivity displaced is expressed relative to the value displaced by cold T3, which was arbitrarily set at 100. See text for more details. The experiment was performed twice with equivalent results; data from one representative experiment are given.

In control experiments, COS-M6 cells were transfected with the parental expression vector. Specific binding of T3 was much lower: approx. 19,000 dpm displaced on a total of 72,000 dpm; this most probably corresponds to endogenous thyroid hormone receptor present in the nuclei of the cells.

When COS-M6 cells were transiently transfected with PPAR no specific binding of T3, either in presence or absence of peroxisome proliferators (ciprofibrate or bezafibrate), could be demonstrated (results not shown).

In order to confirm these results transactivation ex-

Table II

Activation of TR in JEG-3 cells transiently transfected with TR and a TRE-driven CAT reporter

Condition		Fold induction of CAT
Control (DMSO)		1.0
Ciprofibrate	10^{-5} M	0.8
Ciprofibrate	10^{-4} M	0.9
T3	10^{-9} M	5.3
T3	10^{-8} M	29.6

The induction of CAT activity is expressed relative to the control, which is arbitrarily set at 1. See text for more details. The experiment was performed twice with equivalent results; data from one representative experiment are given.

Table III

Activation of TR-PPAR in JEG-3 cells transiently transfected with the chimeric TR-PPAR receptor and a TRE-driven CAT reporter

Condition	Fold induction of CAT
Control (DMSO)	1.0
T3 10^{-8} M	1.0
Methylclofenapate 10^{-5} M	1.7
Methylclofenapate 10^{-4} M	5.3

The induction of CAT activity is expressed relative to the control, which is arbitrarily set at 1. See text for more details. The experiment was performed twice with equivalent results; data from one representative experiment are given.

periments were performed. A TR expression plasmid and a reporter, pTK35BA, containing a CAT gene driven by a thyroid hormone respons element (TRE) derived from the rat growth hormone 5' flanking region [13], were co-transfected in JEG-3 cells. Cells were treated with T3, ciprofibrate, or vehicle (DMSO), for 48h and CAT activity was measured on a cellular extract and normalized to the activity of co-transfected alkaline phosphatase.

As shown in Table II, T3 strongly stimulated the expression of CAT, while ciprofibrate, up to a concentration of 10^{-4} M, had no effect, clearly indicating that the peroxisome proliferator did not activate the TR.

In analogous experiments a chimeric TR-PPAR expression vector, consisting of the ligand binding domain of the PPAR linked to the DNA binding domain of the TR (see section 2), was co-transfected with the same TRE-CAT reporter plasmid and the alkaline phosphatase normalization vector used in the foregoing experiments. The cells were treated with T3, methylclofenapate, or vehicle (DMSO), and CAT activity was measured (Table III). As expected, methylclofenapate stimulated the expression of CAT, in a dose dependent manner, but T3 was without effect.

It can be concluded that there is no evidence for cross-reactivity of TR and PPAR with respect to their activating ligands.

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