Tyrphostin 47 Nonenzymatically Decarboxylates [1-14C]-Pyruvate*

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ABSTRACT

Tyrphostins inhibit tyrosine kinases and have little effect on the activity of serine/threonine kinases. Pyruvate dehydrogenase kinase inactivates pyruvate dehydrogenase by phosphorylating serine residues within the multienzyme complex. This serine/threonine kinase represents a new family of protein kinases, and one (tyrphostin 47) of two tyrphostins tested appeared to activate the pyruvate dehydrogenase kinase as determined by [1-14C]-lactate oxidation to 14CO2. Experiments designed to determine if the tyrphostins altered pyruvate dehydrogenase activity in mitochondria prepared from rat epididymal adipocytes using [1-14C]-pyruvate as the substrate demonstrated a dose dependent increase in enzyme activity in the presence of tyrphostin 47, but not in tyrphostin 23. This apparent stimulation of pyruvate dehydrogenase activity was attributed to tyrphostin 47’s ability to nonenzymatically decarboxylate [1-14C]-pyruvate, the substrate for the pyruvate dehydrogenase assay. Neither tyrphostin directly altered pyruvate dehydrogenase kinase activity. Therefore, assays utilizing [1-14C]-pyruvate and tyrphostin 47 are subject to analytical interference.

Introduction

Tyrphostins are a family of benzylidenemalonitrile derivatives resembling the phenolic group of tyrosine designed to inhibit protein tyrosine kinase with weak or no inhibition of serine/threonine kinases.1,2 Therefore, most tyrphostins are not effective inhibitors of serine/threonine kinases associated with recep-

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Pyruvate dehydrogenase is a multienzyme complex located on the inner mitochondrial membrane and is composed of three major enzymes: pyruvate decarbox-
The activity of this complex is regulated by the degree of phosphorylation of the alpha-subunit of pyruvate decarboxylase. The phosphorlated form of the enzyme is inactive; the dephosphorylated form is active. Three serines are phosphorylated on the alpha-subunit by a cAMP-independent pyruvate dehydrogenase kinase. The activity of this kinase is regulated by the nature of its tight binding to the pyruvate dehydrogenase complex. The pyruvate dehydrogenase kinase is encoded by nuclear deoxyribonucleic acid (DNA). The mature protein is composed of 408 amino acids and has a molecular weight of 47,270 daltons. The gene lacks motifs associated with eukaryotic serine/threonine kinases. The DNA sequence for pyruvate dehydrogenase kinase resembles the prokaryotic histidine kinase family. This enzyme represents the first member of a new eukaryotic family of protein kinases. This paper explores the effect of the tyrphostins on pyruvate dehydrogenase activity.

To determine if two synthetic tyrphostins, tyrphostin 47 (T47) and tyrphostin 23 (T23), alter pyruvate dehydrogenase kinase activity, either T47 or T23 was added to rat epididymal adipocytes and the amount of $^{14}$CO$_2$ generated from $[1-^{14}C]$-lactate oxidation was quantitated: $[1-^{14}C]$-lactate $\rightarrow$ LDH $\rightarrow$ $[1-^{14}C]$-pyruvate $\rightarrow$ PDH $\rightarrow$ $^{14}$CO$_2$. The T47, but not T23, significantly decreased lactate oxidation, suggesting inhibition of pyruvate dehydrogenase through pyruvate dehydrogenase kinase activation. However, pyruvate dehydrogenase activity measured in adipocyte mitochondrial preparations was increased in a dose dependent manner in the presence of T47 but not T23 as measured with $[1-^{14}C]$-pyruvate. This apparent stimulation of pyruvate dehydrogenase by T47 in mitochondrial preparations was shown to be secondary to nonenzymatic decarboxylation of the radiolabeled substrate, $[1-^{14}C]$-pyruvate. Therefore, assays utilizing this radiolabeled metabolite and T47 to determine pyruvate dehydrogenase activity are subject to analytical interference.

**Materials and Methods**

Male Sprague-Dawley rats (120 to 140 g) were obtained.* Additional materials obtained were $[1-^{14}C]$-D,L-lactic acid, sodium salt, $[1-^{14}C]$-pyruvic acid, sodium salt, bovine serum albumin (BSA) fraction V, and tyrphostin 23 (RG-50810) and tyrphostin 47 (RG-50864). Porcine insulin was a gift. Stock solutions were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO per assay did not exceed 5 percent by volume. Additional purchases included bicinchoninic acid protein assay reagent** and complete counting cocktail.† All other reagents were purchased from one company.‡

**Isolation of Adipocytes**

Two to three male Sprague-Dawley rats (120 to 140 g) were used per experiment. The animals were maintained on Purina Laboratory Rodent Chow and water *ad libitum*. Twenty-four hours before sacrifice, food, but not water, was restricted. The animals were sacrificed by cervical dislocation, and the epididymal fat pads removed.

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Intact adipocytes were isolated from the epididymal fat pads by using the collagenase digestion technique of Rodbell as modified by Jarett. The fat pads were incubated for 60 minutes at 37°C in Krebs-Ringer phosphate medium (KRPM), pH 7.4 (128 mM NaCl, 5.2 mM KCl, 1.4 mM MgSO4, 10 mM NaHPO4, 1.4 mM CaCl2, 0.17 mM D-glucose, 3 percent BSA) with 1 mg of Type II collagenase per mL. After collagenase digestion, the cells were filtered through a nylon mesh and washed three times with KRPM. The packed cell volume was recorded and cells were suspended in KRPM (1:75, by volume). One mL of this cell suspension was used per lactate oxidation assay. Protein content was determined from the average weight of rats used.

PREPARATION OF RAT ADIPOCYTE MITOCHONDRIA

Fifty male Sprague-Dawley rats (120 g) were used for each mitochondrial preparation. Intact adipocytes were isolated from epididymal fat pads as previously described. After extensive washing in KRPM, cells were homogenized in 10 mM 4-morpholene propanesulfonic acid, 0.25 M sucrose (MOPS-sucrose), pH 7.4. The homogenate was centrifuged at 20,000 g for 15 minutes at 4°C. The resulting pellet containing mitochondria was resuspended in MOPS-sucrose then fractionated on a discontinuous sucrose gradient of 0.8, 1.06, and 2.02 M sucrose as described by Sykes et al. The mitochondrial fraction isolated at the 1.06 to 2.02 M sucrose interface was phosphorylated for 10 minutes at 37°C in MOPS-sucrose containing final concentrations of 100 μM MgCl2, 1.0 mM dithiothreitol, 2.0 μg/mL oligomycin, and 10 mM ATP. The phosphorylated mitochondrial pellet obtained by centrifugation at 35,000 g for 15 minutes at 4°C was resuspended in 2 mL of 50 mM potassium phosphate buffer, pH 7.4 containing 0.25 M sucrose and 1 mM dithiothreitol, then frozen in aliquots and stored at −70°C. The bicinchoninic acid procedure was used to determine protein concentration in the mitochondrial preparation.

LACTATE OXIDATION

Plastic vials were set up in triplicate for each condition. The final volume consisted of 1 mL reaction mixture plus 1 mL whole cell suspension. The reaction mixture consisted of 0.4 μCi [1-14C]-D,L-lactate, cold lactate (111.2 nmol/assay) and various concentrations of insulin or designated tyrphostin in KRPM. The reaction was initiated by the addition of 1 mL cell suspension to each vial. Vials were capped immediately with air tight stoppers which suspend center wells, with one inch by three inch pieces of rolled filter paper, directly over the assay mixture. Vials were placed into a Dubnoff metabolic shaking incubator and gently mixed at 37°C for 60 minutes. The reaction was stopped by injecting 0.2 mL 6 N H2SO4 into the assay mixture. One molar benzethonium hydroxide (0.2 mL) was applied to each filter paper. Vials were incubated for an additional 45 minutes at room temperature to allow 14CO2 to absorb onto the filter paper. Vials were uncapped and filter papers transferred to scintillation vials containing complete counting cocktail and counted. The amount of 14CO2 liberated was calculated and expressed as nmol L-lactate oxidized to 14CO2 per mg protein in cell suspension per hour.

MITOCHONDRIAL PYRUVATE DEHYDROGENASE ACTIVITY

Pyruvate dehydrogenase activity was assayed as the release of 14CO2 from [1-14C]-pyruvate, using the method described by Kiechle et al with some modifications. A mixture of 25 μL mito-
chondrial preparation (final concentration of 30 μg protein per mL), 25 μL of varying concentrations of T23, T47 or DMSO blank, and 50 μL ion solution containing final concentrations of 500 μM CaCl₂, 250 μM ATP, 20 mM MgCl₂ in 50 mM potassium phosphate buffer, pH 7.4, were incubated in plastic tubes for 10 minutes at 37°C. The reaction was then initiated by the addition of 25 μL reaction mixture containing final concentrations of 0.5 mM β-NAD, 0.1 mM Coenzyme A, 0.1 mM thiamine pyrophosphate, 100 μM/mL phosphotransacetylase, 0.25 mM pyruvate, 1 mM dithiothreitol, and 0.125 μCi [1-¹⁴C]-pyruvate in 50 mM potassium phosphate buffer, pH 7.4, to each tube. The tubes were immediately capped with air tight stoppers with center wells containing filter papers. This incubation continued for another 10 minutes at 37°C. The reaction was stopped by injecting 0.1 mL 6N H₂SO₄ directly into the assay mixture. One molar benzethonium hydroxide (0.2 mL) was applied to each filter paper. Incubation continued at 25°C for 45 minutes. Caps were removed and filter papers transferred to scintillation vials containing complete counting cocktail. Nonezymatic decarboxylation was expressed as pmol of ¹⁴CO₂ generated per minute.

STATISTICAL ANALYSIS

The results were given as the mean ± SEM and changes from basal activity analyzed using the unpaired Student t-test. Significance was determined at p < 0.05.

RESULTS

LACTATE OXIDATION IN ADIPOCYTES

Figure 1 illustrates that the oxidation of [1-¹⁴C]-D,L-lactate to ¹⁴CO₂ in rat epididymal adipocytes is linear with respect to time for at least 80 minutes. Incubation of adipocytes with insulin revealed a significant increase in radiolabeled lactate oxidation at 10 μU/mL insulin or greater (figure 2). This insulin-induced increase in ¹⁴CO₂ production is attributed to stimulation of pyruvate dehydrogenase activity. This assay may be used to study the regulation of pyruvate dehydrogenase activity in intact adipocytes.

TYRPHOSTINS AND INTACT ADIPOCYTES

To determine the effect of tyrphostins on lactate oxidation in adipocytes, increasing concentrations of T23 or T47 were added to intact adipocytes (figure 3). The T47 significantly decreased lactate oxidation at 1 and 10 μM. These results suggest the pyruvate dehydrogenase kinase may be activated or pyruvate dehydrogenase phosphatase inhibited by T47. Both T47 and T23 inhibited the reaction at 500 μM. After 60 minutes incubation with 500 μM T47 and T23, greater than 40% of the adipocytes suf-
Figure 1. Effect of assay time on lactate oxidation in isolated adipocytes. Results are expressed as DPM of $^{14}$C-labeled substrate converted to $^{14}$CO$_2$.

Figure 2. Effect of increasing insulin concentration on lactate oxidation in isolated adipocytes. Cells were incubated for 60 minutes with varying concentrations of insulin. Results are expressed as nmol $^{14}$CO$_2$ per mg protein of cell suspension per hour. *p < 0.05.

ferred membrane damage as assessed by the accumulation of trypan blue in injured cells.

**Tyrphostins and Mitochondrial Pyruvate Dehydrogenase Activity**

To determine the effect of tyrphostins on pyruvate dehydrogenase activity in mitochondria preparations from rat epididymal adipocytes, increasing concentrations of T47 and T23 were added to the mitochondrial pyruvate dehydrogenase assay (figure 4). The T47, but not T23, apparently stimulated pyruvate dehydrogenase activity in an assay with sufficient Mg$^{2+}$ (20 mM) and Ca$^{2+}$ (0.5 mM) to activate the pyruvate dehydrogenase phosphatase$^{16}$ and adenosine triphosphate (ATP), substrate for the pyruvate dehydrogenase Mg$^{2+}$-dependent kinase. Attempts to resolve whether the activation of PDH complex by T47 was secondary to inhibition of the PDH kinase (decreased inactivation by phosphoryla-
tion) or stimulation of the PDH phosphatase (activation by dephosphorylation) lead to equivocal results.

**Tyrphostins and Nonenzymatic Decarboxylation of [1-14C]-Pyruvate**

[1-14C]-pyruvate is relatively unstable and subject to nonenzymatic decarboxylation observed as an increased background level of 14CO2.17,18,19 Experiments were performed to determine if either T23 or T47 nonenzymatically decarboxylated [1-14C]-pyruvate or [1-14C]-D,L-lactate in KRPM without cells or mitochondria (table 1). T23 had no effect on the release of 14CO2 from either [1-14C]-pyruvate or [1-14C]-D,L-lactate compared to the basal level. However, 100 and 250 µM T47 caused significant nonenzymatic decarboxylation of [1-14C]-pyruvate but not [1-14C]-D,L-lactate.

**Figure 3.** Effect of tyrphostin 23 (——) and tyrphostin 47 (-----) on lactate oxidation in isolated adipocytes. Adipocytes were incubated in the presence of tyrphostin 23 or tyrphostin 47 for 60 minutes. Results are expressed in nmole of 14CO2 produced from [1-14C]-D,L-lactate per mg protein of cell suspension per hour. *p < 0.05.

**Figure 4.** The effect of tyrphostin 23 and tyrphostin 47 on adipocyte mitochondrial pyruvate dehydrogenase (PDH) activity in the presence of 20 mM Mg2+, 0.5 mM Ca2+, and 250 µM ATP. *p < 0.001.
TABLE I

Nonenzymatic Decarboxylation of [1-14C]-Pyruvate and [1-14C]-D,L-Lactate in KRPM

<table>
<thead>
<tr>
<th>Tyrophostin Concentration (µM)</th>
<th>[1-14C]-Pyruvate (pmol/min)</th>
<th>T23</th>
<th>T47</th>
<th>[1-14C]-D,L-Lactate (pmol/min)</th>
<th>T23</th>
<th>T47</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>33.5 ±4.1</td>
<td>33.5 ±4.1</td>
<td>0.89 ± 0.04</td>
<td>0.89 ± 0.04</td>
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</tr>
<tr>
<td>10</td>
<td>36.4 ± 6.8</td>
<td>30.7 ± 3.6</td>
<td>0.77 ± 0.05</td>
<td>0.88 ± 0.08</td>
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</tr>
<tr>
<td>100</td>
<td>32.1 ± 3.1</td>
<td>74.1 ± 4.1*</td>
<td>0.92 ± 0.01</td>
<td>0.96 ± 0.11</td>
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<td></td>
</tr>
<tr>
<td>250</td>
<td>39.9 ± 0.6</td>
<td>86.7 ± 6.2*</td>
<td>0.88 ± 0.01</td>
<td>0.92 ± 0.04</td>
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</tr>
</tbody>
</table>

Nonenzymatic decarboxylation of [1-14C]-pyruvate (0.05 µCi/assay) and [1-14C]-D,L-lactate (0.2 µCi/assay) were measured when incubated with varying concentrations of T23 and T47. This representative experiment was performed in triplicate, and the results represent the mean ± SEM.

*P < 0.05.

Discussion

The oxidation of [1-14C]-D,L-lactate to 14CO2 is increased by insulin in human skin fibroblasts, BC3H-1 myocytes, and adipocyte (figure 2). This increase in lactate oxidation is attributed to insulin's stimulation of mitochondrial pyruvate dehydrogenase activity in the intact cells. The lactate oxidation assay is useful for monitoring pyruvate dehydrogenase activity in intact cells. Synthetic tyrphostins are rapidly taken up by cells and inhibit protein tyrosine kinases, including the insulin receptor kinase. They have been used to explore whether or not protein tyrosine kinases are involved in signal transduction in a variety of cells, including smooth muscle. Most tyrphostins are 100 to 10,000 times less effective in inhibiting protein kinase A, protein kinase C or Ca2+/calmodulin-dependent kinases. Both T23 and T47 inhibit insulin receptor kinase activity in isolated insulin receptors. The T23 has also been shown to block effectively insulin-induced effects such as S6 kinase phosphorylation, glucose uptake and incorporation, and α-aminoisobutyric acid uptake in isolated rat adipocytes. Both T47 and T23 are cytotoxic to adipocytes at concentrations greater than 500 µM as assessed by trypan blue exclusion.

Tyrophostins do not directly affect serine/threonine kinases in vitro. However, figure 3 indicates that 1 and 10 µM T47 significantly inhibited lactate oxidation in intact adipocytes. This inhibition may have been secondary to activation of pyruvate dehydrogenase kinase, a novel serine/threonine kinase representing a new family of protein kinases. However, the apparent stimulation of pyruvate dehydrogenase activity in mitochondrial preparations from adipocytes (figure 4) is attributed to the ability of T47 to nonezymatically decarboxylate the substrate, [1-14C]-pyruvate. Consequently, this radiolabeled metabolite should be avoided when T47 is utilized. The effect of nonenzymatic decarboxylation of [1-14C]-pyruvate metabolically converted from [1-14C]-D,L-lactate in intact adipocytes is apparently negligible (figure 3) since T47 alone did not increase 14CO2 production. Dithiothreitol, a cofactor in the pyruvate dehydrogenase assay, is known to cause nonenzymatic decarboxylation of [1-14C]-pyruvate in the absence of cofactors. The absence of these effects in intact adipocytes suggests that the only significant contribution of nonenzymatic decarboxylation in intact adipocytes is from T47.
of tissue.\textsuperscript{16,17} The KRPM, which does not contain dithiothreitol, was used to measure nonenzymatic decarboxylation (table I) of radiolabeled pyruvate by T47 and T23.

In conclusion, the use of oxidation of [\textsuperscript{1-\textsuperscript{14}}C]-D,L-lactate to \textsuperscript{14}CO\textsubscript{2} is a useful model for characterizing pyruvate dehydrogenase activity in intact cells.\textsuperscript{14,15,16} Lactate oxidation is increased in the presence of insulin, consistent with insulin's stimulation of pyruvate dehydrogenase activity through phosphatase activation.\textsuperscript{24} Tyrophostins tested (T47 and T23) do not directly alter pyruvate dehydrogenase kinase activity as suggested by the lactate oxidation assay. The explanation for the decrease in lactate oxidation by 1 and 10 \textmu M T47 remains unknown. However, T47 does nonenzymatically decarboxylate [\textsuperscript{1-\textsuperscript{14}}C]-pyruvate, a substrate frequently used to assay pyruvate dehydrogenase activity in mitochondrial preparations.

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References

20. Buffington CK, Stentz FB, Kitabchi AE. Activation of pyruvate dehydrogenase complex by


