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Research Article

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Leucine Oxidation and Protein Turnover in Clofibrate-induced Muscle Protein Degradation in Rats

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A B S T R A C T Treatment of hyperlipidemia with clofibrate may result in development of a muscular syndrome. Our previous investigation (1979. *J. Clin. Invest.* **64**: 405.) showed that chronic administration of clofibrate to rats causes myotonia and decreases glucose and fatty acid oxidation and total protein of skeletal muscle. In the present experiments we have investigated amino acid and protein metabolism in these rats. Clofibrate administration decreased the concentration of all three branched-chain amino acids without affecting those of others in muscle. Studies to examine the mechanism of decreases in muscle concentrations of branched-chain amino acids showed the following: (a) Plasma concentration of leucine was decreased, whereas there was no significant change in the concentration of isoleucine and valine. (b) Liver concentrations of all three branched-chain amino acids remained unaltered. (c) The uptake of cycloleucine (a nonmetabolizable analogue of leucine) by both muscle and liver was unaffected. (d) The percentage of a trace amount of injected [1-¹⁴C]leucine expired as ¹⁴CO₂ in 1 h was significantly increased. (e) The capacity of muscle homogenate for α -decarboxylation of leucine was enhanced, whereas that of liver was unaffected. (f) The activity of leucine transaminase was unaffected, whereas that of α -ketoisocaproate dehydrogenase was increased in muscle.

Studies of protein synthesis, carried out as incorporation of leucine into protein and corrected for differences in specific activity, showed no alteration in liver but enhanced synthesis in muscle of clofibrate-fed rats. Clofibrate stimulated muscle protein degradation, which was demonstrated by increased tyrosine release from gastrocnemius muscle slices and by increased urinary excretion of 3-methylhistidine.

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We conclude that (a) clofibrate treatment increased branched-chain amino acid oxidation by increasing the activity of branched-chain α -ketoacid dehydrogenase in the muscle, (b) increased oxidation results in selective decreases in the concentration of these amino acids in muscle, and (c) decreases in branched-chain amino acid concentration may be responsible for increased protein degradation in muscle.

INTRODUCTION

Oxidation is an important pathway for the metabolism of branched-chain amino acids. Patients with hereditary disorders such as maple syrup urine disease and isovaleric acidemia, in which the oxidation of these amino acids is impaired, often have life-threatening metabolic derangements (1). Unlike other amino acids, which are oxidized mostly in the liver, the oxidation of branched-chain amino acids is accomplished principally in the skeletal muscle (2-5).

In 1971 Adibi and co-workers (6) reported that starvation increases oxidation of leucine by the skeletal muscle. This observation, which has also been noted by others (7; review), raised the possibility of alteration of branched-chain amino acid oxidation by nutritional and metabolic factors. Indeed, studies in a number of laboratories including our own have identified such factors. For example, ketone bodies (8), carnitine (9-11), hexanoate, and octanoate (12, 13) stimulate, and pyruvate (12) and decanoate (13) inhibit the oxidation of leucine by the muscle in vitro.

The fact that starvation and diabetes increase the oxidation of both fatty acids (5, 14) and branched-chain amino acids (5, 8, 15, 16) by the muscle, suggests that there may be a common modulation for oxidation of both substrates. To examine the existence of such a possibility, it would be important to investigate branched-chain amino acid oxidation by muscle in a situation of impaired fatty acid oxidation by this tissue. We have recently reported that treatment of rats with clofibrate decreases fatty acid oxidation by the skeletal muscle

(17). Therefore, the clofibrate-fed rat provided us with a suitable animal model in which to test whether the oxidation of both substrates follows a similar pattern. In the present studies we examined the effects of clofibrate treatment on concentration, transport, transamination, and in vitro and in vivo oxidation of leucine.

As shown in our previous paper (17), clofibrate treatment decreases total protein of the skeletal muscle. In view of recent suggestions that branched-chain amino acids, particularly leucine, are possible regulators of protein turnover in the muscle (18, 19), the studies of leucine metabolism, described above, were accompanied by studies of protein synthesis and degradation in the muscle.

METHODS

Treatment of animals. Male Sprague-Dawley rats were housed in individual cages in air-conditioned quarters ($\sim 24^{\circ}\text{C}$) and received Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) and drinking water ad libitum. Clofibrate-treated rats received 30 mg clofibrate (Atromid S, Ayerst Laboratories, New York) per 100 g body wt daily for 2 wk. Additional details concerning the treatment are described in our previous publication (17).

Incorporation of leucine into tissue protein in vivo and determination of leucine specific activity. Control and clofibrate-fed rats were injected in the tail vein with 5 μCi of L-[1- ^{14}C]leucine/100 g body wt. After 5, 10, and 15 min the rats were sacrificed by stunning and decapitation, and blood samples were collected in chilled heparinized tubes. Immediately after blood collection, small portions of liver and gastrocnemius muscle were removed in duplicate and freeze-clamped in liquid nitrogen.

For studies of leucine incorporation into protein, the frozen tissues were homogenized (1:9, wt/vol) in cold 10% trichloroacetic acid, which also contained 10 mM L-leucine. The homogenate was centrifuged for 10 min at 1,000 g and the resulting pellet was washed with 5 ml of cold 10% trichloroacetic acid that contained 10 mM L-leucine. The washed pellet was solubilized in 2 ml of 0.3 M NaOH by heating for 20 min at 80°C . The protein in a 0.2-ml aliquot was reprecipitated by the addition of 2 ml of 10% trichloroacetic acid. The precipitate was collected on a disk of Whatman filter paper No. 1 (Whatman Inc., Clifton, N. J.), which was uniformly covered with a layer of celite, and washed successively with 5% trichloroacetic acid, ethanol, and ether. This method of collecting and washing the protein sample has been validated (20). The celite cake containing the trapped protein was transferred to a scintillation counting vial and the protein dissolved by the addition of 0.5 ml of Soluene-350 (New England Nuclear, Boston, Mass.). The radioactivity in each vial was determined in a liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) after addition of 10 ml of Liquifluor (New England Nuclear)-toluene scintillation mixture to each vial. The incorporation of leucine into protein was expressed as disintegrations per minute per milligram of protein.

For determination of leucine specific activity, the frozen tissues were homogenized in 6% sulfosalicylic acid (1 ml/100 mg tissue). Blood samples were centrifuged for 10 min at 600 g to separate plasma, which was deproteinized with 6% sulfosalicylic acid (1:1 vol/vol). The sulfosalicylic acid extracts of tissues and plasma were analyzed for specific activity of leucine by a combination of ion-exchange chroma-

tography (Beckman Instruments Inc., Spinco Div., model 120 C, Palo Alto, Calif.) and liquid scintillation spectrometry. The concentration of leucine was determined by the reported method (21). For the determination of radioactive leucine concentration, an aliquot (1 ml) of sulfosalicylic acid extract was applied to a $0.9 \times 60\text{-cm}$ ion-exchange column of UR-30 resin (Beckman Instruments, Inc.). The column was eluted with 0.2 N sodium citrate buffer, pH 3.20, for 170 min followed by elution with 0.2 N sodium citrate buffer, pH 4.25, for 150 min. Under these elution conditions, leucine emerged from the column between 220 and 250 min. Therefore, fractions were collected every minute during this period to ensure complete recovery of leucine. Fractions were collected directly into counting vials and after adding 10 ml of Aquasol-2 (New England Nuclear), radioactivity was determined in a liquid scintillation spectrometer. Each sample was corrected for efficiency by external standardization and for background counts. Net counts in all fractions were pooled and the results were expressed as disintegrations per minute per nanomole of leucine.

The intracellular specific activity of leucine in muscle and liver was calculated by correcting for the concentration and specific activity of leucine in the extracellular space. The concentration and specific activity of leucine in the extracellular space was considered to be the same as that in plasma. Extracellular space in liver and gastrocnemius muscle was determined using inulin as described (22). The extracellular space in liver or muscle of clofibrate-fed rats did not differ significantly from that in the control rats. The extracellular space in livers of control and clofibrate-treated rats was 28.1 ± 1.2 and $26.3 \pm 1.1\%$, respectively (mean \pm SEM in four rats). The extracellular space in muscle of control and clofibrate-treated rats was 14.5 ± 0.6 and $15.4 \pm 0.9\%$, respectively (mean \pm SEM in four rats).

Determination of urinary constituents. Rat urine was collected daily from control and clofibrate-fed rats for the entire duration of clofibrate treatment (2 wk). Total nitrogen in the urine was determined by the modified micro-Kjeldahl procedure using mercury as a catalyst (23). Urinary creatinine was determined by the picric acid method (24). For analysis of 3-methylhistidine concentration, an aliquot of the pooled urine sample was hydrolyzed with HCl to deacetylate any N-acetyl-3-methylhistidine as outlined by Young et al. (25, 26). The 3-methylhistidine concentration was determined using an amino acid analyzer.

Muscle protein degradation in vitro. The method of Fulks et al. (18) for determining protein degradation in rat diaphragm was modified for the present study. Protein degradation was determined by measuring the release of tyrosine from gastrocnemius muscle slices instead of diaphragm. Gastrocnemius muscle slices weighing ~ 100 mg and 0.5 mm thick were prepared with a Stadie-Riggs microtome. Muscle slices were placed in flasks containing 3 ml of Krebs-Ringer bicarbonate buffer (27), pH 7.4, containing 10 mM glucose and saturated with 95% O₂-5% CO₂. After preincubating the stoppered flasks for 30 min at 37°C , the muscle slices were transferred to flasks containing 3 ml of Krebs-Ringer bicarbonate buffer that contained 0.5 mM cycloheximide and incubated at 37°C . Preliminary experiments were carried out to establish the linearity of tyrosine release from the gastrocnemius muscle slices. The release of tyrosine was linear for up to 3 h. The amount (nmol/mg muscle) of tyrosine released was 0.27 ± 0.01 , 0.50 ± 0.01 , and 0.72 ± 0.03 in 1, 2, and 3 h, respectively (mean \pm SEM, in seven to eight rats). Therefore, in all subsequent studies tyrosine release was determined in muscle slices incubated for 2 h. At the end of 2 h of incubation, a 2-ml aliquot of the medium was combined with 0.5 ml of cold 50% trichloroacetic acid, mixed, and centrifuged. The

muscle slices were blotted and homogenized in 2 ml of cold 10% trichloroacetic acid and centrifuged. The acid soluble fractions of the medium and muscle homogenate were assayed for tyrosine according to the fluorometric method of Waalkes and Udenfriend (28).

Amino acid concentration in tissues and plasma. The amino acid analyses were accomplished by ion-exchange chromatography using an amino acid analyzer as reported (21). Tissues and plasma were prepared for amino acid analysis as described above for specific activity studies.

Cycloleucine uptake by tissues in vivo. The transport of the nonmetabolizable amino acid cycloleucine into liver and gastrocnemius muscle was measured by the tissue accumulation method as reported (22). The accumulation of cycloleucine within the tissues was expressed as distribution ratio (intracellular concentration/extracellular concentration) and was calculated by the described formulae (22).

Leucine oxidation in vivo. Control and clofibrate-fed rats were injected in the tail vein with 2 μ Ci of L-[1-¹⁴C]leucine/100 g body wt. Immediately after the injection, each animal was placed in a metabolic cage (Plas-Labs, Lansing, Mich.) for a period of 1 h and the expired ¹⁴CO₂ was trapped in 5 ml of hydroxide of Hyamine-10X (Rohm & Haas Co., Philadelphia, Penn.) for 12 successive 5-min intervals. A 1-ml aliquot of the trapping solution was transferred to a scintillation counting vial containing 10 ml of Liquifluor-toluene scintillation counting mixture. The radioactivity in each vial was determined in a liquid scintillation spectrometer. Radioactivity in the expired CO₂ was expressed as a percentage of the injected dose expired as ¹⁴CO₂.

Leucine oxidation in vitro. The rate of leucine oxidation was investigated by measuring the rate of ¹⁴CO₂ production when L-[1-¹⁴C]leucine was incubated with tissue homogenates as reported (8). Briefly, the studies were carried out in 5 ml of described incubation medium (8) containing 1 μ mol L-leucine, 5 μ mol α -ketoglutarate, 10 μ mol NAD⁺, 1 μ Ci of L-[1-¹⁴C]leucine, and 50 mg of homogenized tissue (6–8 mg protein), and incubated at 37°C for 60 min.

Leucine transaminase activity. Leucine transaminase activity was determined by measuring the rate of formation of α -ketoisocaproate when L-[1-¹⁴C]leucine was incubated with tissue homogenates as reported previously (8, 29). Briefly, the studies were carried out in 1 ml reaction mixture that contained 15 μ mol leucine, 15 μ mol α -ketoglutarate, 1 μ mol pyridoxal phosphate, 25 μ mol sodium pyrophosphate, pH 8.6, 1 μ Ci L-[1-¹⁴C]leucine, and 100 mg homogenized tissue (12–15 mg protein), and incubated at 37°C for 6 min for muscle and 30 min for liver. α -Ketoisocaproate was separated from leucine by ion-exchange chromatography as reported (29).

α -Ketoisocaproate dehydrogenase activity. α -Ketoisocaproate dehydrogenase activity was determined by measuring the rate of ¹⁴CO₂ production when [α -1-¹⁴C]ketoisocaproate was incubated with muscle homogenates. Incubation studies were carried out with methods similar to those described for leucine oxidation with the exception that 1 μ mol of α -ketoisocaproate and 1 μ Ci of [α -1-¹⁴C]ketoisocaproate were substituted for labeled and unlabeled leucine in the reaction mixture.

Determination of protein. Protein concentration in the tissue homogenates was measured by the method of Lowry et al. (30) using bovine serum albumin as the standard.

Materials. L-[1-¹⁴C]Leucine (56.90 mCi/mmol) was purchased from New England Nuclear. [α -1-¹⁴C]Ketoisocaproate was prepared in this laboratory from L-[1-¹⁴C]leucine by incubation with L-amino acid oxidase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as described by Rudiger et al. (31). About 90% of the labeled leucine was recovered as α -ketoisocaproate. The purity of the product was assessed by

thin-layer chromatography using a solvent system of *tert*-butanol/methyl ethyl ketone/water/formic acid in proportions of 20:20:5:0.12 by volume (32). All of the radioactivity was present at one spot, which corresponded to α -ketoisocaproate. All other chemicals used were of the reagent grade.

Statistics. The *t* test was used for the statistical analysis of the data (33).

RESULTS

As described in our previous paper (17), there was no significant difference between the initial body weight or weight gain of control and clofibrate-fed rats during the 2 wk of treatment. Among the various effects of clofibrate on tissue composition, detailed earlier (17), there was an increase in the protein concentration in the liver and a decrease in protein concentration in the muscle. The following studies were performed to provide insight into the mechanism of these effects.

Protein synthesis in vivo. The rate of incorporation of [¹⁴C]leucine into tissue protein was used as an index of protein synthesis. Clofibrate treatment had no significant effect on rates of incorporation of [¹⁴C]leucine into protein of gastrocnemius muscle (Table I). These rates, however, were reduced in the liver of clofibrate-treated rats (Table I). The rate of incorporation of a ¹⁴C-labeled amino acid into tissue proteins could be affected by a change in specific activity. We, therefore, measured the specific activity of leucine in plasma and intracellular fluid of liver and gastrocnemius muscle of control and clofibrate-treated rats.

As shown in Table I, there was no significant difference between the specific activity of leucine measured either in plasma, muscle, or liver of control and clofibrate-fed rats 5 min after the isotope injection. However, at both 10- and 15-min intervals the specific activities of leucine were generally lower in clofibrate-treated rats. It should be noted that specific activities of leucine, whether in control or clofibrate-fed rats, were always several fold greater in the muscle than in the liver (Table I). Furthermore, the decrease in specific activity with time was more rapid in plasma and tissues of clofibrate-fed than in control rats. When the rates of incorporation of leucine into tissue protein were corrected for differences in specific activity, there was no longer any significant difference between rates of leucine incorporation in the liver of the two groups of rats, but the rates of [¹⁴C]leucine incorporation into protein became significantly greater in the muscle of clofibrate-fed rats (Table I).

Muscle protein degradation in vitro. The amino acid tyrosine is neither synthesized nor degraded by the muscle (34). Therefore, the release of tyrosine has been used as an index of muscle protein breakdown (18). To permit the measurement of protein degradation independently of protein synthesis, cycloheximide was added to the incubation medium to block protein

TABLE I
Effect of Clofibrate on Specific Activity of Leucine and on Rate of Leucine Incorporation into Proteins

Tissue	Time	Specific activity		Rate of leucine incorporation		
		Control	Clofibrate-fed	Control	Uncorrected	Corrected
		min	dpm/nmol	dpm/mg protein		
Gastrocnemius muscle	5	330±43	393±35	141±14	161±29	192±34
	10	271±37	156±12*	206±20	230±22	399±38§
	15	141±8	65±7§	260±8	287±13	623±29§
Liver	5	132±21	79±7	685±101	405±73	677±122
	10	59±7	32±9	1,246±128	649±58§	1,198±106
	15	37±4	17±2§	1,491±50	758±51§	1,650±111
Plasma	5	395±6	429±18	—	—	—
	10	225±12	173±14*	—	—	—
	15	149±17	116±6			

Specific activity of leucine and rate of leucine incorporation into tissue proteins were determined following the injection of a trace amount of [¹⁴C-1]leucine to control and clofibrate-fed rats. The rate of leucine incorporation was corrected based on the differences in specific activity of leucine between control and clofibrate-fed rats. All values mean±SEM, n = 4 rats.

Significantly different from controls at:

* P < 0.05.

§ P < 0.01.

synthesis (18). As shown in Table II, in the presence of cycloheximide, the tyrosine release into the medium by gastrocnemius muscle slices was significantly higher (32%) in clofibrate-fed than in control rats. The total amount of tyrosine released by the muscle slices, as assessed by accumulation of tyrosine in the medium together with changes in the tyrosine pool in muscle slices with time, was also significantly higher in clofibrate-fed than in control rats (Table II). The rate of tyrosine release by gastrocnemius muscle slices of control rats was not only linear for up to 3 h but was also comparable to the rate of tyrosine release by diaphragm (18) and soleus muscle (35) supporting the use of muscle slices for the study of muscle protein degradation in vitro.

TABLE II
Effect of Clofibrate on Tyrosine Release by the Gastrocnemius Muscle Slices

Treatment	In medium	Change in muscle tyrosine in 2 h		Total
		nmol/mg muscle/2 h		
Control	0.50±0.02	0.09±0.01	0.59±0.01	
Clofibrate-fed	0.66±0.05*	0.08±0.01	0.74±0.06*	

All values mean±SEM, n = 6 rats.

* Significantly different from controls, P < 0.05.

Muscle protein degradation in vivo. To investigate the physiological relevance of increased muscle protein degradation in vitro as shown above, we measured the urinary excretion of 3-methylhistidine in control and clofibrate-fed rats. This amino acid is formed by methylation of specific histidine residues of peptide chains and is largely confined to actin and myosin of skeletal muscle (36, 37). Unlike other amino acids, 3-methylhistidine when released by degradation of muscle proteins is not reutilized for protein synthesis nor catabolized as an energy source, but is quantitatively excreted in the urine (25). Based on these considerations, the urinary excretion of 3-methylhistidine has been used as an index of muscle protein breakdown in vivo (26). As shown in Table III, the urinary excretion of 3-methylhistidine was significantly higher (35%) in clofibrate-fed than in control rats. This increase in 3-methylhistidine excretion was not accompanied by any significant alteration in urinary excretion of either nitrogen or creatinine (Table III).

Amino acid concentration in tissues and plasma. To determine whether metabolism of branched-chain amino acids was indeed altered by chronic clofibrate administration, we measured the concentration of these amino acids along with several others in the liver, gastrocnemius muscle, and plasma (Table IV). Among the 14 individual amino acids measured, only the concentrations of branched-chain amino acids in the muscle were significantly altered by clofibrate treat-

TABLE III

Effect of Clofibrate on Urinary Excretion of Metabolites and Muscle Protein Breakdown

Measurement	Control	Clofibrate-fed
Urine volume, ml/2 wk	152±13	150±9
Urinary nitrogen, g/2 wk	5.69±0.80	5.24±1.29
Urinary creatinine, mg/2 wk	212±33	196±22
Urinary 3-methylhistidine, $\mu\text{mol}/2 \text{ wk}$	42.59±2.39	57.44±3.91*
Muscle protein breakdown, g/2 wk	11.06±0.62	14.92±1.01*

All values are mean±SEM, n = 6 rats.

* Significantly different from controls at P < 0.05.

ment. In contrast, the concentrations of these amino acids in the liver of clofibrate-fed rats remained unaffected (Table IV). The concentration of leucine was reduced in plasma, whereas that of isoleucine and valine was not significantly affected (Table IV). Clofibrate treatment significantly reduced concentrations of alanine, threonine, and asparagine-glutamine in plasma and significantly increased concentrations of glutamate and phenylalanine in liver (Table IV).

Among the possible factors responsible for the above selective decreases in muscle concentration of branched-chain amino acids, alterations in tissue uptake and/or oxidation of these amino acids appeared more probable. The following studies were performed

to provide insight into these aspects of branched-chain amino acid metabolism in clofibrate-treated rats.

Cycloleucine uptake in vivo. For studies of uptake, we investigated the accumulation of [¹⁴C]cycloleucine, a nonmetabolizable analogue of leucine, by gastrocnemius muscle and liver. Muscle and liver were singled out for these and the following studies because the metabolism of intravenously administered amino acids is principally accomplished in these tissues (38, 39). The uptake of cycloleucine in vivo, as measured by the distribution ratio was not significantly affected by clofibrate either in gastrocnemius muscle (1.18±0.07 in controls vs. 1.35±0.01 in clofibrate-fed rats, mean±SEM in four rats) or in liver (1.75±0.05 in control vs. 1.70±0.11 in clofibrate-fed rats, four rats)

Leucine oxidation in vivo. To investigate oxidation in vivo, we measured rates of ¹⁴CO₂ production after injection of a trace dose of [1-¹⁴C]leucine in intact rats. As shown in Fig. 1, clofibrate-fed rats expired significantly more ¹⁴CO₂ in comparison to control rats between 15- and 55-min intervals. The percentage of the injected dose expired as ¹⁴CO₂ during the initial 1-h period was also significantly higher in clofibrate-fed than in control rats (23.32±1.54 vs. 13.62±1.20, P < 0.01). It should be noted that due to lower specific activities of leucine in tissues and plasma of clofibrate-fed than in control rats (Table I), the enhancement of leucine oxidation is probably greater than that apparent from the results of the present studies (Fig. 1).

Leucine oxidation in vitro. To determine whether

TABLE IV
Effect of Clofibrate on the Concentrations of Free Amino Acids in Tissues and Plasma

Amino acid	Muscle		Liver		Plasma	
	Control	Treated	Control	Treated	Control	Treated
$\mu\text{mol/g tissue}$						
Aspartic acid	0.38±0.08	0.35±0.06	0.82±0.09	1.21±0.16	0.01±0.01	0.02±0.01
Threonine	0.57±0.10	0.53±0.04	0.30±0.04	0.39±0.03	0.27±0.02	0.19±0.01*
Serine	0.98±0.09	0.85±0.06	0.73±0.14	0.59±0.07	0.23±0.02	0.19±0.02
Asparagine + glutamine	4.18±0.43	4.45±0.30	4.84±0.35	5.85±0.62	0.73±0.04	0.51±0.05*
Glutamic acid	2.08±0.31	2.47±0.09	2.74±0.17	4.18±0.38*	0.05±0.01	0.08±0.01
Glycine	5.11±0.62	5.84±0.42	2.34±0.27	2.46±0.13	0.32±0.02	0.35±0.02
Alanine	2.36±0.21	2.15±0.07	2.45±0.66	3.23±0.30	0.61±0.04	0.44±0.04§
Valine	0.21±0.02	0.11±0.01*	0.20±0.03	0.23±0.01	0.17±0.01	0.14±0.01
Methionine	0.06±0.01	0.05±0.01	0.07±0.01	0.07±0.01	0.05±0.01	0.03±0.01
Isoleucine	0.11±0.01	0.04±0.01*	0.12±0.03	0.13±0.01	0.09±0.01	0.06±0.01
Leucine	0.15±0.01	0.07±0.01*	0.21±0.04	0.25±0.03	0.15±0.01	0.10±0.01*
Tyrosine	0.10±0.01	0.10±0.01	0.06±0.01	0.08±0.01	0.07±0.01	0.05±0.01
Phenylalanine	0.07±0.01	0.07±0.01	0.07±0.01	0.11±0.01§	0.06±0.01	0.06±0.01

All values mean±SEM in five to seven rats.

Significantly different from controls at:

* P < 0.01.

§ P < 0.05.

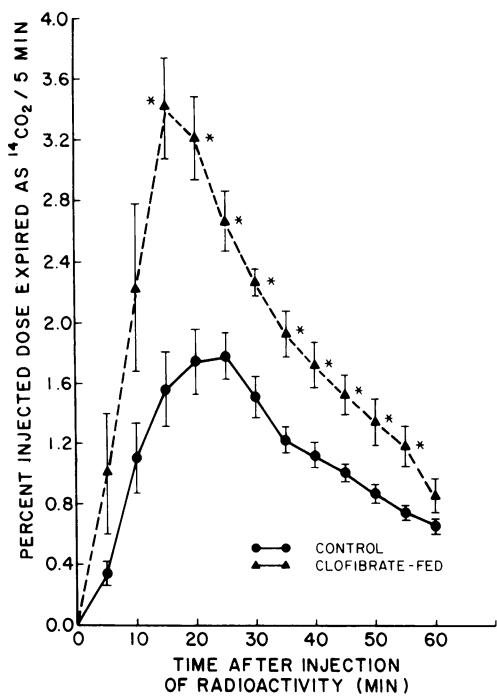


FIGURE 1 Rates (mean \pm SEM in five to six rats) of leucine oxidation in vivo in control and clofibrate-fed rats. Statistical significance for the difference between control and clofibrate-fed rats is shown as an asterisk, $P < 0.01$.

an increase in leucine oxidation by muscle or liver or both accounted for the increased oxidation in vivo, we investigated the effect of clofibrate on the capacity of these tissues to oxidize leucine in vitro. As shown in Table V, the rate of α -decarboxylation of leucine by the gastrocnemius muscle homogenate was 48% higher in clofibrate-fed than in control rats. In contrast to

muscle, the rate of leucine oxidation by the liver homogenate was not significantly affected by clofibrate treatment (Table V). It should be noted that the difference in oxidation was not due to any difference in concentration or specific activity of leucine in the incubation medium. The initial concentration and specific activity of leucine in the medium was identical for both groups of rats. The amounts of endogenous leucine provided by tissue homogenates were trivial (3–8 nmol) as compared to the amount of leucine added.

Leucine transaminase and α -ketoisocaproate dehydrogenase activities. Clofibrate-induced increases in the rate of α -decarboxylation of leucine by the muscle homogenate could be caused by the effect of clofibrate on either leucine transaminase or decarboxylation of α -ketoisocaproate (transamination product of leucine) or on both of these reactions. To investigate these possibilities, we determined the activity of leucine transaminase and α -ketoisocaproate dehydrogenase by the muscle homogenate of control and clofibrate-fed rats. Since clofibrate did not affect leucine oxidation by the liver, this tissue was not included in this study. Clofibrate treatment did not affect the activity of leucine transaminase (Table V), but the treatment significantly increased the activity of α -ketoisocaproate dehydrogenase in clofibrate-treated rats (Table V).

DISCUSSION

A striking finding of these experiments was that clofibrate administration to otherwise adequately fed rats decreased concentrations of branched-chain amino acids without affecting concentrations of other amino acids in the muscle (Table IV). Changes in muscle concentrations of amino acids in response to nutritional

TABLE V
Effect of Clofibrate on Leucine Oxidation, Leucine Transaminase,
and α -Ketoisocaproate Dehydrogenase

Tissue	Measurement	Control	Clofibrate-fed
Liver	Leucine oxidation, $nmol/mg$ protein/ 60 min	1.32 ± 0.08	1.46 ± 0.10
Gastrocnemius muscle	Leucine oxidation, $nmol/mg$ protein/ 60 min	1.52 ± 0.06	$2.26 \pm 0.12^*$
Gastrocnemius muscle	Leucine transaminase, $nmol/mg$ protein/ min	7.25 ± 0.56	7.57 ± 0.32
Gastrocnemius muscle	α -Ketoisocaproate dehydrogenase, $nmol/mg$ protein/ 60 min	2.93 ± 0.10	$4.47 \pm 0.19^*$

Rates of leucine oxidation and activities of leucine transaminase and α -ketoisocaproate dehydrogenase were determined in tissue homogenates of control and clofibrate-fed rats. All values mean \pm SEM in 8–10 rats.

* Significantly different from controls at $P < 0.01$.

and hormonal alterations are not limited to branched-chain amino acids but include others (40, 41). Four different mechanisms could conceivably account for the above decreases in concentrations of branched-chain amino acids. First, there could have been a decrease in the muscle uptake of circulating branched-chain amino acids. This possibility is dismissed by our observation that clofibrate treatment had no significant effect on muscle accumulation of cycloleucine. Second, there could have been increased muscle release of branched-chain amino acids to systemic circulation. This possibility is not supported by the observation that clofibrate treatment lacked significant effect on plasma concentration of either isoleucine or valine, and actually decreased that of leucine (Table IV). Third, there could have been increased incorporation and/or decreased production of branched-chain amino acids from muscle protein. This mechanism does not seem likely since neither altered protein synthesis nor degradation would affect concentrations of branched-chain amino acids alone without affecting those of others. In fact, clofibrate treatment actually increased degradation of muscle protein. Fourth, there could have been increased oxidation of branched-chain amino acids by the muscle. Among the various mechanisms discussed above, this last possibility is well supported by two sets of observations: (a) In comparison to control rats, clofibrate-fed rats displayed a greater rate of $^{14}\text{CO}_2$ production in the expired air after intravenous injection of a trace amount of [^{14}C]-leucine (Fig. 1). (b) Clofibrate treatment increased the capacity of skeletal muscle to oxidize leucine *in vitro* (Table V). Based on these considerations, we conclude that the decreases in branched-chain amino acid concentrations in the muscle are due to their increased oxidation in this tissue. This conclusion receives additional support from the observation that in the liver, where leucine oxidation was not altered (Table V), there was also no significant change in the concentration of branched-chain amino acids (Table IV).

The results of our studies also show that between the two enzymes concerned with α -decarboxylation of leucine by the muscle, the activity of α -ketoisocaproate dehydrogenase is more sensitive to metabolic regulation than that of leucine transaminase. Our previous studies (17), together with the present data, indicate that the activity of α -ketoisocaproate dehydrogenase in the muscle is not related to alteration of fatty acid oxidation in this tissue, in that activity of this enzyme is increased in conditions that either increase (starvation and diabetes) (5, 14) or decrease (clofibrate-treatment) muscle oxidation of fatty acids (17). On the other hand, clofibrate treatment, like starvation and diabetes, results in increased fatty acid oxidation by the liver (17) and increased concentrations of ketone bodies in plasma and tissues (17). Our earlier studies

have shown that ketone bodies, particularly acetoacetate, enhance the activity of α -ketoisocaproate dehydrogenase in muscle (8). Therefore, the enhancement of fatty acid oxidation by the liver with consequent ketogenesis may account for the increased activity of α -ketoisocaproate dehydrogenase in muscle of clofibrate-fed rats.

Protein concentration in the muscle is the result of an equilibrium between rates of synthesis and degradation. Clofibrate treatment appears to stimulate both of these rates (Tables I–III), but changes the equilibrium in favor of degradation. This view is consistent with our earlier observation that clofibrate treatment decreases the protein concentration in the muscle (17). Assuming that the rates of myofibrillar and sarcoplasmic protein breakdown are of approximately the same magnitude (42), and that the 3-methylhistidine content of rat skeletal muscle is $3.85 \mu\text{mol/g}$ protein (43), the rate of muscle protein degradation can be estimated from the urinary 3-methylhistidine excretion data. As shown in Table III, the amount of muscle protein degraded over the entire 2-wk experiment was 35% higher in clofibrate-fed than in control rats.

Based on available evidence, at least two mechanisms can be considered for increased protein degradation in the muscle of clofibrate-fed rats. First, insulin has been shown to have an inhibitory effect on the degradation of muscle protein (44, 45). As reported by others (46) and confirmed by us (17), clofibrate treatment significantly reduced the levels of insulin in plasma and may have been responsible for the increased rate of muscle protein degradation. However, this explanation fails to take into account that insulin inhibits protein degradation in liver (47) and the liver protein content of clofibrate-fed rats was actually increased (17). Second, it has been proposed that branched-chain amino acids, particularly leucine, inhibit the breakdown of muscle protein (18, 19). As shown in Table IV, clofibrate treatment selectively decreased the concentrations of all three branched-chain amino acids in the muscle.

These observations may also provide further insight into the pathogenesis of the muscular syndrome in patients who receive clofibrate therapy for treatment of hyperlipidemias (48, 49). Our previous electromyographic and electron microscopic studies revealed the development of myotonia and dilation of transverse tubules of skeletal muscle fibers of rats similarly treated with clofibrate (50). The transverse tubules, which are extensions of the surface membrane within the muscle fiber, have been implicated to play a key role in the genesis of electrical activity of the muscle (51) and have been shown to be affected in the muscle biopsies of patients with myotonia (52). Although we did not find any evidence of muscle damage with investigation by light microscopy (50), others have found extensive

degeneration and necrosis of muscle fibers using a higher dose of clofibrate for a longer duration (53). Therefore, the alterations of protein and energy metabolism, together with the dilation of transverse tubules, are probably a subtle manifestation of the toxic effect of this drug on the skeletal muscle.

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