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EFFECT OF SUB-ACUTE ADMINISTRATION OF CLOFIBRATE ON THE OXIDATION OF FATTY ACIDS BY LIVER MITOCHONDRIA

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Abstract—Normal and thyroidectomized male rats were fed powdered Rockland Mouse/Rat Diet (complete) with and without 0.3% clofibrate, *ab lib.*, for 7 days. At the end of the feeding period the rats were killed by decapitation, livers were removed, and liver mitochondria were isolated and studied. In normal rats, clofibrate increased liver weight, mitochondrial yield per g of liver, and mitochondrial oxidations, in state 3, of α-glycerophosphate, octanoate, palmitate, octanoylcarnitine and palmitoylcarnitine; however, the oxidation of succinate in states 3 and 4 was not altered. Thyroidectomy resulted in decreased liver weight and decreased mitochondrial oxidation of α-glycerophosphate, succinate, octanoate, and palmitate. Administration of clofibrate to thyroidectomized rats increased mitochondrial oxidations of octanoate and palmitate but did not affect liver weight or oxidations of succinate and α-glycerophosphate.

Clofibrate (ethyl-chlorophenoxyisobutyrate) lowers the serum of triglyceride in both humans and animals [1, 2] but the mechanism is unknown. Many potential modes of action have been suggested including decreased fatty acid release from adipose tissue [3]; increased clearance of serum triglyceride by adipose tissue [4]; inhibition of hepatic triglyceride release [5–7]; and reduction in the rate of hepatic fatty acid synthesis [8]. It it possible that accelerated hepatic fatty acid oxidation could also contribute to the hypotriglyceridemic action of clofibrate by competing with triglyceride biosynthesis for available substrate. In man, chronic administration of clofibrate has been reported to increase the oxidation of palmitate to acetoacetate and CO_2 [9].

However, the literature concerning the effects of clofibrate or CPIB (chlorophenoxyisobutyrate) on hepatic fatty acid oxidation is contradictory. Hassinen and Kähönen [10] reported that the rate of oxygen consumption linked to octanoate oxidation was increased in perfused liver and in isolated hepatic mitochondria from rats which had received 500 mg/kg subcutaneously of clofibrate daily for 7 days. In apparent accord with these findings, Solberg *et al.* [11, 12] and Daae and Aas [13] have reported that the activities of acyltransferase enzymes in rat liver mitochondria are increased by feeding 0.3% clofibrate in the stock diet for 1–6 weeks. However, Cederbaum *et al.* [14] have recently reported that liver mitochondria from rats that were administered 300 mg/kg of clofibrate subcutaneously for 14 or 18 days showed decreased rates of fatty acid oxidation.

An accurate assessment of the relationship between clofibrate administration and the rate of fatty acid oxidation in liver might contribute toward elucidating the hypotriglyceridemic action of this drug. In this regard, the present series of experiments were designed to measure the effect of subacute oral administration of clofibrate, to normal and thyroidectomized male rats, on fatty acid oxidation by isolated rat liver mitochondria.

MATERIALS AND METHODS

Animals and treatment. Normal and thyroidectomized male rats of the CR–CD strain were obtained form the supplier and allowed to stabilize for 1 and 6 weeks, respectively, prior to the beginning of the study. During this period of time the rats were fed pellets of Rockland Mouse/Rat Diet (complete), *ad lib.*; normal rats received tap water and thyroidectomized rats received Hank's Solution [15]. After the stabilization period, 2 normal and 2 thyroidectomized rats were entered into a feeding study (each day) in which the pelleted diet was replaced with powdered diet or powdered diet containing 0.3% clofibrate (w/w). Rat weight was determined daily and food consumption every other day, After 7 days of feeding, and without prior fasting, 1 rat from each group was killed by decapitation in the morning and 1 in the afternoon. Blood was collected from the wound for analysis of T_4, livers were quickly removed, and liver mitochondria were isolated by differential centrifugation and finally suspended in 0.25 M sucrose [16]. Mitochondrial protein was determined by a modification of the biuret method of Gornal *et al.*[17] in which turbidity corrections were made by removing the blue color with a small amount of powdered KCN.

The normal control and clofibrate treated rats had mean T_4 levels of 6.1 and 4.3 μg/ml, respectively, whereas serum from thyroidectomized rats always contained less than 1 μg T_4/ml.

Oxygen consumption. Oxygen consumption of intact liver mitochondria was determined polarographically at 30° as previously described [18]. The incubation media (1.8 ml, pH 7.4) contained the following constituents at the indicated concentrations: Tris buffer, 65 mM; KCl, 75 mM; $MgCl_2$, 5 mM; phosphate buffer, 12 mM; EDTA, 1 mM; and 18–20 mg of fatty acid free albumin [19, 20]. ADP, succinate, α-glycerophosphate, acetate, octanoate, palmitate, octanoylcarnitine, palmitoylcarnitine, carnitine, sucrose, and

Table 1. Effects of clofibrate treatment on rat growth, liver weight and yield of liver mitochondria*

Treatment	Rat weight (g) day 0	Rat weight (g) day 7	Rat weight (g) gain	liver weight (g)/ 100 g body weight	Mitochondrial yield (mg protein/g liver)
Normal					
Control (7)	277 ± 6	309 ± 7	33.7 ± 4.0	4.13 ± 0.12	49.5 ± 0.20
+ Clofibrate (7)	272 ± 6	315 ± 8	44.1 ± 4.8	6.22 ± 0.22	57.9 ± 0.25
	N.S.	N.S.	N.S.	$P < 0.001$	$P < 0.05$
Thyroidectomized					
Control (10)	226 ± 15	239 ± 12	13.0 ± 5.1	3.08 ± 0.13	55.8 ± 0.28
+ Clofibrate (11)	228 ± 15	235 ± 11	7.2 ± 7.6	3.13 ± 0.21	56.5 ± 0.20
	N.S.	N.S.	N.S.	N.S.	N.S.

* Values represent means ± S.E.M. Statistical evaluations were performed by Student's *t*-test for unpaired data; N.S. means not significant ($P > 0.05$). The number of animals for which data was obtained is given in parentheses.

mitochondria were added at various concentrations and combinations during each run bringing the final volume to approximately 2.0 ml. Further experimental details are given below. In all cases when anions were added potassium was the counterion. Tris and phosphate buffers (pH 7.4) were prepared by mixing aliquots of stock solutions containing Tris and Tris–HCl, and KH_2PO_4 and K_2HPO_4, respectively. The concentration of O_2 in the medium equilibrated with air at 30° was experimentally determined. Frozen-thawed mitochondria, added to the medium at 1 mg protein/ml, were used to oxidize known amounts of $NADH_2$ to completion. The O_2 concentration was then calculated from the equation, $2NADH_2 + O_2 \rightarrow 2H_2O + 2NAD^+$, and found to be 0.21 mM.

Materials. Rats were purchased from Charles River Breeding Laboratories, Wilmington, MA. Total serum T_4 (test no. 1597) was determined by Mason-Barron Laboratories, Inc., Chicago, IL. Acetic acid, succinic acid, and inorganic salts were purchased from Mallinckrodt Chemical Co., St. Louis, MO; ADP, α-glycero phosphate, Tris, and Tris–HCl from Sigma Chemical Co., St. Louis, MO; palmitic acid from Fisher Scientific Co., Fairlawn, NJ; DL-carnitine·HCl from General Biochemicals, Chagrin Falls, Ohio; octanoic acid from Aldrich Chemical Co., Inc., Milwaukee, WI; sucrose from Schwarz/Mann, Orangeburg, NY; $NADH_2$ from P-L Biochemicals, Inc., Milwaukee, WI; and bovine serum albumin (Pentex, Fraction V) from Miles Laboratories, Inc., Kankakee, IL. Palmitoyl-L-carnitine and octanoyl-L-carnitine were gifts from the Otsuka Pharmaceutical Co., Tokoshima, Japan.

RESULTS

Thyroxine is involved in mediating caloriginesis as well as rat organ and body growth. Among many other changes, thyroidectomy produces reduced growth rate [21–23], decreased liver: body weight ratio [23], and decreased metabolic rate [24]. Mitochondrial α-glycerophosphate dehydrogenase [25] and succinate dehydrogenase [26] are 2 enzymes involved in the cellular process of calorigenesis with activities that reflect the thyroxine level and the corresponding level of energy metabolism.

As expected, the thyroidectomized rats used in the present studies showed reduced growth rate, decreased liver weight:body weight ratio (Table 1), and decreased rates of α-glycerophosphate and succinate oxidation by liver mitochondria (Table 2). Also, mitochondrial yield per g of liver was not altered. The effects of clofibrate on most of these parameters in both normal and thyroidectomized rats have been reported previously and the present work confirms these earlier findings as indicated. For normal rats, clofibrate administration increased the liver weight: body weight ratio [27] (Table 1), mitochondrial yield per g of liver [28] (Table 1) and mitochondrial oxidation of α-glycerophosphate [23] (Table 2). However, these effects were not seen in thyroidectomized rats [23] (Tables 1 and 2). Clofibrate treatment did not affect the oxidation of succinate by mitochondria

Table 2. Effects of clofibrate on oxidation of α-glycero phosphate and succinate by rat liver mitochondria*

Treatment	α-Glycero phosphate: state 3 (/ng at. 0/min/mg P)	Succinate: state 3 (ng at. 0/min/mg P)	Succinate: state 4 (ng at. 0/min/mg P)	Succinate: RCR	Succinate: ADP:0
Normal	5.59 ± 0.20 (6)	161 ± 6 (7)	25.7 ± 0.73 (7)	6.16 ± 0.14 (7)	1.80 ± 0.04 (7)
+ Clofibrate	17.28 ± 1.74 (6)	159 ± 5 (7)	28.4 ± 1.45 (7)	5.80 ± 0.13 (7)	1.76 ± 0.03 (7)
	$P < 0.001$	N.S.	N.S.	N.S.	N.S.
Thyroidectomized	0.68 ± 0.13 (7)	129 ± 13 (6)	21.4 ± 3.45 (6)	6.50 ± 0.73 (6)	—
+ Clofibrate	0.99 ± 0.12 (6)	143 ± 12 (7)	23.3 ± 4.22 (7)	6.67 ± 0.60	—

* Reaction mixtures contained the basic constituents described in Methods as well as 2.0 mg of mitochondrial protein, 25 mM sucrose and 0.4 mM ADP. Substrates were 20 mM DL-α-glycerophosphate or 10 mM succinate. Values represent means ± S.E.M. Statistical evaluations were performed by Student's *t*-test for unpaired data; N.S. means not significant ($P > 0.05$). The number of animals for which data was obtained is given in parentheses.

Table 3. Effects of clofibrate administration on oxygen uptake linked to the oxidation of fatty acids by liver mitochondria from normal rats*

Additions	Rate of oxygen uptake (ng at 0/min/mg protein) Control	Clofibrate treated	Level of significance
None	4.70 ± 0.47 (7)	4.89 ± 0.36 (7)	N.S.
ADP	9.24 ± 0.64 (7)	12.8 ± 0.81 (7)	$P < 0.005$
ADP + acetate	9.24 ± 0.85 (7)	15.5 ± 1.3 (7)	$P < 0.01$
ADP + acetate + carnitine	12.4 ± 1.2 (7)	19.0 ± 1.3 (7)	$P < 0.001$
ADP + acetate + carnitine + malate	27.0 ± 1.3 (7)	32.7 ± 2.2 (7)	$P < 0.05$
ADP + octanoate	25.3 ± 1.65 (7)	36.5 ± 4.57 (5)	$P < 0.05$
ADP + octanoate + carnitine	26.5 ± 1.89 (7)	53.8 ± 6.99 (5)	$P < 0.005$
ADP + octanoate + carnitine + malate	59.7 ± 2.66 (7)	80.9 ± 8.47 (5)	$P < 0.05$
ADP + palmitate	12.8 ± 0.65 (7)	21.5 ± 1.47 (7)	$P < 0.001$
ADP + palmitate + carnitine	22.0 ± 1.08 (7)	39.1 ± 3.32 (7)	$P < 0.001$
ADP + palmitate + carnitine + malate	49.2 ± 2.71 (7)	64.9 ± 4.80 (7)	$P < 0.025$

* Reaction mixtures contained the basic constituents described in Methods as well as 2.0 mg of mitochondrial protein and 25 mM sucrose. Other additions were as follows: 1.1 mM ADP, 10 mM malate, 10 mM acetate, 2 mM octanoate, 1 mM palmitate, and 2 mM carnitine. Values represent means ± S.E.M. Statistical evaluations were performed by Student's *t*-test for unpaired data; N.S. means not significant ($P > 0.05$).

The number of animals for which data was obtained is given in parentheses.

of either normal [29] (Table 2) or thyroidectomized rats (Table 2). It is important to note that these various effects were produced at a clofibrate dose (0.3%, w/w) that did not affect rat weight gain (Table 1) since fasting can affect the parameters measured in this study.

The results reported above indicate that the mitochondria were characteristically altered by clofibrate and were, therefore, suitable for evaluating the effects of clofibrate on other mitochondrial processes such as fatty acid oxidation. In the present investigation, acetic, octanoic, and palmitic acids representing short chain, medium chain, and long chain groups, respectively, were chosen for study of their rates of oxidation by mitochondria from normal and thyroidectomized rats treated with clofibrate. Rates of mitochondrial oxygen uptake were studied under the following conditions: (1) unstimulated, endogenous rates or rates in the presence of fatty acid; (2) stimulated, state 3 rates obtained in the presence of fatty acid and ADP (3) carnitine augmented, state 3 rates obtained in the presence of fatty acid, ADP, and carnitine; and (4) maximal, state 3 rates obtained in the presence of fatty acid, ADP, carnitine, and malate. The rates of oxygen uptake in each experimental condition differed with the fatty acid chosen; uptake was highest with octanoate as substrate, slightly lower with palmitate, and the lowest with acetate. In normal rats, clofibrate treatment did not enhance the endogenous rate of oxygen uptake but markedly increased uptake in all of the other conditions (Table 3) and similar, although less dramatic, effects were obtained with liver mitochondria from thyroidectomized rats (Table 4). In agreement with the work of Hassinen and Kähönen [10] the clofibrate stimulation of β-oxidation revealed a carnitine requirement for octanoate

Table 4. Effects of clofibrate administration on oxygen uptake linked to the oxidation of fatty acids by liver mitochondria from thyroidectomized rats*

Additions	Rate of oxygen uptake (ng at 0/min/mg protein) Control	Clofibrate treated	Level of significance†
None	3.80 ± 0.37 (7)	4.00 ± 0.29 (7)	N.S.
ADP	7.45 ± 0.97 (7)	9.4 ± 1.03 (7)	$P < 0.01$
ADP + acetate	7.76 ± 0.88 (6)	9.82 ± 1.11 (6)	$P < 0.01$
ADP + acetate + carnitine	8.73 ± 1.22 (6)	12.2 ± 1.6 (6)	$P < 0.025$
ADP + acetate + malate	18.0 ± 3.4 (6)	25.3 ± 3.4 (6)	$P < 0.001$
ADP + octanoate	22.5 ± 3.7 (9)	29.7 ± 4.6 (9)	$P < 0.05$
ADP + octanoate + carnitine	19.6 ± 2.9 (9)	28.3 ± 4.3 (9)	$P < 0.01$
ADP ± palmitate	14.4 ± 3.0 (8)	19.9 ± 2.6 (8)	$P < 0.025$
ADP + palmitate + carnitine	15.4 ± 2.2 (8)	24.7 ± 4.0 (8)	$P < 0.05$
ADP + palmitate + carnitine + malate	32.8 ± 6.6 (6)	43.0 ± 7.6 (6)	$P < 0.05$

* Experimental design as in Table 3.

† Because of large day to day variations seen with mitochondria from thyroidectomized rats statistical evaluations were performed by Student's *t*-test for paired data.

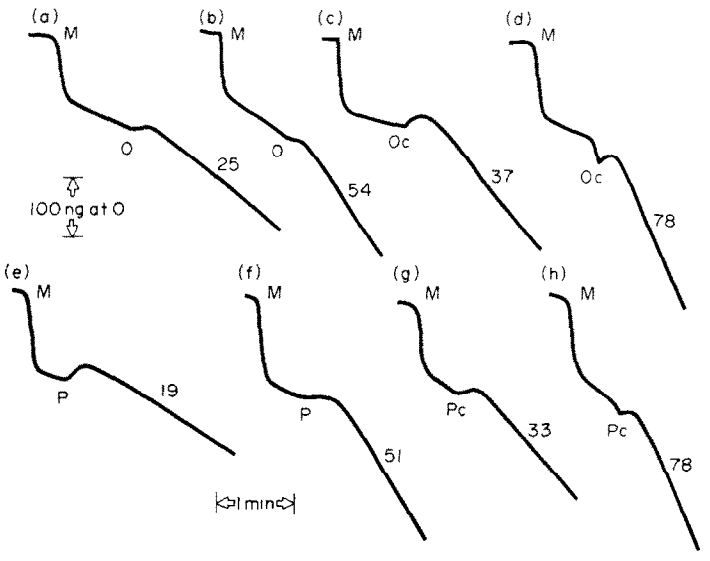

Fig. 1. The stimulatory effect of clofibrate treatment on the oxidations of octanoate (O), octanoylcarnitine (Oc), palmitate (P), and palmitoylcarnitine (Pc) by rat liver mitochondria. Mitochondria from 3 control (a,c,e,g) and 3 clofibrate treated rats (b,d,f,h) were pooled and adjusted to 22 mg protein/ml with 0.25 M sucrose. The 2 ml reaction mixture always contained 55 mM Tris buffer (pH 7.4), 63 mM KCl, 4.2 mM $MgCl_2$, 10 mM phosphate buffer (pH 7.4), 1 mM EDTA, 18 mg of albumin, 25 mM sucrose, 1.1 mM ADP, and 4.4 mg of mitochondrial protein (M). Other additions included 0.5 mM palmitoylcarnitine (g,h), 1.0 mM octanoylcarnitine (c,d), 2 mM octanoate (a,b), 1 mM palmitate (e,f), and 10 mM carnitine (a,b,e,f). Values represent oxygen uptake expressed as ng. atoms oxygen/min/mg protein.

oxidation (Table 3) that was masked at the lower rates of oxidation seen in liver mitochondria from normal control and thyroidectomized rats.

In a separate experiment, oxygen uptake linked to the oxidation of the activated fatty acids octanoylcarnitine and palmitoylcarnitine was studied in mitochondria from normal rats and from normal rats treated with clofibrate in order to determine if fatty acid oxidation was enhanced by clofibrate at sites beyond the latent CoA-carnitine acyltransferase associated with the inner mitochondrial membrane. Acylcarnitine appears to readily enter the mitochondrial matrix bypassing the activation and carnitine transferase steps [30]. Non-latent CoA-carnitine acyltransferase is believed to be rate limiting for long chain fatty acid oxidation that begins with activation in the intra-cristal space [31]. As shown in Fig. 1, liver mitochondria from clofibrate treated rats oxidized the free fatty acids and the corresponding carnitine derivatives at considerably faster rates than did mitochondria from control rats. However, the fatty acids were oxidized at about one-half the rates of the acylcarnitines. Malonate, a succinate dehydrogenase inhibitor, was added in one series of experiments to determine if inhibition of the TCA cycle would alter the stimulatory effects of clofibrate treatment. Malonate slightly reduced all of the rates of oxygen consumption but the results were essentially the same as those presented in Fig. 1.

When fatty acids are used as substrates it is possible to convert the rates of oxygen consumption into carbon flux through the β-oxidation pathway when the end products are known [31–34]. With coupled mitochondria oxidizing octanoate, palmitate, and their carnitine derivatives in state 3, in the absence of other citric acid cycle intermediates, almost all of the acetyl-CoA derived from β-oxidation is converted to acetoacetate [31, 32, 34]. Under these conditions, fatty acid or fatty acylcarnitine consumption can be determined from the following equations:

palmitate (or palmitoylcarnitine)
$$+ 7O_2 \rightarrow 4 \text{ acetoacetate}$$
octanoate (or octanoylcarnitine)
$$+ 3O_2 \rightarrow 2 \text{ acetoacetate}$$

The addition of malate to the reaction mixture results in decreased acetoacetate synthesis and enhanced citrate synthesis and oxygen uptake. At initial malate concentrations of at least 4 mM almost all of the acetyl-CoA enters the citric acid cycle through citrate and almost no acetoacetate is formed [34]. Under these conditions a maximal value for fatty acid or fatty acylcarnitine consumption can be estimated from the following equations:

palmitate (or palmitoyl-carnitine)
$$+ 11O_2 + 8 \rightarrow 8 \text{ citrate}$$
octanoate (octanoylcarnitine)
$$+ 5O_2 + 4 \text{ malate} \rightarrow 4 \text{ citrate}$$

However, in the presence of malate the rate of β-oxidation is overestimated because no correction is made for oxygen uptake associated with further oxidation of citrate.

Table 5 shows the results of our experiments with the data expressed in terms of fatty acid consumption. Similar calculations could not be applied to the acetate values because acetate does not undergo β-oxidation but is activated and either converted to acetoacetate or condensed with oxaloacetate to form citrate [31]. Presumably, oxygen uptake in state 3, in the presence of acetate, actually represented the oxidation of endogenous substrates and stimulation by malate

Table 5. Effects of clofibrate on octanoate and palmitate comsumption by liver mitochondria from normal and thyroidectomized rats

Additions	Rate of fatty acid consumption (nmoles/min/mg protein)			
	Normal rats		Thyroidectomized rats	
	Control	Clofibrate treated	Control	Clofibrate treated
ADP + octanoate + carnitine*	4.4 ± 0.3	9.0 ± 1.2	3.3 ± 0.5	4.7 ± 0.7
ADP + octanoate + carnitine + malate*	6.0 ± 0.3	8.1 ± 0.8		
ADP + palmitate + carnitine*	1.6 ± 0.08	2.8 ± 0.2	1.1 ± 0.2	1.8 ± 0.4
ADP + palmitate + carnitine + malate*	2.2 ± 0.1	3.0 ± 0.2	1.5 ± 0.3	2.0 ± 0.3
ADP + octanoate + carnitine†	4.2	9.0		
ADP + octanoylcarnitine†	12.0	13.0		
ADP + palmitate + carnitine†	1.4	3.6		
ADP + palmitoylcarnitine†	2.4	5.6		

* Values derived from data of Tables 3 and 4.
† Values derived from data of Fig. 1.

resulted in the synthesis of citrate which was further oxidized *via* the TCA cycle. In this regard, the clofibrate stimulated increase of oxygen uptake seen in the presence of acetate (Tables 3 and 4) may represent only the stimulation of endogenous respiration that occurred when ADP alone was added to mitochondria from clofibrate treated rats.

DISCUSSION

It is believed that the rate limiting step in the oxidation of long chain and perhaps medium chain length fatty acids is the synthesis of acylcarnitine via non latent Co-A carnitine acyltransferase [31]. In accord with this concept both octanoate and palmitate were oxidized at considerably slower rates than were the corresponding carnitine derivatives by mitochondria from both control and thyroidectomized rats (Fig. 1). The stimulation of β-oxidation after clofibrate treatment (Table 5) probably involved both increased activity of nonlatent CoA-carnitine acyltransferase and increased activity of enzymes within the β-oxidation pathway itself. Effects of clofibrate on the carnitine acyltransferase enzymes have been well documented. The activity of total liver acylcarnitine transferases [11–13, 35, 36] and of liver mitochondrial acylcarnitine transferases of the inner and outer compartments [12] are increased after clofibrate treatment. Increased activity of enzymes within the β-oxidation pathway after clofibrate treatment is indicated by the faster rates of oxidation seen with the carnitine derivatives of octanoate and palmitate (Fig. 1); these acylcarnitines penetrate the inner membrane readily and maximal oxidation rates are obtained. The marked stimulation of both octanoate and palmitate oxidations (Fig. 1) by clofibrate could not have resulted from increased activity of nonlatent carnitine acyltransferase alone since the rates of octanoate and palmitate oxidations by mitochondria from the clofibrate treated rats exceeded the rates of octanoyl and palmitoylcarnitine oxidations by mitochondria from the untreated control rats.

Thyroidectomy did not prevent the clofibrate induced enhancement of fatty acid oxidation. The rates of palmitate and octanoate oxidations by mitochondria from both control and clofibrate treated rats were lower than in the corresponding normal animals but the stimulatory effect of clofibrate was still clearly evident (Table 5) indicating that this effect in isolated mitochondria was not thyroxine dependent. However, the effect of clofibrate on the total liver capacity for fatty acid oxidation was reduced by thyroidectomy because in the normal rat the effects on the mitochondria were further amplified by an increased number of mitochondria/g of liver (Table 1) and by an increased liver size (Table 1).

It is important to consider that although the results of this study show that the maximum capacity for liver oxidation of fatty acids is enhanced by clofibrate treatment, whether this increased capacity is actually utilized is dependent upon the blood level of fatty acids, competition between fatty acid oxidation and other energy yielding reactions, and the turnover of ATP within the liver cell.

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