Dichloroacetate Inhibits Glutamine Oxidation by Decreasing Pyruvate Availability for Transamination

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We have shown that dichloroacetate (DCA) inhibits growth, glutamine oxidation, and pyruvate and alanine production in a concentration-dependent manner in PQXB 1/2 hybridoma cells. The use of inhibitors indicates that glutamine oxidation proceeds by an aminooxyacetate-sensitive transamination reaction in this cell line. Addition of pyruvate to DCA-treated cells restored glutamine oxidation to control values. Our data suggest that DCA inhibits glutamine oxidation by decreasing the availability of pyruvate for transamination, which in turn results in glutamate accumulation and a consequent inhibition of glutaminase activity. Impaired glutamine catabolism in the presence of DCA has subsequent effects on overall metabolic balance and cell maintenance and growth.

DICHLOROACETATE (DCA) is well established as an activator of pyruvate dehydrogenase (PDH) and activates the PDH enzyme complex in a number of mammalian tissues.[1-6] Significant interest has focused on the potential of DCA for the treatment of conditions such as diabetes and lactic acidosis.[5,6] Recently, we demonstrated that PQXB 1/2 hybridoma cells grown in media supplemented with DCA exhibited a lower specific glutamine consumption rate than cells grown in the absence of DCA.[7] Interest in the regulation of glutamine metabolism of mammalian cells and its interrelationship with glycolysis is largely associated with the fact that the malignant phenotype is characterized by increased glutamine consumption.[8-10] A direct correlation between the rate of glutamine consumption and the degree of malignancy has been shown in a series of rat hepatoma cell lines.[9,10] Glutamine is an important energy source for cultured tumor cell lines, where it can provide up to 65% of cellular energy requirements.[11] Following conversion of glutamine to glutamate by glutaminase, the next step in catabolism of "glutamine" can occur by one of two possible reactions involving either glutamate dehydrogenase (GDH) or an aminotransferase, both of which would yield α-ketoglutarate for subsequent catabolism (Fig 1). In cells such as hybridoma cell lines, which exhibit approximately equimolar amounts of glutamine consumption and ammonia production and produce significant quantities of alanine, it would seem that aminotransferases such as alanine aminotransferase (AAT) are quantitatively more important than GDH for the conversion of glutamate to α-ketoglutarate. Here, we show that DCA inhibits glutamine oxidation of PQXB 1/2 hybridoma cells by decreasing the availability of pyruvate for transamination.

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MATERIALS AND METHODS

Reagents

All cell culture media and supplements were obtained from Gibco-BRL (Paisley, Scotland, UK). DCA, EDTA, methanol, and perchloric acid were from BDH Chemicals (Liverpool, UK). NAD^+, NADH, alanine dehydrogenase, and lactate dehydrogenase were from Boehringer (London, UK). L-[U-^{14}C]glutamine was obtained from Amersham International (Amersham, UK), and EcoScint A was purchased from National Diagnostics (Atlanta, GA). Aminooxyacetate, glutarate, and all other chemicals were purchased from Sigma Chemical (Poole, UK). The PQXB 1/2 hybridoma cell line (no. 89050503) was obtained from the European Collection of Animal Cell Cultures (PHLS, Salisbury, UK).

Cell Culture

PQXB 1/2 hybridoma cells were routinely grown in RPMI 1640 medium supplemented with glutamine (2 mmol/L), sodium bicarbonate (25 mmol/L), and heat-inactivated fetal calf serum (10% vol/vol). To determine the effect of DCA on growth, cells in mid-log phase were harvested by centrifugation (100 × *g* for 5 minutes at room temperature) and resuspended in fresh culture medium to a density of 1×10^5 cells/mL. Sterile DCA (sodium salt, pH 7.4) was added from a freshly prepared 1-mol/L stock to produce the required final concentration. Cells were cultivated in static T 25-cm^2 flasks (working vol, 10 mL) at 37°C in an atmosphere of 5% (vol/vol) CO_2 in air. Total and viable cell numbers were determined by trypan blue exclusion and counting in a hemocytometer.[12]

Metabolic Analyses

PQXB 1/2 hybridoma cells at mid-phase were harvested by centrifugation (100 × *g* for 5 minutes at room temperature) and resuspended in fresh culture medium at a concentration of 5×10^6 cells/mL. The cell suspension was distributed in 2-mL portions into 10-mL Konte flasks containing additives as noted, and these were immediately stoppered with a Subaseal. The flasks were gassed with O_2:CO_2 (19:1 vol/vol) and incubated in a 37°C reciprocal shaking water bath at 80 cycles/min. At appropriate time points, incubations were terminated by addition of perchloric acid (2% vol/vol final), and acidified extracts were centrifuged (12,000 × *g* for 5 minutes at room temperature). The supernatants were removed and neutralized with 2 mol/L KOH containing 0.5 mol/L triethanolamine. Measurements of pyruvate[13] and alanine[14] levels were performed on the neutralized extracts.

For analysis of glutamine oxidation, cells were incubated in Konte flasks as described earlier in medium containing L-[U-^{14}C]glutamine (0.25 μCi/μmol). Flasks were stoppered with Subaseals containing a center well with a filter-paper wick. After the appropriate incubation period, 300 μL of a mixture of 2-phenylethylamine:methanol (1:1 vol/vol) was injected through the Subaseal into the center well to trap

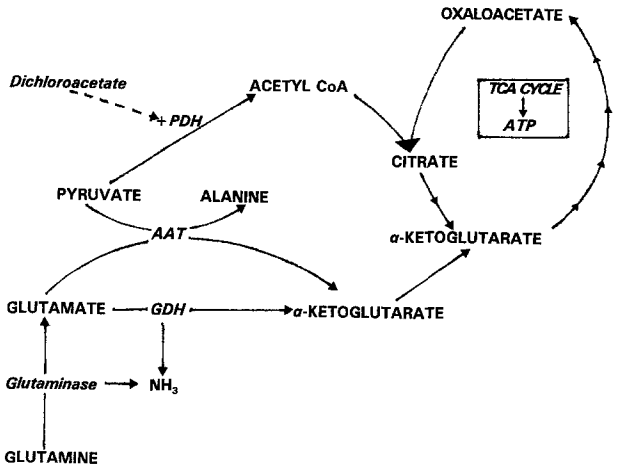

Fig 1. Schematic diagram of the interrelationships between glutamine and pyruvate metabolism. Shown are the 2 possible means for generation of α-ketoglutarate for oxidation in the TCA cycle from glutamine-derived glutamate, GDH, and AAT, and the relationship to the influence of DCA on pyruvate availability for transamination. In addition, the stoichiometry of NH_3 production in relation to utilization of glutamine via GDH or AAT can be defined.

$^{14}CO_2$. Immediately afterward, $^{14}CO_2$ dissolved in the medium was liberated by injection of 200 μL perchloric acid (20% vol/vol) into the cell suspension. Addition of perchloric acid also served to terminate the reactions. The flasks were shaken for another 1 hour at room temperature, after which the center wells were removed and placed into scintillation vials containing 3 mL Ecoscint A, and then counted for radioactivity by liquid scintillation counting.

Statistical Analysis

As indicated, replicate analyses were made with several independent batches of cells, and values are reported as the mean ± SEM. Where appropriate, the significance of differences between treatments was assessed by two-tailed Student's *t* test.

RESULTS

DCA Inhibits Glutamine Oxidation

The rates of L-[U-^{14}C]glutamine oxidation for control, 1-mmol/L DCA–treated and 10-mmol/L DCA–treated PQXB 1/2 hybridoma cells were constant for at least 4 hours (Fig 2). The control rate of glutamine oxidation (0.224 ± 0.013 μmol/10^7 cells/h, n = 6 separate cell batches) is almost identical to that obtained by Jenkins et al[15] for the same cell line. DCA inhibited glutamine oxidation in a concentration-dependent manner (Figs 2 and 3). DCA at 1 mmol/L decreased the rate of glutamine oxidation by 16% (significantly different from control by Student's *t* test, $P < .003$), and at the higher concentration of 10 mmol/L, DCA decreased the rate of glutamine oxidation by 62%. All cell batches used in these experiments were from cells in the mid-log phase of growth and had viabilities greater than 95% (as assessed by trypan blue staining). Over the short period of these experiments (2 hours), cell viability was not decreased by addition of DCA over the entire range of concentrations used.

Glutamine Oxidation Occurs via Transamination

To define the relative importance of aminotransferases and GDH in glutamine catabolism, the effects of inhibitors of these enzymes on CO_2 release from L-[U-^{14}C]glutamine were studied. Addition of glutarate to incubations at a final concentration of 2 mmol/L, a level at which this compound is a potent inhibitor of GDH,[16] had no effect on glutamine oxidation (results not shown). In contrast, 0.6 mmol/L aminooxyacetate, which acts as a general aminotransferase inhibitor at this concentration,[17] decreased glutamine oxidation over a 2-hour period by 63% (from 0.205 ± 0.014 μmol/10^7 cells/h in control conditions to 0.076 ± 0.009 μmol/10^7 cells/h, significantly different from control by Student's *t* test, $P < .001$, n = 4 separate cell batches). The effect of DCA is similar to that reported for this compound on glutamine oxidation in bovine adrenocortical cells.[18] These results suggest that aminotransferase activity is quantitatively more important than GDH activity for glutamine catabolism in PQXB 1/2 hybridoma cells.

DCA Decreases Pyruvate and Alanine Production

DCA decreased the production of pyruvate and alanine from PQXB 1/2 cells in a concentration-dependent manner (Fig 4). These results are consistent with those of other studies in vivo and in vitro, which describe the effect of DCA on these metabolites in various rat tissues.[2,3,19]

Pyruvate Addition Reverses the Inhibitory Actions of DCA on Glutamine Oxidation

Evidence provided here and elsewhere[7] suggests that oxidation of glutamine requires AAT for the conversion of glutamine-derived glutamate to α-ketoglutarate. Since DCA decreased pyruvate production from PQXB 1/2 hybridoma cells, one mechanism by which DCA might inhibit glutamine oxidation

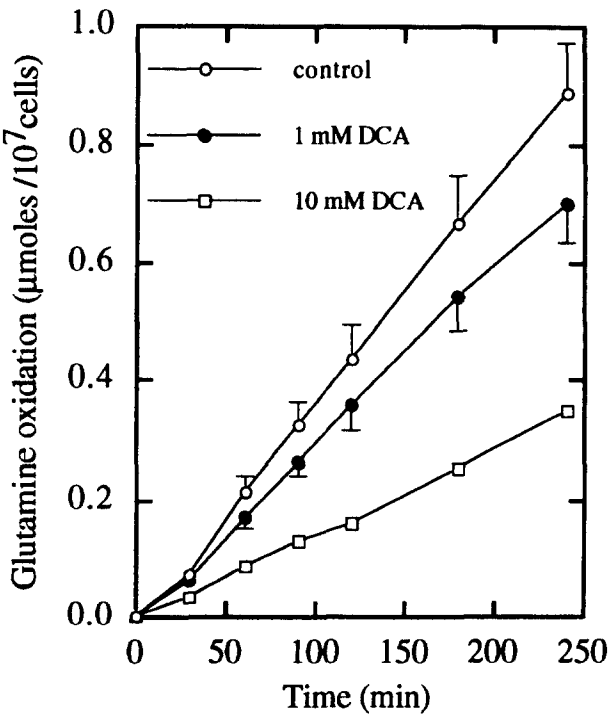

Fig 2. Effect of DCA on the time course of L-[U-^{14}C]glutamine oxidation by PQXB 1/2 hybridoma cells. Results are the mean ± SEM (n = 3 separate batches of cells).

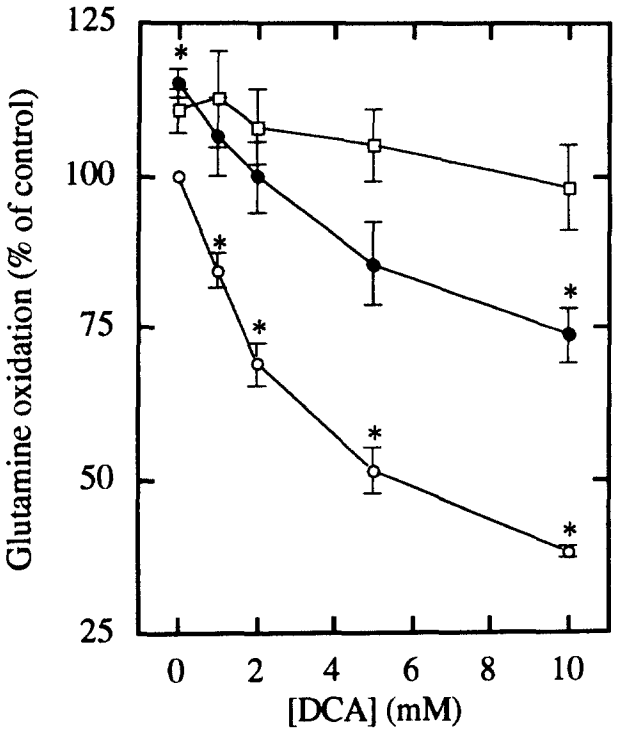

Fig 3. Pyruvate addition restores L-[U-^{14}C]glutamine oxidation in DCA-treated PQXB 1/2 hybridoma cells. L-[U-^{14}C]glutamine oxidation was determined in cells incubated in the presence or absence of DCA (at the indicated final concentration) for 2 hours in the absence (○) or presence (●) of 1 mmol/L pyruvate and 10 mmol/L pyruvate (□). Results are the mean ± SEM (n = 4 separate cell batches). *Significantly different (Student's *t* test) from the 100% control (without DCA or pyruvate), $P < .05$.

would involve decreased availability of pyruvate for transamination. We show that pyruvate and, consequently, alanine production were decreased by DCA (Fig 4), but to test this hypothesis directly, we examined the interaction of exogenous pyruvate and pyruvate on glutamine oxidation (Fig 3). Addition of pyruvate at a concentration of 1 mmol/L abolished the inhibitory action on glutamine oxidation at all but the highest concentration of DCA tested (10 mmol/L). When pyruvate was added at a concentration of 10 mmol/L, DCA had no significant effect on glutamine oxidation at any concentration tested. It is of interest that in control conditions (ie, in the absence of DCA; Fig 3), inclusion of pyruvate at 1 and 10 mmol/L, stimulated glutamine oxidation by 14.8% ± 1.6% (significance testing *v* samples untreated with DCA by Student's *t* test, $P < .01$, n = 3 separate cell batches) and 10.6% ± 3.2% (*v* samples untreated with DCA, $P < .06$, n = 3 separate cell batches), respectively, suggesting that under control conditions, the rate of glutamine oxidation may be limited by AAT activity as a consequence of suboptimal medium pyruvate concentration.

DCA Inhibits Growth of PQXB 1/2 Hybridoma Cells

The observation that DCA decreased glutamine oxidation had longer-term consequences for cell growth. Decreased glutamine oxidation in the presence of DCA correlated with a concentration-dependent inhibition of the growth of PQXB 1/2 hybridoma cells (Fig 5). This finding is similar to that of Shireman et al,[20] who found that concentrations of DCA above 0.5 mmol/L inhibited the growth of fibroblasts, presumably due to limitation of adenosine triphosphate generation in conditions of diminished glutamine oxidation.[9-11]

DISCUSSION

In this report, we have demonstrated for the first time that DCA inhibits glutamine oxidation in a tumor-derived cell line of mammalian origin. Glutamine oxidation in PQXB 1/2 hybridoma cells appears to proceed via a transamination reaction. Several other studies have suggested that aminotransferases are more important than GDH for glutamine catabolism in hybridomas,[7,12,21] lymphocytes,[22] and HeLa and Chinese hamster ovary cells.[23] More specifically, our results suggest that AAT is involved, and that DCA inhibits glutamine oxidation by decreasing the availability of pyruvate for transamination (Fig 1). DCA has been shown to decrease pyruvate concentration in various rat tissues.[2,3,19] Since pyruvate is a substrate for both PDH and AAT, it is likely that DCA, by stimulation of PDH activity, decreases the amount of pyruvate available for transamination. In addition, decreased availability of pyruvate, and thus transamination of glutamate, may lead to an accumulation of glutamate. We have shown that glutamate does indeed accumulate in batch cultures of PQXB 1/2 hybridoma cells treated with

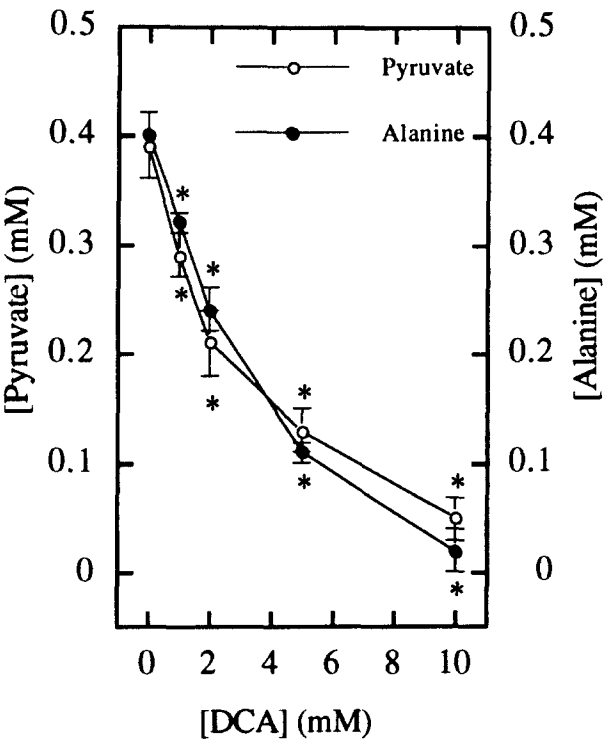

Fig 4. Effect of DCA on pyruvate and alanine production by PQXB 1/2 hybridoma cells. Measurements were made on media extracts after a 2-hour incubation with the concentrations of DCA as indicated. Results are the mean ± SEM (n = 4 separate cell batches). *Significantly different (Student's *t* test) from the control (minus DCA), $P \pm .05$.

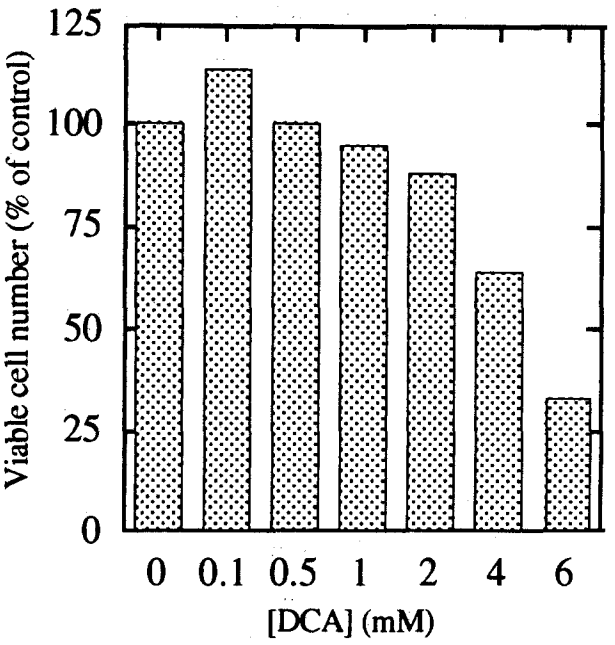

Fig 5. Effect of DCA on growth of PQXB 1/2 hybridoma cells. Results are the mean of duplicate flasks, with cells incubated in medium in the absence of DCA considered as 100%. Initial cell density was 1 × 10^5/mL, and after 72 hours of culture, cell density under control conditions (absence of DCA) was 8.7 × 10^5/mL. Values for viable cell numbers are presented (as assessed by trypan blue exclusion), since >95% of all cells were viable by this criterion and inclusion of DCA did not impair viability.

DCA.[7] Hence, glutamate accumulation as a consequence of DCA action may also play a role in decreasing glutamine oxidation, since glutamate has been reported to inhibit glutaminase.[24] An inhibition of glutaminase activity would be consistent with our finding that DCA decreases ammonia production from glutamine by PQXB 1/2 hybridoma cells in batch cultures.[7] As a consequence, a change in pyruvate availability as a result of DCA actions may modify glutamine (via glutamate) oxidation through two means, by a direct effect on transamination and by a consequent indirect effect on glutaminase activity.

The dependency of glutamine oxidation on pyruvate is significant in relation to several previously unexplained findings. For example, it may explain why pyruvate is required for culturing some cell types at low cell densities and under cloning conditions.[25,26] It has been suggested that exogenously added pyruvate limits the loss of the intracellular pyruvate pool to the medium at low cell densities, whereas high cell densities maintain effective intracellular pools.[25] Therefore, cells grown at low cell densities in the absence of added pyruvate may not survive, as a consequence of being unable to maintain their energy status due to inefficient glutamine utilization. Also, in some cell types such as rat lymphocytes, the presence of glucose stimulates the oxidation and consumption of glutamine.[27] It is possible that this observation may be due to an increased supply of pyruvate, derived from glycolysis, for transamination.

Finally, our finding that DCA inhibits glutamine oxidation and consequently has long-term consequences for the growth (Fig 5) of hybridoma cells suggests that DCA or its derivatives may define new avenues for selective chemotherapeutic regimens.

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