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Thyromimetic Action of the Peroxisome Proliferators Clofibrate, Perfluorooctanoic Acid, and Acetylsalicylic Acid Includes Changes in mRNA Levels for Certain Genes Involved in Mitochondrial Biogenesis

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The effects of three peroxisome proliferators on the mRNA levels for some mitochondrial inner-membrane proteins in rat liver were investigated. Clofibrate, perfluorooctanoic acid, and acetylsalicylic acid all increased the mRNA levels for the mitochondrial-encoded respiratory-chain components cytochrome *c* oxidase subunit I and NADH dehydrogenase subunit I. Mitochondrial 16S rRNA was also induced by clofibrate. The mRNA levels for the nuclear-encoded mitochondrial inner-membrane proteins adenine nucleotide translocator and cytochrome *c*1 were selectively induced by the different peroxisome proliferators. Malic enzyme, which is induced by thyroid hormone, was also induced by the three peroxisome proliferators tested. These effects are in some ways similar to those obtained with thyroid hormone.

Key Words: **thyromimetic action; peroxisome proliferators; mitochondria; mRNA.**

Peroxisome proliferators activate a member of the steroid hormone receptor superfamily, the so-called peroxisome proliferator-activated receptor (PPAR)[2] (1, 2). PPAR binds to a specific element (PPRE) found in several genes known to be invariably activated by peroxisome proliferators, i.e., the genes encoding peroxisomal acyl-CoA oxidase and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, which are involved in peroxisomal β-oxidation (3–5), and the gene encoding cytochrome P450IVA6, involved in the ω-hydroxylation of fatty acids (6). It has been reported that the retinoid receptor (RXR) is required for PPAR to bind to the PPRE (7) and that RXR is also an auxiliary protein for binding the thyroid hormone receptor (THR) to its hormone-responsive element (TRE) (8).

Thus, RXR apparently plays a central role in mediating the actions of both peroxisome proliferators and thyroid hormone. Furthermore, Bogazzi *et al.* (9) reported that PPAR can activate thyroid hormone responses through formation of a heterodimer with the thyroid hormone β receptor, thereby providing direct evidence for coupling of the THR and PPAR signaling pathways. Analysis of the basic chemical structures of peroxisome proliferators and thyroid hormone shows that they both have a hydrophobic backbone linked to a carboxylic group (or in the case of peroxisome proliferators, a group that may be converted into a carboxylic group *in vivo*).

Thyromimetic effects of peroxisome proliferators have been reported earlier. Both peroxisome proliferators (10, 11) and thyroid hormone (12) are potent hypolipidemic agents. In rats several peroxisome proliferators can induce malic enzyme, mitochondrial glycerol-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, and S14, i.e., enzymes which are classically considered as being thyroid hormone-dependent (13).

In addition, thyroid hormone and peroxisome proliferators both affect mammalian mitochondria. Thyroid hormone regulates the volume and cytochrome content of the inner membrane (14, 15) and the rate of mitochondrial protein synthesis (16, 17), etc. Peroxisome

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[2] Abbreviations used: COXI, cytochrome *c* oxidase subunit I; NDI, NADH dehydrogenase subunit I; ANT2, adenine nucleotide translocator 2; PFOA, perfluorooctanoic acid; ASA, acetylsalicylic acid; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator responsive element; RXR, retinoid receptor; THR, thyroid hormone receptor; SDS, sodium dodecyl sulfate; TRE, thyroid hormone-responsive element; T3, thyroid hormone; RE, responsive element.

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proliferators also alter mitochondrial morphology and enzyme composition (18–20). These morphological changes include elongation and flattening, an increase in the number of mitochondria, and a decrease in their size.

Considerable work has been done to define the mechanism by which thyroid hormone regulates mitochondrial biogenesis (14–17, 21–22). The results indicate that transcription of the mitochondrial genome and mitochondrial protein synthesis are increased *in vivo*. In addition, specific nuclear genes encoding for proteins involved in oxidative phosphorylation are highly regulated, both *in vivo* and *in vitro*. These findings show that a selected set of T3-responsive genes are somehow involved in the increase in mitochondria.

In the present experiment we address the question as to whether peroxisome proliferators increase mitochondrial biogenesis by examining the influence of clofibrate, perfluorooctanoic acid, and acetylsalicylic acid on the mRNA levels for certain mitochondrial inner-membrane proteins. The levels of the mRNA species chosen for study are known to be regulated by thyroid hormone, an endogenous regulator of mitochondrial biogenesis. Perfluorooctanoic acid and clofibrate are equally potent peroxisome proliferators in terms of the maximal effects obtained. However, the dose of perfluorooctanoic acid required to obtain maximal effects is about 50 times less than the corresponding dose for perfluorooctanoic acid. The maximal effects obtained with acetylsalicylic acid are considerably less than with the other two peroxisome proliferators examined here.

MATERIALS AND METHODS

Animals and treatment. Male Spraque–Dawley rats (ALAB, Sollentuna, Sweden) weighing 180 g were used in this investigation. The animals were housed in groups of three in plastic cages with steel bottoms with a 12-h light/dark cycle at 25°C. They were given free access to commercial food R3 containing 5% fat, 24% protein, 49% carbohydrate, and 22% water (ALAB) before initiation of the experiments.

The 0.05% perfluorooctanoic acid-, 0.5% clofibrate-, or 1% acetylsalicylic acid-containing diets (w/w) were prepared as described previously (20). Control diet was prepared in the same manner, except that no peroxisome proliferator was added. These diets were stored at −20°C prior to use. Rats were administered these diets for 10 days. Both control and exposed rats consumed about 10 g food per day. This resulted in mean daily drug intakes of 5 mg perfluorooctanoic acid, 50 mg clofibrate, or 100 mg acetylsalicylic acid.

cDNA probes. The plasmids used for hybridization included the following: human cDNA clones for adenine nucleotide translocator protein 2 (ANT2) (23) and for cytochrome *c*1 (24), a mouse γ-actin clone (25), and a clone for NADH dehydrogenase subunit I (NDI) from rat (26). An *Eco*RI fragment c of rat liver mitochondrial DNA (26) was used to probe the cytochrome *c* oxidase subunit I (COXI) transcript. The transcripts of COXII and subunit 2 of NADH dehydrogenase are also detected by this fragment c, but 80% of the total hybridization is accounted for by COXI. Fragment D of rat liver mitochondrial DNA (26) was used as a probe for mitochondrial 16S rRNA transcript; a short piece of the NDI sequence is also present in this clone D.

Plasmids containing the cDNA probes were labeled with [α-^{32}P]-dCTP using random-primer labeling.

RNA extraction and preparation of subcellular fractions. At the end of the treatment period, the animals were killed by cervical dislocation and the livers were removed. Samples of liver tissue (2 g) were taken from each rat and subcellular fractions were prepared as described previously (20). The remaining tissue was frozen in liquid nitrogen and stored at −80°C for later use. Total RNA was extracted by the methods of Chomczynski and Sacchi (27). Poly(A)-rich RNA was isolated by two passages through an oligo(dT) column (28).

Northern blot analysis. RNA was denatured in 50% formamide and 6% formaldehyde by heating for 10 min at 65°C, with subsequent cooling on ice. Electrophoresis was performed using 2% agarose gels containing 2.2 M formaldehyde. RNA was transferred to Hybond-N membranes (Amersham) by capillary blotting. The filters were baked for 2 h at 80°C and then hybridized with the ^{32}P-labeled cDNA probes described above at 42°C in 50% formamide. The filters were washed four times at room temperature for 10 min with 2× NaCl/Cit. (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) and 0.1% SDS, once at 65°C for 15 min with 1× NaCl/Cit. and 0.1% SDS, and twice for 10 min at 65°C in 0.1× NaCl/Cit. and 0.1% SDS. Autoradiographs were performed using X-ray film (Fuji RX) with intensifying screens at −70°C for 1–24 h and the signal intensities were measured using an LKB laser scanner (Bromma, Sweden).

Enzyme assays. All enzyme assays were performed with saturating substrate concentrations and conditions of linearity with time and protein, as determined using fractions from control and treated rats.

The activities of malic enzyme (29), cytochrome *c* oxidase (30), lauroyl-CoA oxidase (31), and NADH oxidase (30) were all determined according to the literature. Protein was measured by the method of Lowry and co-workers (32) with bovine serum albumin as standard.

Statistical analysis. Data are presented as means ± SD and, wherever appropriate, statistical significance has been tested for using Student's *t* test.

RESULTS

10 days treatment of rats with 0.05% perfluorooctanoic acid, 0.5% clofibrate, or 1% acetylsalicylic acid in their diet resulted in 30.9-, 26.5-, and 5.2-fold inductions of lauroyl-CoA oxidase activity (a marker enzyme for peroxisome proliferation), respectively (Table I), confirming the earlier findings that perfluorooctanoic acid and clofibrate are strong peroxisome proliferators, while acetylsalicylic acid is a moderately potent peroxisome proliferator (20, 33, 34).

As a marker for the thyromimetic status in liver, malic enzyme activity was also measured in the cytosolic fraction (Table I). This activity was induced 3.2- and 5.1-fold by 0.05% perfluorooctanoic acid and 0.5% clofibrate, respectively, and only 1.1-fold by 1% acetylsalicylic acid. Thus, the thyromimetic potency, as measured by malic enzyme induction, reflects the relative peroxisome proliferation potency as measured by lauroyl-CoA oxidase induction. An increase in liver malic enzyme activity in clofibrate-treated rats has been reported previously (20), but the induction of this activity by perfluorooctanoic acid is first reported here.

Measurement of the activities of cytochrome *c* oxidase and NADH oxidase showed that 10 days of treat-

TABLE I

Effects of Dietary 0.05% Perfluorooctanoic Acid (PFOA), 0.5% Clofibrate, or 1% Acetylsalicylic Acid (ASA) for 10 Days on Some Enzyme Activities in Rat Liver

	Enzyme activities			
Treatment	Lauroyl-CoA oxidase (nmol/min g liver) in mitochondria	Malic enzyme (μmol/min/g liver) in cytosol	Cytochrome *c* oxidase (μmol O_2/min/g liver) in mitochondria	NADH-oxidase (nmol/min/g liver) in mitochondria
Control	57.6 ± 12.9	1.23 ± 0.44	7.05 ± 1.35	140 ± 16.1
0.05% PFOA	1530 ± 549** (26)	3.88 ± 0.87** (3.2)	6.32 ± 0.30 (0.9)	206 ± 49.2* (1.5)
Control	43.4 ± 3.23	0.97 ± 0.09	7.43 ± 1.83	164 ± 80.9
0.5% clofibrate	1340 ± 186*** (31)	4.99 ± 1.12*** (5.1)	11.0 ± 2.14* (1.5)	369 ± 140* (2.3)
Control	47.7 ± 4.94	1.19 ± 0.07	9.10 ± 0.60	178 ± 10.0
1% ASA	247 ± 63.9*** (5)	1.36 ± 0.08* (1.1)	10.1 ± 0.95 (1.1)	354 ± 28.8*** (2.0)

Note. All values are means ± SD for four animals.
The data in parentheses represent the fold induction.
* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control group.

ment of rats with 0.5% clofibrate only resulted in a 1.5-fold increase in cytochrome *c* oxidase activity (Table I). No increase in this activity was observed with 1% acetylsalicylic acid or 0.05% perfluorooctanoic acid. NADH oxidase activity was increased 1.5-, 2.3-, and 2.0-fold by 0.05% perfluorooctanoic acid, 0.5% clofibrate, and 1% acetylsalicylic acid, respectively (Table I). Upon comparison with the Northern blots (Table II), the correlation between the inductions of the mRNA levels for the cytochrome *c* oxidase and the NADH dehydrogenase subunit and increases in their enzyme activities is seen to be poor.

Ten days of treatment of rats with these different peroxisome proliferators resulted in an increase in the transcript levels for certain mitochondrial- and nuclear-encoded mitochondrial inner-membrane proteins (Fig. 1). The quantitative densitometric analysis is presented in Table II. The mRNA level for mitochondrial-encoded COXI was increased 2.3-, 4.5-, and 1.5-fold by 0.5% clofibrate, 0.05% perfluorooctanoic acid, and 1% acetylsalicylic acid, respectively. After stripping COXI from the filters, NDI, the gene for which is also present in mitochondrial DNA, was probed on the same filters. NDI mRNA was increased 4.6- and 2.2-fold by 0.5% clofibrate and 1% acetylsalicylic acid, respectively. By contrast, NDI mRNA was found to be highly responsive to 0.05% perfluorooctanoic acid, which increased its level 24.5-fold. Subsequent hybridization of the same filters with a probe for mitochondrial 16S rRNA revealed that this transcript was increased 3.3-fold by 0.5% clofibrate. Surprisingly, no increase in the 16S rRNA was obtained with 0.05% perfluorooctanoic acid or 1% acetylsalicylic acid (data not shown).

ANT2 and cytochrome *c*1 are nuclear-encoded mitochondrial inner-membrane proteins. The ANT2 mRNA level was increased 3.7- and 1.8-fold by 0.5% clofibrate and 1% acetylsalicylic acid, respectively; while the level of mRNA for cytochrome *c*1, which was probed after ANT2 on the same filters, was induced 2.9-fold by the moderate peroxisome proliferator acetylsalicylic acid.

γ-Actin mRNA was measured as a control nonmitochondrial protein. This probe was hybridized after cytochrome *c*1 using the same filters. The level of this mRNA was unchanged by 1% acetylsalicylic acid or 0.05% perfluorooctanoic acid.

DISCUSSION

Effects of peroxisome proliferators on mitochondria have been observed previously (18–20), but it is not known whether these changes reflect mitochondrial biogenesis. Since thyroid hormone is known to be an endogenous regulator of mitochondrial biogenesis, we compared the thyromimetic action of three peroxisome proliferators of varying potencies—i.e., clofibrate, perfluorooctanoic acid, and acetylsalicylic acid—with respect to up-regulation of malic enzyme [the expression of which is regulated through a TRE present in the enzyme's promoter (35, 36)] and the levels of certain mitochondrial and nuclear transcripts involved in mitochondrial biogenesis (26, 37–41).

In agreement with earlier studies (13), we show that malic enzyme activity is induced by these three peroxisome proliferators. Our studies extend these findings by showing that malic enzyme induction is correlated with peroxisome proliferator potency, as reflected in lauroyl-CoA oxidase activity. A recent study identified a RXR/PPAR-responsive element which is a direct repeat separated by one nucleotide on the malic enzyme promoter which may render this gene responsive to peroxisome proliferators (42). The "thyromimetic" response of malic enzyme to peroxisome proliferators and

TABLE II

Quantitative Changes in the Transcript Levels for Some Mitochondrial- and Nuclear-Encoded Mitochondrial Inner-Membrane Proteins Responsive to Peroxisome Proliferators in Rat Liver

Probe	Sample							
	Control	0.5% Clofibrate	T/C	Control	0.05% PFOA	T/C	1% ASA	T/C
COXI	596 ± 67	13740 ± 191	2.3***	155 ± 98	691 ± 173	4.5**	690 ± 212	1.5
NDI	202 ± 49	924 ± 50	4.6***	20 ± 11	492 ± 120	24.5**	726 ± 264	2.2*
16S rRNA	233 ± 47	764 ± 149	3.3***	390 ± 193	427 ± 162	1.1	ND	
ANT2	294 ± 78	1090 ± 438	3.7*	93 ± 18	87 ± 43	0.94	831 ± 150	1.8*
Cyt *c*1	159 ± 27	189 ± 233	1.2	204 ± 67	203 ± 61	1.0	1280 ± 22	2.9***
γ-Actin	ND	ND		57 ± 9.4	78 ± 30	1.4	315 ± 44	1.1

Note. Rats were given food containing the peroxisome proliferator for 10 days. Densitometric scanning of Northern blots is described under Materials and Methods and arbitrary units are presented. The data come from one experiment representative of two parallel experiments. Means ± SD ($n = 4$) are given. T/C, treated with peroxisome proliferator/control. ND, not determined.

Significantly different from control: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

the correlation between peroxisome proliferator potency and malic enzyme induction reported here most likely reflect usage of a peroxisome proliferator-regulated PPRE (43), while up-regulation of malic enzyme by T3 involves TRE on the malic enzyme promoter.

The more interesting response to the three peroxisome proliferators studied here is that of the mitochondrial transcripts. The levels of both COXI and NDI mRNAs were significantly increased by clofibrate and perfluorooctanoic acid and were only slightly increased by acetylsalicylic acid. Thus, increases in mitochondrial transcripts also reflect to some extent peroxisome proliferator potency. The responses to these peroxisome proliferators are of the same magnitude as that to thyroid hormone (26). However, it is difficult to compare these effects in a quantitative fashion, since effects of thyroid hormone are usually examined using hypothyroid animals, whereas our investigation has been performed on euthyroid rats.

It is not certain that T3 and the three peroxisome proliferators studied here are operating via the same mechanism in this case either. At least three mechanisms can be responsible for changes in the levels of mitochondrial transcripts, i.e., an increased rate of transcription initiation, differential transcription of the rRNA and protein-encoding genes, and altered mRNA stability. Thyroid hormone enhances transcription initiation (26, 39, 44, 45) and, most probably, differential transcription of the rRNA genes (26). Progression of transcription from the rRNA genes into the downstream protein coding region is regulated by an attenuation response which would be expected when transcription initiation becomes activated.

Altered mRNA stability could also contribute to changes in the steady-state levels of mitochondrial transcripts. Even though these levels are normally controlled at the level of transcription (46), we know nothing about alterations in mRNA stability in cells perturbed by hormone or peroxisome proliferators. Clearly, the large variation in NDI transcripts induced by T3 (26) and perfluorooctanoic acid is best explained by differential transcript stability.

The weak correlation between increases in mitochondrial COXI and NDI transcripts and COX and NADH oxidase activities is worth noting. Transcript levels increase significantly more than the corresponding enzyme activities. Similar results have been observed for thyroid hormone, i.e., liver mitochondrial transcripts can increase severalfold (26, 40), whereas the levels of the individual subunits in the inner membrane increase no more than two- to threefold (47). Also of interest in this connection is the observation by Latruffe and co-workers (48) that long-term treatment of Fischer rats with ciprofibrate increases the level of mRNA coding for (*R*)-3-hydroxybutyrate dehydrogenase (located on the inner mitochondrial membrane),

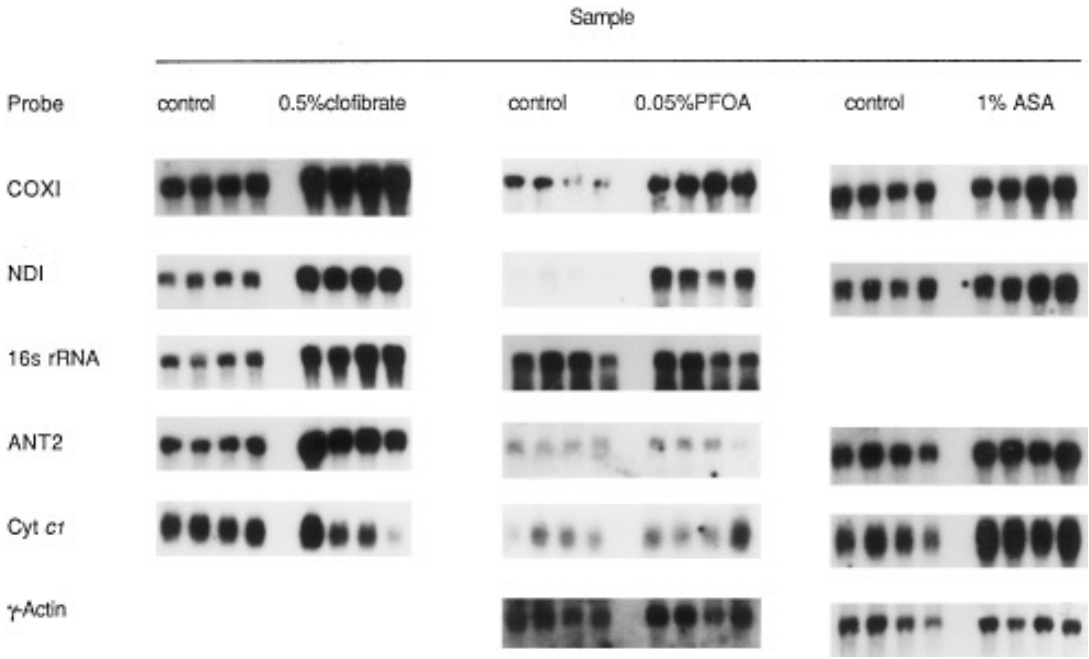

FIG. 1. Levels of the mRNAs for certain mitochondrial- and nuclear-encoded mitochondrial inner-membrane proteins after exposure to peroxisome proliferators. Rats were treated with three different peroxisome proliferators for 10 days. RNA was prepared in parallel from the livers of four control and four peroxisome proliferator-treated rats. Ten micrograms of total RNA was placed in each lane for quantitating the transcripts of cytochrome *c* oxidase subunit I, NADH dehydrogenase subunit I, and 16S rRNA. Equal amounts of poly(A)-rich RNA (4 μg) were placed in each lane for measuring the transcripts of adenine nucleotide translocator protein 2, cytochrome *c*1, and γ-actin. Electrophoresis and Northern blot analysis were performed as described under Materials and Methods. Each lane represents the RNA from an individual animal. These results are from one experiment, with very similar results being obtained in another identical experiment. (Note: There are technical difficulties involved in presenting Northern blots when there are large differences between control and treated samples. For instance, we could have chosen to expose the blots for NDI in the PFOA group for a longer period of time so that the control blots would be clearly visible, but in this case the blots from treated animals would simply be a dark blob. The quantitation of the blots presented in Table II was, of course, performed with exposure times which gave reasonable bands for densitometric analysis.)

as well as the level of this protein, while actually inhibiting enzyme activity.

These data are consistent with experiments showing that mitochondrial transcripts and translation products are produced in excess in rapidly growing cells (49). However, the transcripts for the subunits of complex I respond strongly to many different agents, including T3 (26), neoplastic transformation (50), growth activation (51), and peroxisome proliferators (this study), suggesting an important and, perhaps, global requirement for increased production of these subunits. This suggestion is strengthened by observations in this study, and with T3 (26), that increases in mitochondrial transcript levels are correlated with a slightly greater increase in NADH oxidase than in cytochrome oxidase activity.

The mRNA levels for nuclear-encoded mitochondrial inner-membrane proteins cytochrome *c*1 and ANT2 are increased in hypothyroid adult rat liver as much as 30- to 50-fold by thyroid hormone (52). This regulation occurs at the transcriptional level. The present studies demonstrate that the mRNA levels for ANT2 and cytochrome *c*1 were selectively induced in euthyroid rats by the three peroxisome proliferators employed and to a much lesser extent than by thyroid hormone in hypothyroid rats. No correlation between peroxisome proliferator potency and increased cytochrome *c*1 and ANT2 transcripts was observed. The results here are also in agreement with other studies on rodent liver showing some common responses to all proliferators (e.g., increases in the number and size of peroxisomes, cytochrome P-450 IVA, peroxisomal fatty acid β-oxidation, and soluble epoxide hydrolase activity), as well as individual responses to specific peroxisome proliferators (e.g., induction of UDP-glucuronosyltransferase and inhibition of glutathione transferase) (20, 33, 34).

The common effects of the three peroxisome proliferators employed here and of thyroid hormone on the mRNA levels for mitochondria-encoded transcripts and on malic enzyme might thus be explained by the interaction of PPAR and TRE or RXR on different responsive elements (REs), resulting in activation of differently related genes. Of course, it remains to be established whether these effects are direct effects common for all peroxisome proliferators and whether PPAR is actually present in mitochondria. Further studies on the nature of the REs and of the interaction of PPAR and TRE or RXR will help us to understand how the signal path-

ways involving PPAR and THR are coupled, as well as how peroxisome proliferators and thyroid hormone mediate their effects on mitochondrial biogenesis and lipid metabolism.

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