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Branched Chain Amino Acid Oxidation in Cultured Rat Skeletal Muscle Cells

SELECTIVE INHIBITION BY CLOFIBRIC ACID

WILLIAM M. PARDRIDGE, DELIA CASANELLO-ERTL, and LUIZA DUDUCIAN-VARTAVARIAN, Department of Medicine, Division of Endocrinology/Metabolism, University of California Los Angeles School of Medicine, Los Angeles, California 90024

ABSTRACT Leucine metabolism in skeletal muscle is linked to protein turnover. Since clofibrate is known both to cause myopathy and to decrease muscle protein content, the present investigations were designed to examine the effects of acute clofibrate treatment on leucine oxidation. Rat skeletal muscle cells in tissue culture were used in these studies because cultivated skeletal muscle cells, like muscle in vivo, have been shown to actively utilize branched chain amino acids and to produce alanine. The conversion of [1-14C]-leucine to [14CO_2] or to the [14C]keto-acid of leucine (a-keto-isocaprate) was linear for at least 2 h of incubation; the production of [14CO_2] from [1-14C]leucine was saturable with a $K_m = 6.3 \text{ mM}$ and a maximum oxidation rate ($V_{max}$) = 31 nmol/mg protein per 120 min. Clofibrate acid selectively inhibit the oxidation of [1-14C]leucine ($K_i = 0.85 \text{ mM}$) and [U-14C]iso-leucine, but had no effect on the oxidation of [U-14C]glutamate, -alanine, -lactate, or -palmitate. The inhibition of [1-14C]leucine oxidation by clofibrate was also observed in the rat quarter-diaphragm preparation. Clofibrate primarily inhibited the production of [14CO_2] and had relatively little effect on the production of [14C]-keto-acid of leucine. A physiological concentration—3.0 g/100 ml—of albumin, which actively binds clofibrate acid, inhibited but did not abolish the effects of a 2-mM concentration of clofibrate acid on leucine oxidation. Clofibrate treatment stimulated the net consumption of pyruvate, and inhibited the net production of alanine. The drug also increased the cytosolic NADH/NAD+ ratio as reflected by an increase in the lactate/pyruvate ratio, in association with a decrease in cell aspartate levels. The changes in pyruvate metabolism and cell redox state induced by the drug were delayed compared with the nearly immediate inhibition of leucine oxidation. These studies suggest that clofibrate acid, in concentrations that approximate high therapeutic levels of the drug, selectively inhibits branched chain amino acid oxidation, possibly at the level of the branched chain keto-acid dehydrogenase.

INTRODUCTION

Clofibrate is known to cause myopathy in both man (1) and experimental animals (2). Recent studies with rats suggest that the drug may impair protein turnover in skeletal muscle because clofibrate-treated animals have shown diminished total muscle protein (3). Protein turnover in skeletal muscle is believed to be linked to the metabolism of leucine (4), a branched chain amino acid. Therefore, if clofibrate does induce myopathic changes by altering protein turnover in skeletal muscle, one possible mechanism for these events might be a drug-induced alteration in leucine metabolism in muscle. Another observation that suggests a possible linkage between clofibrate and branched chain amino acid metabolism in skeletal muscle is the inhibition of alanine synthesis associated with therapeutic doses of the drug (5). Skeletal muscle is the major site of alanine production (6-9), and alanine synthesis in muscle cells is coupled to the transamination of the branched chain amino acids (10, 11). Therefore, a putative inhibition of branched chain amino acid utilization by clofibrate would be expected to decrease the production of alanine.

The effects of clofibrate treatment on leucine oxida-

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1 Clofibrate (Atromid-S) is the ethyl ester of the active compound clofibracid, or $p$-chlorophenoxyisobutyric acid.

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tion in isolated rat skeletal muscle cells in tissue culture are described in this paper. Similar to intact skeletal muscle (6-11), cultivated skeletal muscle cells have been shown in previous studies (12-14) both to actively use the branched chain amino acids and to produce alanine.

METHODS

All tissue culture supplies, enzymes, and reagents were obtained as previously described (12). All isotopes were purchased from New England Nuclear, Boston, Mass. Clofibrate acid was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

The maintenance of stock cultures of cells of the L4 myogenic line (15) and the preparation of 10-d-old myotubes (on 60- or 100-mm petri dishes) were exactly as described previously (12), except that the cells were grown in a humidified atmosphere of 95% air:5% CO₂, and the medium bicarbonate concentration was reduced by one-half, to 22 mM. Experimental incubations for the determination of metabolite levels were initiated by discarding old medium and adding to 100-mm dishes 3 ml of Dulbecco’s modified Eagle’s medium containing fresh fetal calf serum and adjusted to 0.4 mM pyruvate, 2.5 mM lactate, and 0.5 mM glucose; the levels of other nutrients in this medium have been listed previously (12). Dishes with or without clofibrate acid (2 mM) were incubated up to 4 h. At the end of the incubation, neutralized perchloric acid extracts of cold-washed cells and of medium were prepared as reported previously, with special storage precaution under acid pH for the α-keto-acids, pyruvate and α-ketoglutamate (12, 16).

All metabolite levels were determined in duplicate with enzymic fluorometric or spectrophotometric methods as reported previously (12, 16). Clofibrate levels in the medium were determined spectrophotometrically (17).

CO₂ collection studies were performed with 10-d-old myotube preparations grown on 60-mm dishes. The incubation was started by discarding old medium and adding 2.5 ml of Dulbecco’s modified Eagle’s medium; this medium contained no serum and was adjusted to 0.1 mM pyruvate, 0.4 mM glutamate, and 0.1 μCi/ml of 14C-metabolite. The 60-mm dishes without lids were placed in small crystallizing dishes, which were tightly capped with a rubber stopper that encased a serum stopper and filter-paper-lined plastic cup (Kontes Co., Vineland, N. J.). The dishes were gassed for about 1 min with 95% O₂:5% CO₂ and incubated at 37°C for up to 2 h with slow agitation. At the end of the incubation, 0.4 ml NCS base (Amersham Corp., Arlington Heights, Ill.) was added to the cup, the medium was acidified by injection of 0.5 ml of N perchloric acid, and CO₂ was collected over the next 60 min. The cup containing labeled CO₂ was placed in a glass vial for liquid scintillation counting. The [1-14C]-keto-acid of leucine (α-keto-isocaprate) remaining in the medium was measured by removing 0.5 ml of the acidified medium to a 25-ml flask. This flask was stoppered and 0.5 ml of 15% hydrogen peroxide was added, and the labeled CO₂ produced by peroxide treatment was collected as described above. Odessy and Goldberg (18) have shown that peroxide treatment quantitatively decarboxylates the branched chain keto-acids. The total [1-14C]leucine transaminated equaled the 14CO₂ fraction plus the [1-14C]keto-isocaprate fraction (18).

Rates of CO₂ or keto-acid formation (nanomoles per milligram protein per minute) were calculated by dividing incorporation rates (disintegrations per minute per milligram protein per minute) by medium specific activity (disintegrations per minute per nanomole). Since the leucine level in tissue culture medium is high—0.8 mM—it is assumed that the specific activity of leucine (and the keto-acid of leucine) in the cell is equal to the specific activity of leucine in the medium. Hutson et al. (19) have shown that these relationships hold for the perfused hindlimb when the medium leucine level is 0.5 mM. The effects of clofibrate acid (2 mM) on the oxidation of [1-14C]leucine were also investigated in a few studies in the rat quarter-diaphragm preparation. Diaphragms were obtained from 300-g Sprague-Dawley fed rats and incubated in a Krebs-Ringer buffer gassed with 95% O₂:5% CO₂ at 37°C. The final 2-ml incubation media contained 11 mM glucose, 0.1 mM L-leucine, and 0.1 μCi/ml of [1-14C] leucine.

 Cultures were shown to be free of mycoplasma contamination in our laboratory by two independent methods (20, 21). Data are reported as mean±SEM. Quadruplicate dishes were studied at all time points for CO₂ collection studies and triplicate dishes were used for metabolite measurements. Background disintegrations per minute in all CO₂ collection studies were determined by incubating dishes without cells containing medium labeled with the 14C-compound. Statistical significance was assessed using t test.

RESULTS

As skeletal muscle cells in tissue culture have not been heretofore used as a model system for the investigation of amino acid oxidation, a total of 17 U-14C-amino acids were assayed for rates of CO₂ production during a 2-h incubation (Table I). In general, the amino acids found to be converted to CO₂ in diaphragm—e.g., the branched chain amino acids alanine, glutamate, and aspartate (22)—are the same amino acids metabolized

<table>
<thead>
<tr>
<th>Table I</th>
<th>Oxidation of Amino Acids, Lactate, and Glucose by L₄ Skeletal Muscle Cells in Tissue Culture and the Effect of Clofibrate Acid (2 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate (nM)</td>
<td>CO₂ production* (nmol/mg protein/120 min)</td>
</tr>
<tr>
<td>[U-14C]Lactate (1.0)</td>
<td>9.0±1.2</td>
</tr>
<tr>
<td>[U-14C]Glucose (5.0)</td>
<td>8.8±2.0</td>
</tr>
<tr>
<td>[U-14C]Glutamate (0.4)</td>
<td>10.7±1.0</td>
</tr>
<tr>
<td>[U-14C]Aspartate (0.4)</td>
<td>7.3±1.0</td>
</tr>
<tr>
<td>[1-14C]Leucine (0.8)</td>
<td>5.2±0.2</td>
</tr>
<tr>
<td>[U-14C]Glutamine (0.4)</td>
<td>3.3±1.0</td>
</tr>
<tr>
<td>[U-14C]Alanine (0.04)</td>
<td>2.8±0.3</td>
</tr>
<tr>
<td>[U-14C]Isoleucine (0.8)</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>[U-14C]Leucine (0.8)</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>[U-14C]Arginine (0.4)</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>[U-14C]Valine (0.8)</td>
<td>0.3±0.1</td>
</tr>
</tbody>
</table>

* Data are mean±SEM. The following U-14C-amino acids at a concentration of 0.4–0.8 mM produced immeasurably low amounts of 14CO₂ (<0.1 nmol/mg protein/120 min): histidine, phenylalanine, tyrosine, tryptophan, lysine, threonine, proline, serine, and glycine.
† P < 0.005 difference from control.

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by L6 muscle cells. In addition, L6 cells oxidize glutamine as a carbon source (12 and Table I). Although CO₂ production studies using [¹⁴C]glutamine in diaphragm have apparently not been reported, we have recently observed a linear rate of CO₂ production from [U-¹⁴C]glutamine in rat diaphragm for at least 2 h of incubation.²

The oxidation of several ¹⁴C-amino acids, [¹⁴C]glucose, and [¹⁴C]lactate was measured in the presence or absence of a 2-mM concentration of clofibrate. As shown in Table I, the oxidation of the branched chain amino acids was selectively inhibited by clofibrate. In addition, the effect of 2 mM clofibrate on the oxidation of [U-¹⁴C]palmitate (0.1 μCi/ml) was measured. In these experiments, the medium was supplemented with 10% fetal calf serum, which maintained the labeled free fatty acid in aqueous solution. The rate of ¹⁴CO₂ production was 461±128 and 529±78 dpm/mg protein per 120 min in the absence or presence of 2 mM clofibrate; this difference was not statistically significant (P < 0.25).

The clofibrate inhibition of [¹⁴C]leucine oxidation was detectable as early as 30 min of incubation and persisted for at least 2 h (Fig. 1). The total leucine transaminated was inhibited an average of 52% by 2 mM clofibrate, due primarily to a 78% inhibition in the ¹⁴CO₂ fraction. In the absence of the drug, 65% of the total [¹⁴C]leucine transaminated was recovered in the CO₂ fraction, whereas only 31% of the radioactivity was in the CO₂ fraction in the presence of clofibrate. The inhibition of [¹⁴C]leucine oxidation by clofibrate was also observed in diaphragm muscle tissue obtained from adult rats (Fig. 2), as well as in skeletal muscle cells obtained from adult rats and grown in primary tissue culture.³

With increasing leucine concentrations, leucine oxidation was saturable (Fig. 3); a double reciprocal plot (data not shown) of the data in Fig. 3 was linear (r = 0.98) and indicated that the Kₘ = 0.3 mM and maximal oxidation rate (Vₘₐₓ) = 31 nmol/mg protein per 120 min. Levels of leucine up to 10 mM did not overcome the inhibitory effects of 1.5 mM clofibrate, i.e., the Vₘₐₓ was lowered with relatively little change in Kₘ (Fig. 3). Additional experiments in which the clofibrate concentration was varied demonstrated that the clofibrate level at 50% inhibition (Kᵢ) was 0.85 mM (Fig. 4).

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⁴ Abbreviation used in this paper: Vₘₐₓ maximum oxidation rate.
a concentration that approximates high therapeutic levels of the drug (23).

The effect of albumin, which actively binds clofibrate acid (24), was also investigated. In these studies, the oxidation of [1-14C]leucine in the absence or presence of 3.0 g/100 ml albumin was 4.2±0.3 nmol/mg protein per 120 min, and this was decreased to 1.2±0.2 and 2.5±0.2 nmol/mg protein per 120 min by 2 mM clofibrate acid in the absence or presence, respectively, of 3.0 g/100 ml albumin. Therefore, a physiologic concentration of albumin did not abolish the effect of clofibrate, but did result in a 43% diminution in the inhibition of leucine oxidation caused by 2 mM clofibrate.

To determine whether or not the inhibition of leucine transamination is associated with a decrease in the production of alanine by muscle, L6 cells were incubated for up to 4 h with clofibrate, and metabolite measurements were obtained at 1, 2, 3 and 4 h of incubation. Cell protein in these studies averaged 2.3±0.2 mg protein/100-mm dish. The metabolite data at 0 and 4 h are shown in Tables II and III. A 50% decrease in net alanine production was observed in association with an increase in net pyruvate consumption. The onset of pyruvate consumption occurred after the block in branched chain amino acid utilization; e.g., at 1 h of incubation, when leucine oxidation was already inhibited (Fig. 1), there was no difference in medium pyruvate (0.32±0.02 mM control, 0.31±0.02 mM treated). Relatively little change in medium glucose, lactate, or glutamine was observed in the treated cells (Table II).

However, a significant increase in medium lactate/pyruvate ratio was observed (Table II) in association with a decrease in cell aspartate (Table III). A similar inverse relationship between the medium lactate/pyruvate ratio and cell aspartate has been observed previously (14, 16). No significant differences in medium lactate/pyruvate ratio or cell aspartate were observed at 1 or 2 h of incubation; e.g., at 2 h the lactate/pyruvate ratio was 8±1 in control cells and 9±1 in treated cells, and the cell aspartate level was 5.3±0.3 nmol/mg protein in control cells and 5.9±0.5 nmol/mg protein in treated cells. In parallel to medium alanine, cell alanine was decreased in the drug-treated cells (Table III). No significant differences in glutamine, ATP, or Krebs cycle intermediates—malate, citrate, or α-ketoglutarate—were observed in the clofibrate-treated cells (Table III).

In addition, glycogen was not significantly different in the control (16±1) vs. the treated (13±2) cells after 4 h of incubation.

Clofibrate was not metabolized by the cells; the medium drug concentration was 1.9±0.1 and 2.0±0.1 mM at 0 and 4 h of incubation, respectively.

**DISCUSSION**

The present studies demonstrate that the acute administration of clofibrate to muscle cells results in a

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**TABLE II**

*Effect of Clofibrate Acid (2 mM) on Medium Metabolite Levels*

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Incubation time*</th>
<th>0 h</th>
<th>4 h (control)</th>
<th>4 h (clofibrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>5.8±0.1</td>
<td>5.3±0.1</td>
<td>5.4±0.1</td>
</tr>
<tr>
<td>Lactate</td>
<td></td>
<td>2.9±0.1</td>
<td>3.4±0.1</td>
<td>3.3±0.1</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.41±0.02</td>
<td>0.21±0.02</td>
<td>0.091±0.0021</td>
<td></td>
</tr>
<tr>
<td>Lactate/pyruvate</td>
<td>7±1</td>
<td>16±1</td>
<td>36±11</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>0.02±0.01</td>
<td>0.12±0.01</td>
<td>0.07±0.011</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.52±0.01</td>
<td>0.65±0.02</td>
<td>0.69±0.04</td>
<td></td>
</tr>
</tbody>
</table>

* Mean±SEM (three dishes measured in duplicate).  
† P < 0.025 difference from 4 h (control).

---

**TABLE III**

*Effects of Clofibrate Acid (2 mM) on Intracellular Metabolite Levels*

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Incubation time*</th>
<th>0 h</th>
<th>4 h (control)</th>
<th>4 h (clofibrate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>1.2±0.1</td>
<td>1.6±0.2</td>
<td>1.4±0.1</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>2.5±0.1</td>
<td>2.0±0.2</td>
<td>1.5±0.2</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>1.3±0.2</td>
<td>1.7±0.1</td>
<td>1.9±0.2</td>
<td></td>
</tr>
<tr>
<td>Aspartate</td>
<td>5.7±0.2</td>
<td>3.3±0.4</td>
<td>1.2±0.2†</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>25±1</td>
<td>6.5±0.7</td>
<td>2.3±0.4†</td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>50±2</td>
<td>22±2</td>
<td>20±4</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>102±2</td>
<td>42±5</td>
<td>58±7</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>24±1</td>
<td>26±1</td>
<td>24±2</td>
<td></td>
</tr>
</tbody>
</table>

* Mean±SEM (three dishes measured in duplicate).  
† P < 0.005 difference from 4 h (control).
rapid inhibition of branched chain amino acid oxidation. Other metabolic effects of clofibrate that may be secondary to the block in branched chain amino acid utilization are an increase in pyruvate consumption associated with a decrease in net alanine production, and a probable alteration in the NADH/NAD+ redox state of the cytosol, as reflected by the medium lactate/pyruvate ratio. These latter two effects of acute clofibrate administration have also been observed in skeletal muscle cells obtained from adult rats and grown in primary tissue culture.

The inhibition by clofibrate of the conversion of \([1-{^{14}C}]\)leucine to \({^{14}C}\)O\(_2\) might occur at one of at least four steps: (a) cell membrane transport of leucine; (b) leucine transamination, which occurs primarily in the cytosol (18); (c) transport of \([1-{^{14}C}]\)keto isocaproic acid from the cytosol into the mitochondria; and (d) the \(\alpha\)-decarboxylation of the keto acid to \(\text{CO}_2\), which is mediated by the mitochondrial branched chain keto-acid dehydrogenase (18). An inhibition by clofibrate at steps (a) or (b) would be expected to inhibit the production of \(\text{CO}_2\) in proportion to a decrease in \(\text{14C}\)-keto-acid. Conversely, a block in steps (c) or (d) would be expected to decrease the \(\text{14C}\)-keto-acid fraction to a greater extent than the \(\text{14C}\)-keto-acid fraction. The data in Fig. 1 demonstrate that clofibrate selectively inhibits the production of \(\text{CO}_2\), not the \(\alpha\)-keto-acid of leucine. Therefore, it is likely that the drug is an inhibitor of the \(\alpha\)-decarboxylation of the branched chain keto-acids, although a site of action at a mitochondrial anion transport step cannot be excluded. The mechanism by which clofibrin acid might inhibit the \(\alpha\)-decarboxylation of branched chain keto-acids could be either a direct or indirect action of the drug. Clofibrinate possibly inhibits the branched chain keto-acid dehydrogenase directly, via a noncompetitive mechanism (Fig. 3). Alternatively, the site of action of the drug might be at a distal step in the pathway of branched chain keto-acid oxidation; under these conditions the product of the \(\alpha\)-decarboxylation pathway (e.g., isovaleryl coenzyme A [CoA] in the case of the leucine pathway) would accumulate. Since the branched chain keto-acid dehydrogenase is subject to product inhibition (18), an accumulation of acyl CoA derivatives would be expected to inhibit the \(\alpha\)-decarboxylation of branched chain keto-acids. A similar sequence of events is believed to occur in rare forms of hypoglycemia caused by either inborn errors of metabolism or hypoglycin ingestion (25); in these conditions, a block in the metabolism of acyl CoA derivatives leads to an inhibition of both branched chain amino acid or free fatty acid metabolism. However, there is no apparent effect of acute clofibrate administration on free fatty acid oxidation in \(L_5\) muscle cells (see Results). The above two mechanisms of clofibrate action—direct and indirect inhibition of dehydrogenase activity—might be distinguished by future studies in which isovaleryl CoA concentrations are measured after drug treatment.

Another mechanism by which clofibrate might inhibit branched chain amino acid oxidation is a drug-induced increase in the mitochondrial NADH/NAD+ ratio. Odessey and Goldberg (18) have shown that NADH, an end-product of the branched chain keto acid dehydrogenase reaction, inhibits the enzyme. However, at least two observations argue against this mechanism of drug action. Firstly, the clofibrate-induced increase in lactate/pyruvate ratio, which is assumed to reflect the cytosolic NADH/NAD+ ratio, lags behind (see Results) the prompt reduction in leucine oxidation (Fig. 1). Secondly, if the drug did inhibit the dehydrogenase by raising the mitochondrial NADH/NAD+ ratio, then a similar inhibition of other mitochondrial dehydrogenases would be expected. However, the oxidation of alanine or lactate via pyruvate dehydrogenase or glutamate via \(\alpha\)-ketoglutarate dehydrogenase was not altered by clofibrate (Table 1).

Finally, skeletal muscle appears to adapt to chronic clofibrate therapy. Paul and Adibi (3) have studied rats on 0.3 g/kg per d of clofibrate for 3 wk, a dose that would be expected to achieve a plasma clofibrate level of about 1.5 mM (17). Preliminary data indicate that the leucine oxidizing activity in gastrocnemius homogenate is increased 50% (26). Therefore, the level of the branched chain keto-acid dehydrogenase may possibly be induced under conditions of chronic drug inhibition so that the rate of branched chain amino acid oxidation is normalized. Further evidence that metabolic adaptations to chronic clofibrate treatment occur is the observation that the lactate/pyruvate ratio is normal in skeletal muscle of rats fed clofibrate for 3 wk (27). Whether man is able to undergo similar adaptations to chronic clofibrate therapy is not known. Moreover, recent studies by Khatra et al. (28) indicate that skeletal muscle in man may be more dependent on the branched chain amino acids as carbon sources than that in the rat. The majority (76%) of total body branched chain keto-acid dehydrogenase activity in the rat is in liver, whereas in man the majority (62%) of total body dehydrogenase activity is in skeletal muscle. Therefore, it is possible that the extent to which skeletal muscle in man adapts to chronic clofibrate inhibition of branched chain amino acid oxidation is related in part to the development of myopathy.

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