monoamine uptake by cocaine, methylphenidate and amphetamine. *Res Comm Chem Pathol Pharmacol* **21**: 67–75, 1978.
19. Norman AB, Ford LM, Kolmonpunporn M and Sanberg PR, Chronic treatment with MK-801 increases the quinolinic acid-induced loss of D-1 dopamine receptors in rat striatum. *Eur J Pharmacol* **176**: 363–366, 1990.

Biochemical Pharmacology, Vol. 41, No. 4, pp. 652–655, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Thyroid hormone-independent regulation of mitochondrial glycerol-3-phosphate dehydrogenase by the peroxisome proliferator clofibric acid

(*Received* 18 *July* 1990; *accepted* 18 *September* 1990)

Mitochondrial glycerol-3-phosphate dehydrogenase [EC 1.1.99.5] (*sn*-glycerol-3-phosphate:acceptor 2-oxidoreductase) is regulated by thyroid hormone *in vivo* [1–5] and *in vitro* [6, 7]. Thyroid hormone treatment of rats stimulates glycerol-3-phosphate dehydrogenase activity in a number of tissues [1–3], with the largest increase occurring in the liver. This activity is also increased by treatment of animals with a number of hypolipidemic drugs [8–11] and structurally unrelated compounds such as di(2-ethylhexyl)phthalate (DEHP)* [12]. The term peroxisomal proliferator is applied to these hypolipidemic drugs [11, 13] and DEHP [14] since they increase peroxisomal β-oxidation activity.

From the *in vivo* results, it was inferred that peroxisomal proliferators increase hepatic glycerol-3-phosphate dehydrogenase activity through thyroid hormone based on the known thyroid hormone-dependent regulation and the observation that the stimulation with either clofibrate [9] or DEHP [12] was attenuated in hypothyroidism. Interpretation of *in vivo* data is hazardous, however, since assessment as to whether hepatic effects are primary or secondary to actions of thyroid hormone (or its lack) on other tissues is problematic. Primary cultures of hepatocytes have been used to show that clofibric acid (the active metabolite formed upon absorption of clofibrate [15]) stimulates glycerol-3-phosphate dehydrogenase activity [16], but the culture medium employed was supplemented with fetal bovine serum which contains thyroid hormone so that rigorous testing of a thyroid hormone requirement was not undertaken.

We have previously used a fully defined medium with primary cultures of rat hepatocytes to show that thyroid hormone is not required for stimulation of peroxisomal β-oxidation activity by clofibric acid [17] and now have applied this system to the study of the role of thyroid hormone in the stimulation of mitochondrial glycerol-3-phosphate dehydrogenase by clofibric acid and the relationship of this activity to that of peroxisomal β-oxidation.

Materials and Methods

Biochemical reagents including collagenase (Sigma Type IV) were obtained as previously described [17]. Hepatocytes from adult (200–300 g) male Fischer F344 rats (Charles River, Wilmington, MA) were isolated and cultured as reported previously [17] with some modifications. Primaria-coated culture dishes (Baxter Scientific, Romulus, MI) were used. The attachment medium consisted of the basal medium, described below, with the following changes. Dimethyl sulfoxide was omitted, 10% (v/v) fetal bovine serum was added, and antibiotic levels were 100 units/mL penicillin G, 100 μg/mL streptomycin, and 50 μg/mL gentamicin. After 1 hr in 7 mL of attachment medium, nonadhering cells and medium were removed, and culturing was continued using 10 mL of basal medium.

The basal medium consisted of L-15 (modified) with L-glutamine supplemented as follows: 75 units/mL of penicillin G, 75 μg/mL of streptomycin, 30 μg/mL of gentamicin, 1 μM insulin, 1 μM dexamethasone, 10 mM glucose, 1 mM succinate, 2 mM L-carnitine, 0.4% (v/v) dimethyl sulfoxide, and 25 mM Hepes (pH 7.4). A sample of cells was collected after attachment and designated as Time 0. Unless noted otherwise, clofibric acid was added immediately following attachment using dilutions of a 250 mM clofibric acid stock solution in 500 mM sodium carbonate. 3,5,3′-Triiodothyronine (T_3) additions were made using a 1 mM stock solution of the sodium salt in 0.5 mM NaOH. Control media contained equivalent volumes of vehicle. All media were replaced every 24 hr. The harvesting of cells and isolation of an organellar pellet are described elsewhere [17].

Glycerol-3-phosphate dehydrogenase activity was assayed in organellar fractions as described previously [17]. Data are expressed as the ratio of glycerol-3-phosphate dehydrogenase : citrate synthase rates (GPD : CS). The ratio was used since organellar specific activity of citrate synthase declined with time in culture whereas homogenate citrate synthase specific activities were constant, indicating that the proportion of non-mitochondrial protein in the organellar fraction increased with time in culture. Citrate synthase and fatty acyl-CoA oxidase (the rate-limiting enzyme for peroxisomal fatty acid β-oxidation [18]) activities and protein were determined as described previously [17].

Results and Discussion

Addition of clofibric acid increased mitochondrial glycerol-3-phosphate dehydrogenase activity in a time-dependent manner in the absence of exogenous thyroid hormone and serum (Fig. 1). The time course paralleled that for the peroxisomal marker, fatty acyl-CoA oxidase, with both activities reaching their maximum after 144 hr of exposure.

Although thyroid hormone was not added, the possibility

* Abbreviations: DEHP, di(2-ethylhexyl)phthalate; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; GPD:CS, ratio of glycerol-3-phosphate dehydrogenase activity to citrate synthase activity; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane; and T_3, 3,5,3′-triiodothyronine.

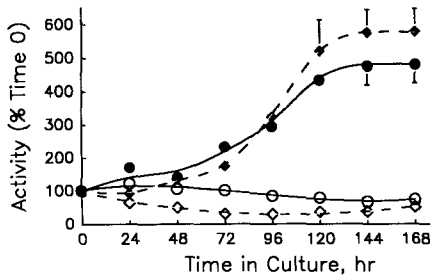

Fig. 1. Time course for the effects of clofibric acid on fatty acyl-CoA oxidase and glycerol-3-phosphate dehydrogenase activities. Cells were cultured for the indicated times either in the presence (●, ◆) or in the absence (○, ◇) of 300 μM clofibric acid. Data are expressed as percent of the value at Time 0 and are plotted as mean ± range for two separate hepatocyte preparations. Key: (◇, ◆) fatty acyl-CoA oxidase activity, and (○, ●) GPD : CS ratio in the organellar fraction. The cellular specific activity [nmol·min^{-1}·(mg protein)$^{-1}$] at Time 0 was 9.15 ± 0.65 for fatty acyl-CoA oxidase, and the organellar GPD : CS ratio at Time 0 was 0.0348 ± 0.0039. Citrate synthase activity was unchanged with either time in culture or treatment with clofibric acid.

existed that the effects of clofibric acid could have been mediated by endogenous thyroid hormone. Because primary cultures of hepatocytes rapidly metabolize T_3 ($T_{1/2}$ of 3.5 hr [6]), culturing for 24 hr should allow for essentially complete metabolism of residual T_3. Whether clofibric acid was added immediately or after 24 hr in culture did not alter either the extent or the kinetics of stimulation of glycerol-3-phosphate dehydrogenase activity (data not shown). These results indicate that thyroid hormone does not mediate, and is not required for, the clofibric acid-induced stimulation of glycerol-3-phosphate dehydrogenase activity.

To characterize further the effects of clofibric acid on glycerol-3-phosphate dehydrogenase activity, the concentration-dependence of the stimulation by clofibric

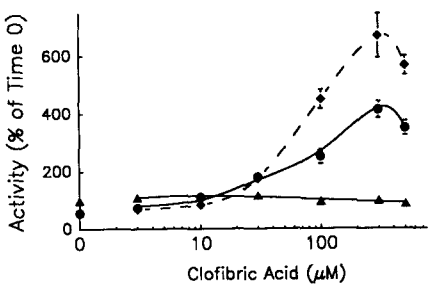

Fig. 2. Effect of various concentrations of clofibric acid on fatty acyl-CoA oxidase, glycerol-3-phosphate dehydrogenase, and citrate synthase activities at 144 hr. The cells were cultured in the indicated concentrations of clofibric acid for 144 hr. Data are expressed as percent of the value at Time 0 and plotted as mean ± SEM for three hepatocyte preparations. The fatty acyl-CoA oxidase (◆) specific activity at Time 0 was 7.93 ± 0.58 nmol·min^{-1}·(mg protein)$^{-1}$. The organellar GPD : CS ratio (●) at Time 0 was 0.0493 ± 0.0038. The cellular citrate synthase (▲) specific activity at Time 0 was 105 ± 7 nmol·min^{-1}·(mg protein)$^{-1}$.

Table 1. Effect of combined treatment with T_3 and clofibric acid on glycerol-3-phosphate dehydrogenase activity

	GPD : CS ratio (% of Time 0)		
Clofibric acid (μM)	T_3 (μM) 0.00	0.01	10.0
0	85.7 (1.0x)	146 (1.7x)	375 (4.4x)
500	274.0 (3.2x)	465 (5.4x)	796 (9.3x)

Cells were cultured for 96 hr in basal medium with the indicated concentrations of T_3 and clofibric acid. Data are the result of a typical experiment and are expressed as percent of GPD : CS ratio at Time 0. Values in parentheses show data expressed as fold of control activity (absence of both clofibric acid and T_3). The value of the GPD : CS ratio at Time 0 was 0.0545.

acid was studied in the absence of thyroid hormone. Peroxisomal fatty acyl-CoA oxidase was also studied since the time courses for the clofibrate-dependent stimulations had been found to be similar (Fig. 1). An exposure duration of 144 hr was chosen since the increases in both activities were complete by this time. As shown in Fig. 2, the stimulations of glycerol-3-phosphate dehydrogenase and fatty acyl-CoA oxidase activities showed similar concentration dependencies, with EC_{50} values around 70 μM.

The decreases in these activities at clofibric acid concentrations exceeding 300 μM were not due to a generalized cytotoxicity as indicated by the lack of effect on citrate synthase activity (Fig. 2). In addition, no concentration-dependent effects of clofibric acid on total protein or DNA were observed (data not shown). Although the cause is unknown, biphasic concentration–response curves have been observed previously with the peroxisomal proliferators, mono(2-ethylhexyl)phthalate and DEHP [19].

To test for independence of actions on glycerol-3-phosphate dehydrogenase, T_3 and clofibric acid alone and in combination were studied. If these agents act independently, addition of either agent to a maximally stimulating concentration of the other should yield a further increase in activity; no further increase should be seen if a single, saturable mechanism is shared by the agents.

Clofibric acid at 300 μM was found to yield the observed maximal stimulation of glycerol-3-phosphate dehydrogenase activity (Fig. 2). As shown in Table 1, when 10 μM T_3, a maximally stimulating concentration [7, 17], was combined with 500 μM clofibric acid, the resulting activity was greater than that produced by either agent alone at its maximally stimulating concentration. These results strongly suggest that peroxisomal proliferators and thyroid hormone stimulate glycerol-3-phosphate dehydrogenase activity through independent mechanisms.

As noted above, previous results had been interpreted as indicating that peroxisomal proliferators stimulate glycerol-3-phosphate dehydrogenase activity through actions on thyroid hormone because the absolute level of activity attained after treatment with these agents was reduced considerably in hypothyroid animals. However, hypothyroidism itself caused a large decrease in basal activity. Our results in Table 1 indicate that the level of activity is the sum of the thyroid hormone-dependent and the peroxisomal proliferator-dependent stimulations. Thus, to evaluate effects of a peroxisomal proliferator under differing thyroid conditions, the data should be expressed as fold-stimulation of the appropriate basal activity. When the previous data [9, 12] were expressed in this manner as shown in Table 2, clofibrate and DEHP were seen to produce 3- to 5-fold stimulations of dehydrogenase activity in both hypothyroid and euthyroid states, in agreement

Table 2. Comparison of fold-increases in hepatic, mitochondrial glycerol-3-phosphate dehydrogenase activity induced by peroxisomal proliferators with differing thyroid states

Treatment	Glycerol-3-phosphate dehydrogenase activity		
	Basal	Treated	Fold-increase
Data of Westerfeld *et al.* [9] with clofibrate			
None	25	118	4.7x
Thyroidectomy	4 (3–5)	12 (5–18)	3.0x
Thyroidectomy + T_3	35 (25–45)	103 (80–125)	3.0x
Triac	130	184	1.4x
Data of Nair and Kurup [12] with DEHP			
None	17.3	60.3	3.5x
Thyroidectomy	8.2	29.2	3.6x
Propylthiouracil	6.7	30.8	4.6x
Data of this report with clofibric acid			
0 nM T_3	85.7	274	3.2x
10 nM T_3	146	465	3.2x
10 μM T_3	375	796	2.1x

Data of Westerfeld *et al.* [9] were obtained in male Holtzman rats fed 0.3% (w/w) clofibric acid for 2 weeks. With thyroidectomy, the midpoint of the range in parentheses was used to calculate fold-increase. Nair and Kurup [12] used inbred, male, albino rats fed 2% (w/w) DEHP for 21 days. Our *in vitro* data are taken from Table 1. Other units are as follows: Westerfeld *et al.*, μL O_2 consumed·(150 mg tissue)$^{-1}$·(10 min)$^{-1}$; Nair and Kurup, nmol·min^{-1}·(mg mitochondrial protein)$^{-1}$.

with our observations *in vitro*. When basal activity was greatly elevated as with triac or 10 μM T_3, the lower fold-stimulation seen may be an indication that another factor (such as the maximal velocity for transcription) eventually limits the absolute response. The observation that the peroxisomal proliferators produced similar fold-increases under varying thyroid hormone conditions is consistent with the notion that these agents ultimately act through a nuclear peroxisomal receptor separate from the thyroid receptor regulating the glycerol-3-phosphate dehydrogenase gene [20].

Our results support the previous suggestion [16] that stimulation of mitochondrial glycerol-3-phosphate dehydrogenase activity is part of the primary response to peroxisomal proliferators. This conclusion is based on similarities in both the time-courses (Fig. 1) and concentration–response curves (Fig. 2) for the stimulations of the peroxisomal marker fatty acyl-CoA oxidase and glycerol-3-phosphate dehydrogenase activities by clofibric acid. A strong correlation between these activities has been found with a large number of peroxisomal proliferators including the hypolipidemic drugs [8–11], the plasticizer DEHP [12, 19] and its metabolite mono(2-ethylhexyl)-phthalate [16, 19], and perfluorocarboxylic acids [21]. In an apparent exception, Kähönen and Ylikahri [22] found no effect of the hypolipidemic drug gemfibrozil on glycerol-3-phosphate dehydrogenase activity, but the data of Foxworthy and Eacho [23] suggest that the doses used were below those needed to stimulate peroxisomal β-oxidation and glycerol-3-phosphate dehydrogenase activities.

Because only enzymatic activity was measured in this investigation, the molecular basis for the increased glycerol-3-phosphate dehydrogenase activity with peroxisomal proliferators cannot be determined. Stimulation of transcription appears most likely, however, since this has been demonstrated to be the primary mechanism leading to increased β-oxidation activities with peroxisomal proliferators [24–26]. With T_3 treatment, an increase in translatable mRNA for glycerol-3-phosphate dehydrogenase has been demonstrated [27].

In summary, primary cultures of adult rat hepatocytes using a serum-free, fully defined medium were used to show that the peroxisomal proliferator clofibric acid stimulates mitochondrial glycerol-3-phosphate dehydrogenase activity by a mechanism distinct from the well-known thyroid hormone-dependent response. Addition of clofibric acid produced an increase in enzyme activity in the absence of added thyroid hormone; experimental conditions designed to deplete endogenous thyroid hormone did not alter the response to the peroxisomal proliferator. In the presence of T_3 concentrations yielding maximal stimulation of glycerol-3-phosphate dehydrogenase activity as determined previously, addition of clofibric acid produced a further increase in glycerol-3-phosphate dehydrogenase activity. Both the concentration-dependence and time-course for the stimulation of mitochondrial glycerol-3-phosphate dehydrogenase by clofibric acid were found to be similar to those of the peroxisomal marker, fatty acyl-CoA oxidase. These results indicate that mitochondrial glycerol-3-phosphate dehydrogenase activity is controlled independently by thyroid hormone and the peroxisomal regulatory system.

Acknowledgements—This research was supported in part by a grant from the American Heart Association of Michigan. Technical assistance in the dietary treatment experiments was ably supplied by G. Barbara Schade. Helpful discussions with Bernard Marks and Ronald Hines are gratefully acknowledged.

Department of Pharmacology RANDALL L. SHOEMAKER*
Wayne State University School of Medicine RUSSELL K. YAMAZAKI†
Detroit
MI 48201, U.S.A.

* Present address: Sinai Hospital, Center for Cell Biology, 6767 West Outer Drive, Detroit, MI 48235.

† Address correspondence to: Dr. Russell K. Yamazaki, Department of Pharmacology, Wayne State University School of Medicine, 540 East Canfield, Detroit, MI 48201.

REFERENCES

1. Lee Y-P, Takemori AE and Lardy H, Enhanced oxidation of α-glycerophosphate by mitochondria of thyroid-fed rats. *J Biol Chem* **234**: 3051–3054, 1959.
2. Ruegamer WR, Newman GH, Richert DA and Westerfeld WW, Specificity of the α-glycerophosphate dehydrogenase and malic enzyme response to thyroxine. *Endocrinology* **77**: 707–715, 1965.
3. Lee Y-P and Lardy HA, Influence of thyroid hormones on 1-α-glycerophosphate dehydrogenases and other dehydrogenases in various organs of the rat. *J Biol Chem* **240**: 1427–1436, 1965.
4. Tarentino AL, Richert DA and Westerfeld WW, The concurrent induction of hepatic α-glycerophosphate dehydrogenase and malate dehydrogenase by thyroid hormone. *Biochim Biophys Acta* **124**: 295–309, 1966.
5. Lee K-L, Sellinger OZ and Miller ON, Induction of mitochondrial α-glycerophosphate dehydrogenase by thyroid stimulating hormone and thyroid hormone analogues. *Proc Soc Exp Biol Med* **126**: 169–173, 1967.
6. Ismail-Beigi F, Bissell DM and Edelman IS, Thyroid thermogenesis in adult rat hepatocytes in primary monolayer culture (direct action of thyroid hormone *in vitro*). *J Gen Physiol* **73**: 369–383, 1979.
7. Wilson EJ and McMurray WC, Regulation of malic enzyme and mitochondrial α-glycerophosphate dehydrogenase by thyroid hormones, insulin, and glucocorticoids in cultured hepatocytes. *J Biol Chem* **256**: 11657–11662, 1981.
8. Hess R, Stäubli W and Riess W, Nature of the hepatomegalic effect produced by ethyl-chlorophenoxyisobutyrate in the rat. *Nature* **208**: 856–858, 1965.
9. Westerfeld WW, Richert DA and Ruegamer WR, The role of the thyroid hormone in the effect of *p*-chlorophenoxyisobutyrate in rats. *Biochem Pharmacol* **17**: 1003–1016, 1968.
10. Krishnakantha TP and Kurup CKR, Increase in hepatic catalase and glycerol phosphate dehydrogenase activities on administration of clofibrate and clofenapate to the rat. *Biochem J* **130**: 167–175, 1972.
11. Holloway BR and Orton TC, The effect of hypolipidaemic agents on hepatic peroxisomal and microsomal drug metabolising enzymes in relation to thyroxine modulation. *Br J Pharmacol* **69**: 318P–319P, 1979.
12. Nair N and Kurup CKR, Are the hypocholesterolemic action and metabolic effects of diethylhexyl phthalate mediated by thyroxine? *Indian J Biochem Biophys* **23**: 76–79, 1986.
13. Lazarow PB and De Duve C, A fatty acyl-CoA oxidizing system in rat liver peroxisomes; Enhancement by clofibrate, a hypolipidemic drug. *Proc Natl Acad Sci USA* **73**: 2043–2046, 1976.
14. Osumi T and Hashimoto T, Enhancement of fatty acyl-CoA oxidizing activity in rat liver peroxisomes by di-(2-ethylhexyl)phthalate. *J Biochem (Tokyo)* **83**: 1361–1365, 1978.
15. Thorp JM, Experimental evaluation of an orally active combination of androsterone with ethyl chlorophenoxyisobutyrate. *Lancet* **1**: 1323–1326, 1962.
16. Mitchell AM, Bridges JW and Elcombe CR, Factors influencing peroxisome proliferation in cultured rat hepatocytes. *Arch Toxicol* **55**: 239–246, 1984.
17. Shoemaker RL and Yamazaki RK, Peroxisomal fatty acyl-CoA oxidase is not regulated by triiodothyronine. *Biochim Biophys Acta* **1044**: 169–172, 1990.
18. Inestrosa NC, Bronfman M and Leighton F, Detection of peroxisomal fatty acyl-coenzyme A oxidase activity. *Biochem J* **182**: 779–788, 1979.
19. Mitchell FE, Bridges JW and Hinton RH, Effects of mono (2-ethylhexyl)phthalate and its straight chain analogues mono-*n*-hexylphthalate and mono-*n*-octyl phthalate on lipid metabolism in isolated hepatocytes. *Biochem Pharmacol* **35**: 2941–2947, 1986.
20. Oppenheimer JH, Coulombe P, Schwartz HL and Gutfeld NW, Nonlinear (amplified) relationship between nuclear occupancy by triiodothyronine and the appearance rate of hepatic α-glycerophosphate dehydrogenase and malic enzyme in the rat. *J Clin Invest* **61**: 987–997, 1978.
21. Just WW, Gorgas K, Hartl F-U, Heineman P, Solzer M and Schimassek H, Biochemical effects and zonal heterogeneity of peroxisome proliferation induced by perfluorocarboxylic acids in rat liver. *Hepatology* **9**: 570–581, 1989.
22. Kähönen MT and Ylikahri RH, Effect of clofibrate and gemfibrozil on the activities of mitochondrial carnitine acyltransferases in rat liver. *Atherosclerosis* **32**: 47–56, 1979.
23. Foxworthy PS and Eacho PI, Conditions influencing the induction of peroxisomal β-oxidation in cultured rat hepatocytes. *Toxicol Lett* **30**: 189–196, 1986.
24. Furuta S, Miyazawa S and Hashimoto T, Biosynthesis of enzymes of peroxisomal β-oxidation. *J Biochem (Tokyo)* **92**: 319–326, 1982.
25. Chatterjee B, Demyan WF, Lalwani ND, Reddy JK and Roy AK, Reversible alteration of hepatic messenger RNA species for peroxisomal and non-peroxisomal proteins induced by the hypolipidemic drug WY-14,643. *Biochem J* **214**: 879–883, 1983.
26. Reddy JK, Goel SK, Nemali MR, Carrino JJ, Laffler TG, Reddy MK, Sperbeck SJ, Osumi T, Hashimoto T, Lalwani ND and Rao MS, Transcriptional regulation of peroxisomal fatty acyl-CoA oxidase and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase in rat liver by peroxisome proliferators. *Proc Natl Acad Sci USA* **83**: 1747–1751, 1986.
27. Taylor VJ and Ragan CI, The induction of mitochondrial L-3-glycerophosphate dehydrogenase by thyroid hormone. *Biochim Biophys Acta* **851**: 49–56, 1986.