Biochemical Pharmacology, Vol. 32, No. 1, pp. 97–100, 1983.
Printed in Great Britain.

0006-2952/83/010097-04 $03.00/0

EFFECTS OF DICHLOROACETATE ON THE LACTATE/PYRUVATE RATIO AND ON ASPARTATE AND LEUCINE METABOLISM IN CULTURED RAT SKELETAL MUSCLE CELLS

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(*Received* 20 *March* 1982; *accepted* 25 *May* 1982)

Abstract—Dichloroacetate accelerates pyruvate and lactate metabolism in many tissues and has often been shown to increase the lactate/pyruvate (L/P) ratio, which is assumed to be in equilibrium with the cytosolic NADH/NAD^+ ratio. The cause of the drug-induced increase in L/P ratio is not known, but abnormalities in the malate-aspartate hydrogen shuttle have been implicated in perfused skeletal muscle. In addition, indirect studies in perfused skeletal muscle suggest that dichloroacetate inhibits branched chain amino acid oxidation. In the present studies, cultured rat skeletal muscle cells were used to examine the effect of dichloroacetate on the L/P ratio and on the metabolism of aspartate and of leucine. Dichloroacetate increased the L/P ratio after only 60 min of incubation, and the process was saturable with an ED_{50} of 1.0 mM. Drug treatment resulted in marked decreases in cell lactate, pyruvate, and alanine, but no significant differences were observed in cell ATP or in cell levels of compounds of the malate-aspartate shuttle (e.g. malate, aspartate, glutamate or α-ketoglutarate). In addition, dichloroacetate had no consistent effect on aspartate oxidation or on aspartate conversion into glutamate. However, action of the drug did result in an inhibition of leucine oxidation, an effect which contrasts with dichloroacetate action in heart or liver, wherein the drug stimulates leucine oxidation. These results confirm other studies showing that dichloroacetate raises the L/P ratio in skeletal muscle, but do not provide evidence for drug-induced alterations of the malate-aspartate cycle, the principal cytosolic hydrogen shuttle system.

Dichloroacetate activates the pyruvate dehydrogenase complex in cells, and this action results in a reduction of plasma alanine and lactate in normal animals [1, 2] and in normal human subjects [3]. In addition, the drug lowers plasma glucose in diabetic patients [4] by a mechanism that is presently unclear but which may involve either an inhibition of hepatic gluconeogenesis [5–7] or a stimulation of peripheral glucose utilization [8, 9]. Neuropathic side effects [10] of the drug restrict the use of dichloroacetate in the routine control of blood glucose, but the drug may still prove to be useful in the treatment of acute disorders such as lactic acidosis [11, 12]. However, lactic acidosis is frequently caused by, or at least associated with, an abnormality in the NADH redox state of cells. Moreover, the lactate/pyruvate (L/P) ratio, a parameter which frequently parallels the cytosolic NADH/NAD^+ redox state [13], is increased by dichloroacetate in perfused rat skeletal muscle [14], in isolated rat liver cells [9], and in plasma in the dog *in vivo* [2, 15]. The drug also causes a reduction in cell aspartate and malate that is usually associated with an increase in cell glutamate [14, 16]. These metabolites are the primary components of the malate-aspartate cycle, the principal hydrogen shuttle system for the transport of NADH reducing equivalents into the mitochondria, and alterations in this shuttle have been implicated in regard to the dichloroacetate-induced increases in L/P ratio [14]. However, direct isotopic studies of aspartate-glutamate interconversions in the presence of dichloroacetate have apparently not been reported. Therefore, the present studies were undertaken to characterize the effects of dichloroacetate on the L/P ratio and on the rates of conversion of [^{14}C]aspartate into [^{14}C]glutamate in primary cultures of rat skeletal muscle cells. In addition, the effects of dichloroacetate on leucine oxidation were examined. Studies in rat skeletal muscle suggest that the drug may inhibit branched chain amino acid oxidation [14], although recent studies indicate that the drug actually stimulates leucine oxidation in cardiac muscle [17].

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MATERIALS AND METHODS

Chemicals and reagents. All reagents, enzymes, and tissue culture materials were obtained from sources previously reported [18, 19]. Dichloroacetate was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Labeled L-[U-^{14}C]aspartate, 225 μCi/μmole, and L-[1-^{14}C]leucine, 55 μCi/μmole, were obtained from the New England Nuclear Corp. (Boston, MA). Dinitrophenyl (DNP)-amino acids and Dowex 50-X8 resin were purchased from the Sigma Chemical Co. (St. Louis, MO).

Cell culture. Myoblasts were obtained from hind-limb muscle of male 300–400 g Sprague–Dawley rats and were grown in culture as described previously

[18], except that rats were injected with an 0.25-ml mixture of bupivacaine (0.5%) and hyaluronidase (50 μg/ml) in each hindlimb for 4 consecutive days and then were killed on day 5. This treatment results in a chemical injury to the muscle and greatly enhances the yield of myogenic cells, as opposed to fibroblasts [18]. By 17–23 days in culture, well-differentiated, contractile, multinucleated myotubes were formed [19] and were used for all experiments. Incubations were carried out in serum free, glutamine-free, pyruvate-free Dulbecco's modified Eagle's medium supplemented with 0.4 mM pyruvate and 2 mM lactate at 37° in a humidified atmosphere of 95% air:5% CO_2.

Analytical methods. Intracellular and medium metabolite levels in perchloric acid extracts were determined with enzymatic fluorometric or spectrophotometric assays exactly as described previously [18]. Samples for pyruvate and α-ketoglutarate determinations were stored at pH < 2 [18]. Rates of formation of $^{14}CO_2$ from [^{14}C]aspartate and [^{14}C]*l*-leucine were measured as reported previously [18, 20]. Medium amino acid specific activities were determined as previously described [19]. Briefly, 1 ml of perchlorate-acidified medium was placed on an 0.9 × 4 cm Dowex 50-X8 column (H^+ form), and the the neutral-anion fraction was eluted with water and discarded. The amino acid fraction was eluted with 3 M NH_3 and evaporated to dryness, and DNP-amino acid derivatives were formed with dinitrofluorobenzene [21]. The DNP-amino acids were separated on activated 500 μm silica gel G plates (Analtech, Newark, DE) using the solvent systems of Smith and co-workers [22], e.g. solvent I: ether 250, methanol 50, ammonia 10, water 10; solvent II: ether 100, acetic acid 10, water 10. The yellow spots were eluted with 0.01 N $NaHCO_3$, quantitated at λ = 360 nm relative to DNP-amino acid standards, and counted for ^{14}C-radioactivity. In our hands, the present solvent systems provide excellent separation of glutamate, aspartate, alanine, and glutamine [19]. No other amino acids overlap these four amino acids, with the exception of asparagine which co-migrates with glutamine. However, since only trace amounts of asparagine are released into the medium over the 4-hr incubation period [23], the asparagine content was considered negligible.

Calculations. Data are reported as means ± S.E.M. (N = 3–4 dishes per point). All assays were performed in duplicate. Statistical significance was assessed by Student's *t*-test. Myoblasts were obtained from four to six rats and pooled for each experiment.

RESULTS

The medium L/P ratio in control cells increased linearly with time from 5 ± 1 to 21 ± 1 between 0 and 4 hr of incubation (Fig. 1). However, in the presence of 2 mM dichloroacetate, the medium L/P ratio rose from 5 ± 1 to 60 ± 15 between 0 and 4 hr, and substantial differences from control plates were observed as early as 1 hr (Fig. 1). The dose–response curve for the dichloroacetate effect on the 4-hr L/P ratio is shown in Fig. 2. The drug action was saturable, with 50% effect observed at 1 mM dichloroacetate. The L/P ratio reached values in excess of 100 at a 5 mM drug concentration, and significant increases in the L/P ratio were observed at dichloroacetate levels as low as 0.2 mM (Fig. 2). Intracellular L/P ratios were measured at all points shown in Figs. 1 and 2, and the correlation between medium and intracellular L/P ratios was linear ($r = 0.98$) with a slope of 0.96 (data not shown).

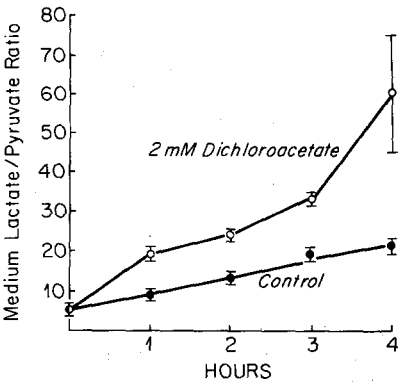

Fig. 1. Medium lactate/pyruvate ratio plotted versus incubation time in control and dichloroacetate-treated cells.

The effect of an 0.5 mM concentration of dichloroacetate on cell metabolite levels after 4 hr of incubation is shown in Table 1. The drug markedly lowered intracellular pyruvate, lactate, and alanine, but it had no significant effect on cell levels of ATP or on levels of components of the malate-aspartate shuttle (Table 1). The 40% decrease in cell aspartate was not statistically significant and decreases in cell aspartate concentrations were not observed at 1, 2, or 3 hr of incubation (data not shown).

Since flux through the malate-aspartate shuttle could be grossly altered without appreciable changes in pool sizes of the respective metabolites, the rates of utilization of [^{14}C]aspartate were examined in two ways. First, the conversion of [^{14}C]aspartate to $^{14}CO_2$ was examined. As shown in Fig. 3, a 2 mM level of dichloroacetate had no consistent effect on the rate of oxidation of labeled aspartate. Second, the rate of conversion of [^{14}C]aspartate into [^{14}C]glutamate was examined. Under conditions of constant precursor specific activity, the flow of carbon from

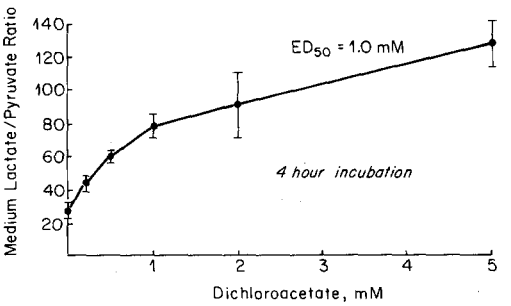

Fig. 2. Medium lactate/pyruvate (L/P) ratio after 4 hr of incubation plotted versus the concentration of dichloroacetate (DCA) added to the incubation medium. The effective concentration (EC_{50}) which caused a 50% increase in L/P ratio was determined from a double-reciprocal plot ($r = 0.99$) of 1/DCA vs 1/(ΔL/P), where ΔL/P = the change in L/P ratio above the control value at DCA = 0 mM.

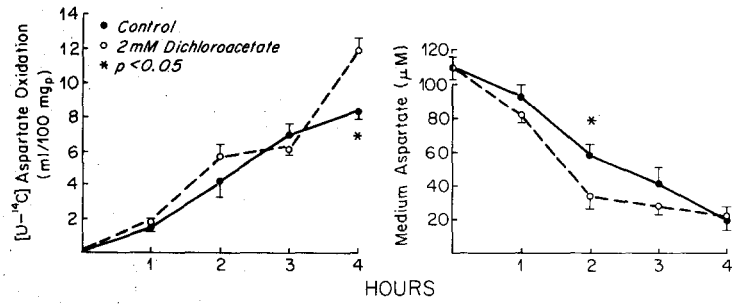

Fig. 3. Normalized amount of $^{14}CO_2$ production from [U-^{14}C]aspartate plotted versus incubation time in either control or dichloroacetate-treated cells. Due to the fall with time in medium aspartate concentration, the actual amount of $^{14}CO_2$ produced was divided by the medium aspartate concentration (nmoles/ml) to give the normalized amount of $^{14}CO_2$ production (ml/100 mg protein). In these experiments, the usual experimental medium (Materials and Methods) was made 0.1 mM aspartate prior to incubation.

Table 1. Cell metabolite levels after 4 hr of incubation with either 0 (control) or 0.5 mM dichloroacetate (DCA)*

Metabolite	Cell metabolite levels (nmoles/mg protein)	
	Control	DCA
ATP	35 ± 3	32 ± 2
Pyruvate	1.4 ± 0.2	0.59 ± 0.06†
Lactate	55 ± 3	36 ± 2†
Malate	2.8 ± 0.5	2.3 ± 0.3
α-Ketoglutarate	1.3 ± 0.2	1.3 ± 0.1
Citrate	5.8 ± 0.9	4.7 ± 0.2
Aspartate	3.5 ± 0.4	2.0 ± 0.7
Glutamate	23 ± 5	23 ± 2
Alanine	19 ± 4	1.2 ± 0.1†

* Data are means ± S.E.M. N = 3 dishes measured in duplicate.
† $P < 0.01$.

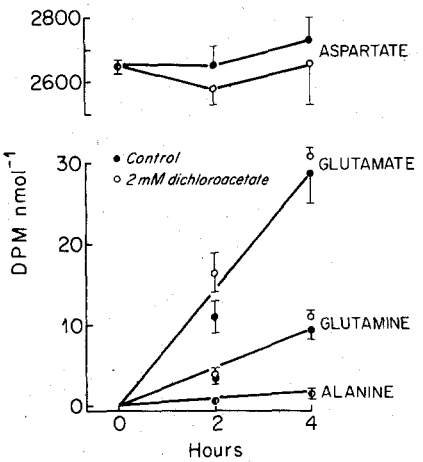

Fig. 4. Medium specific activities (dpm/nmole) of precursor [U-^{14}C]aspartate and product [^{14}C]glutamate, -glutamine, and -alanine, plotted versus incubation time. No significant differences in specific activity of any of the four amino acids were observed between control and dichloroacetate-treated cells. The incubation medium in these experiments was made 0.4 mM aspartate, glutamate, alanine, and glutamine, to facilitate the visual localization of amino acids on the thin-layer chromatograms.

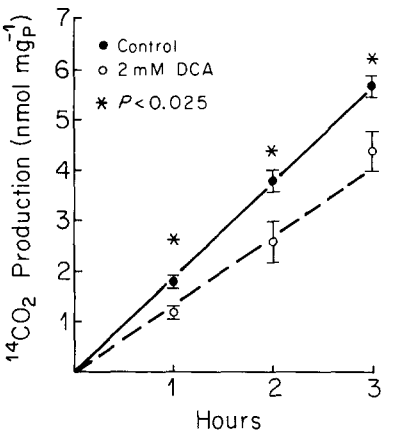

Fig. 5. Rate of $^{14}CO_2$ production from [^{14}C]*l*-leucine plotted versus incubation time in control and dichloroacetate-treated cells. The rate was not normalized (see Fig. 3) for change in medium amino acid concentration, since medium leucine concentration is constant over a 3-hr incubation period [18].

aspartate to glutamate, or to glutamine, or to alanine, was unaffected by a 2 mM concentration of dichloroacetate (Fig. 4).

Finally, the effects of dichloroacetate on the oxidation of [^{14}C]*l*-leucine were examined. As shown in Fig. 5, a 2 mM concentration of dichloroacetate resulted in 25–33% inhibition of leucine oxidation at 1, 2 or 3 hr of incubation.

DISCUSSION

The present studies indicate that dichloroacetate treatment results in marked increases in the L/P ratio in primary cultures of adult rat skeletal muscle cells. The EC_{50} of 1 mM dichloroacetate approximates the peak plasma level in man after a 30-min intravenous infusion of 35 mg/kg dichloroacetate [3].

Owing to rapid rates of glycolysis, the L/P ratio normally rises in cultured skeletal muscle [18]; this suggests that the transport into the mitochondria of reducing equivalents derived from glycolysis is normally rate-limiting in the cultured cells. In contrast, the L/P ratio is normally constant, and the transport of reducing equivalents into the mitochondria is not rate-limiting *in vivo*. While cultured skeletal muscle cells clearly differ in several respects from muscle

in vivo, these cells provide a model system for replicating the elevation in the L/P ratio normally caused by dichloroacetate and for investigating the role of aspartate metabolism in the drug-induced rise in L/P ratio. Moreover cultured skeletal muscle cells provide an ideal model system for investigating amino acid metabolism with isotopic techniques. Owing to the high ratio of extracellular medium to intracellular water space [18, 19], the specific activity of a labeled amino acid precursor, e.g. [^{14}C]aspartate, is maintained at a constant level for several hours (Fig. 4).

The relationship between the L/P and NADH/NAD^+ ratios is a function of lactate dehydrogenase which mediates the following reversible reaction:

$$\text{pyruvate} + \text{NADH} + H^+ \leftrightharpoons \text{lactate} + NAD^+.$$

The activity of lactate dehydrogenase in cells is so high that the reactants are maintained at a near-equilibrium position [13]. Consequently, dichloroacetate-induced increases in pyruvate consumption should be paralleled by proportionate increases in lactate consumption such that the L/P ratio is constant. Conversely, an increased L/P ratio reflects either an increased NADH/NAD^+ ratio or an intracellular acidosis. A primary change in intracellular H^+ is unlikely to be the explanation for the elevated L/P ratio, since dichloroacetate caused a more than 20-fold increase in the L/P ratio (Fig. 2) and a 20-fold increase in H^+ would result in a reduction in ATP levels [24]. Moreover, Dennis *et al.* [25] have shown that dichloroacetate does cause an increase in the NADH/NAD^+ ratio in cardiac muscle.

An elevation in the cytosolic NADH/NAD^+ ratio may be due to an alteration in the transport of cytosolic reducing equivalents into the mitochondria via the malate-aspartate shuttle, e.g. aspartate (cytosol) → oxaloacetate (cytosol) → malate (cytosol) → malate (mitochondria) → oxaloacetate (mitochondria) → aspartate (mitochondria) → aspartate (cytosol). The flux through the malate-aspartate cycle would be slowed if mitochondrial oxaloacetate were diverted from aspartate regeneration and into other pathways such as (a) flux across the Krebs cycle to α-ketoglutarate, followed by synthesis of glutamate or glutamine, or (b) decarboxylation of oxaloacetate to pyruvate via phosphoenolpyruvate carboxykinase [26] or decarboxylation of malate, via malic enzyme [27], to pyruvate followed by the formation of alanine. However, the conversion rate of [^{14}C]aspartate into $^{14}CO_2$ or into [^{14}C]glutamate, [^{14}C]glutamine, or [^{14}C]alanine, was not consistently altered in the presence of 2 mM dichloroacetate (Figs. 3 and 4). Therefore, if flux through the malate-aspartate shuttle is impaired by dichloroacetate, the mechanism does not appear to be diversion of aspartate into other metabolic pathways. The results of the present study, however, do not exclude the interaction of dichloroacetate and the malate-aspartate shuttle by a mechanism not involving aspartate metabolism.

Finally, the present results confirm the suggestion of Goodman *et al.* [14] that dichloroacetate inhibits branched chain amino acid oxidation in skeletal muscle. Therefore, skeletal muscle contrasts with cardiac muscle, where dichloroacetate stimulates leucine oxidation by activating the branched chain keto acid dehydrogenase [17], or with liver, where the drug stimulates leucine catabolism by increasing leucine transamination [28].

Acknowledgements—This work was supported by NIH Grant AM-21762 and a grant from the American Diabetes Association. William M. Pardridge is the recipient of Research Career Development Award Am-00783. The authors are indebted to Victoria Viole for preparation of the manuscript.

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