ABSTRACT

Exposure of L929 cells to hyperthermia in the presence of 2-cyanocinnamic acid, an inhibitor of mitochondrial pyruvate transport, markedly enhanced killing at temperatures as low as 41°C. The inhibitor also reduced the oxidation of both glucose and exogenous pyruvate while increasing lactate production from glucose but not from pyruvate. These results are consistent with previous observations that glucose and pyruvate enhance survival after hyperthermia and point to a role for pyruvate in protecting against hyperthermic cytotoxicity. Heat shock protein synthesis was also inhibited by 2-cyanocinnamic acid under some conditions, suggesting that a function of pyruvate may also be involved in heat shock protein induction.

INTRODUCTION

The potential usefulness of hyperthermia for cancer therapy has aroused interest in the mechanism by which heat kills cells and in the means by which the sensitivity of tumor cells to killing by this modality might be increased. Several mechanisms, including membrane damage (1) and energy depletion (2) have now been advanced to explain the lethal effects of temperatures in the 41°-45°C range. Also, energy source deprivation has aroused interest in the mechanism by which heat kills cells and in the means by which the sensitivity of tumor cells to killing by this modality might be increased. Several mechanisms, including membrane damage (1) and energy depletion (2), have now been advanced to explain the lethal effects of temperatures in the 41°-45°C range. Also, energy source deprivation, including membrane damage (1) and energy depletion (2), has been suggested as a mechanism by which heat kills cells and in the means by which the sensitivity of tumor cells to killing by this modality might be increased. Several mechanisms, including membrane damage (1) and energy depletion (2), have now been advanced to explain the lethal effects of temperatures in the 41°-45°C range. Also, energy source deprivation, including membrane damage (1) and energy depletion (2), has been suggested as a mechanism by which heat kills cells and in the means by which the sensitivity of tumor cells to killing by this modality might be increased. Several mechanisms, including membrane damage (1) and energy depletion (2), have now been advanced to explain the lethal effects of temperatures in the 41°-45°C range. Also, energy source deprivation, including membrane damage (1) and energy depletion (2), has been suggested as a mechanism by which heat kills cells and in the means by which the sensitivity of tumor cells to killing by this modality might be increased. Several mechanisms, including mem-

MATERIALS AND METHODS

Cells and Culture Conditions. L929 cells were obtained from MA Bioproducts (Bethesda, MD) and were routinely maintained in bacteriologic polystyrene Petri dishes containing DMEM and 10% newborn calf serum. This unsupplemented DMEM contained 25 mM glucose, 4 mM glutamine, and 1 mM pyruvate. Cells were removed from the stock cultures by gentle pipetting and plated in 35-mm plastic tissue culture dishes at a density of 1 × 10^5/cm². One to 2 days later, the monolayers had reached a final density of 2–3 × 10^5/cm². Cultures were washed twice with Dulbecco's phosphate-buffered saline, and 2 ml of the medium of interest were added and changed daily. Experiments designed to isolate the effects of 2-CC and related compounds (Aldrich Chemical Co., Milwaukee, WI, or Pfaltz & Bauer, Inc., Waterbury, CT) on pyruvate metabolism utilized serum-free DMEM lacking glutamine and pyruvate, i.e., a medium containing glucose as the only significant source of energy and pyruvate.

Exposure to Hyperthermia. After maintenance for 3 days in the desired medium, inhibitors were added from 0.2 ml aqueous stock solutions titrated to pH 7.4 with NaOH. Cultures were immediately placed on an aluminum sheet in a water-jacketed CO₂ incubator which had been equilibrated at the desired hyperthermic temperature. When treated in this manner, medium in the dishes reached the indicated temperature within 10 min. Cultures were returned to 37°C after various periods of exposure. Cells were then dispersed with 0.25% trypsin (Sigma), plated at appropriate dilutions, and maintained in DMEM plus 10% newborn calf serum for 7 days. Plates were then examined after staining with methylene blue, and clones containing more than 10 cells were counted. Previous work with this cell line has shown that counting clones containing 50 cells yields similar survival values compared to the method used here (4). Surviving fraction was the ratio of the number of clones formed from cultures exposed to hyperthermia divided by the number formed from control cultures maintained in medium of the same composition. Data points represent the mean values for duplicate cultures that were trypsinized and plated separately. Each experiment was repeated from 2 to 4 times with similar results.

Pyruvate Oxidation and Lactate Production. These determinations were made simultaneously on monolayer cultures maintained in 25-cm² tissue culture flasks using an apparatus similar to that described by Reitzer et al. (10). The culture medium was removed and replaced with 0.5 ml of DMEM (25 mM glucose; 4 mM glutamine; 11 mM sodium pyruvate) containing the appropriate ¹⁴C-labeled substrate (Amersham: 1.0 µCi/ml). Each flask was then quickly fitted with a serum stopper into which had been inserted a perforated polylammonium tube closed with a rubber stopper. Incubation was for 2 h at either 37°C or 42°C with agitation every 30 min to ensure that the monolayer was uniformly exposed to the medium. The rubber stopper was then removed, a 10-µl aliquot of medium was taken for lactate determination, and 0.5 ml of 0.5 N NaOH was added to the polylammonium tube. The stopper was replaced after 1.5 ml of 6 N H₂SO₄ was injected into the flask to kill the cells and release CO₂ from the medium. After incubation for 2 h at room temperature, the NaOH was replaced, and incubation continued for an additional 18 h. Both NaOH solutions were dispersed in an aqueous fluor, and radioactivity was determined in a scintillation counter.

The aliquots for lactate determination were chromatographed on Redi-foil cellulose thin-layer plates (Schleicher & Schull) using a butanol:acetic acid:water (15:17:50) solvent system, dried at room temperature, and autoradiographed. The lactate and precursor spots were cut out and counted as above. The results are expressed as nmol of substrate converted to product per mg of protein per h.

hsps Synthesis. Cultures were labeled at 37°C with [³⁵S]methionine (Amersham; 20 µCi/ml) in methionine-free DMEM for 2 h immediately following exposure to hyperthermia. After labeling, monolayers were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (11, 12). Cells were dried and autoradiographed using Kodak XAR-5 film.

RESULTS

Effects of 2-CC on Cell Survival after Exposure to Hyperthermia. Colony formation of L929 cells maintained in DMEM
lacking glutamine and pyruvate was virtually unaffected by 500 μM 2-CC (data not shown). However, Fig. 1 shows that concentrations of this agent ranging from 50–500 μM progressively increased sensitivity to killing at 42°C. This effect was also seen in complete DMEM, but it was not as dramatic. Fig. 2 shows that, in a separate experiment, sensitization was also apparent at 41°C and increased even further at 43°C.

Several variations of these experiments were performed to define the conditions under which 2-CC would be most effective and to gain some insight into its mechanism of action. Thus, the effect appeared to be both rapid and easily reversible, since incubation with 500 μM 2-CC for 30–60 min prior to exposure did not further increase sensitivity, and changing to fresh medium lacking 2-CC reverted survival to that of the controls.

Interestingly, addition of 10 mM pyruvate had no effect; i.e., it neither increased nor decreased sensitization by 2-CC. This result will be discussed below. Finally, a variety of analogues, including cinnamic acid, 2-cyano-3-hydroxycinnamic acid, 2-cyano-4-hydroxycinnamic acid, and 4-hydroxycinnamic acid, was completely ineffective, in terms of modifying cell survival responses, at concentrations of 500 μM.

Effects of 2-CC on Pyruvate Oxidation and Lactate Production. Table 1 shows that, with glucose as the major source of energy, approximately twice as much glucose was oxidized than was converted to lactate, and this partitioning of glucose carbon was not greatly altered by heat shock. Exposure to 2-CC reduced glucose oxidation while increasing lactate production, effectively reversing the ratio of glycolysis to oxidation. Heat shock in the presence of 2-CC increased glucose oxidation with only a small increase in lactate production. Pyruvate, added to the glucose-containing medium, was mainly converted to lactate at 37°C. In this situation, 2-CC also inhibited pyruvate oxidation, but with little effect on lactate production. Similar effects of 2-CC on CO₂ and lactate production were seen using [1-¹⁴C]-pyruvate and [6-¹⁴C]glucose as precursors (data not shown).

Effects of 2-CC on Heat Shock Protein Synthesis. Hsp synthesis was examined over the range of 2-CC concentrations found to enhance hyperthermic cytotoxicity. Fig. 3 shows that hsp synthesis was essentially normal in both DMEM and in a comparable medium containing no glutamine or pyruvate. Exposure to 2-CC during heat shock suppressed hsp synthesis, but only at the highest concentration used (500 μM) and only in medium lacking glutamine and pyruvate. Synthesis of high-molecular-weight heat shock proteins, including hsp85, was inhibited to a greater extent than that of hsp70. Total protein synthesis, based on trichloroacetic acid-precipitable [³⁵S]-methionine incorporation, was markedly inhibited by 2-CC following heat shock and to a lesser extent at 37°C. However, this effect was similar in medium containing only glucose and in complete DMEM, i.e., 74 and 59% inhibition, respectively, after exposure to 500 μM 2-CC at 42°C.

**DISCUSSION**

The inference that an inhibitor of pyruvate metabolism should enhance hyperthermic cytotoxicity appears to be correct, since we now show that one such agent, 2-CC, is able to markedly enhance killing. The effect is most apparent when glucose is the only energy source, but it is also seen in complete DMEM containing glucose, glutamine, and pyruvate. From a practical standpoint, several points must be considered. For example, 2-CC was effective only at concentrations that were relatively high compared to the Kᵢ measured using isolated mitochondria (13). In fact, 150–500 μM is in the same range as the 200 μM concentration used to inhibit pyruvate transport.

![Fig. 1. Effect of 2-CC on the survival of L929 cells. Cultures were maintained in complete DMEM (A) or DMEM lacking glutamine and pyruvate (B). Points, mean cloning efficiencies of duplicate cultures heated at 42°C with the indicated concentrations of 2-CC relative to the corresponding controls maintained at 37°C. Absolute values of duplicates differed by 20% or less.](image1)

![Fig. 2. Sensitization of L929 cells to killing by exposure to 2-CC at various temperatures in DMEM lacking glutamine and pyruvate (see Fig. 1 legend).](image2)

**Table 1: Effects of 2-CC on L929 cell glycolysis and respiration**

<table>
<thead>
<tr>
<th>Condition</th>
<th>U-¹⁴C Glucose (nmol/mg protein/h)</th>
<th>[³⁵S]Pyruvate (nmol/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO₂, Lactate</td>
<td>CO₂, Lactate</td>
</tr>
<tr>
<td>37°C, control</td>
<td>12.4, 5.8</td>
<td>0.74, 22.4</td>
</tr>
<tr>
<td>42°C, control</td>
<td>15.6, 5.5</td>
<td></td>
</tr>
<tr>
<td>37°C, 500 μM 2-CC</td>
<td>4.9, 12.1</td>
<td>0.20, 20.3</td>
</tr>
<tr>
<td>42°C, 500 μM 2-CC</td>
<td>9.5, 13.5</td>
<td></td>
</tr>
</tbody>
</table>

* Medium lacked glutamine and pyruvate.

**Downloaded from cancerres.aacrjournals.org on April 12, 2017. © 1986 American Association for Cancer Research.**
What do the results tell us about the mechanism by which 2-CC increases hyperthermic cytotoxicity? Considering the protection afforded by pyruvate or other keto acids (4, 14) and the known action of 2-CC on pyruvate transport, this agent most likely interferes with a function of pyruvate that is essential for survival following heat shock. Although lactate production from pyruvate is increased by 2-CC when glucose is the sole energy source, even the increased rate is still relatively low since both glutamine and insulin are absent (15). Exogenous pyruvate enhances lactate production while protecting glucose-deprived cells against killing (4). These observations, taken together with the observation that 2-CC does not enhance lactate production when exogenous pyruvate is available, strongly suggest that elevated intracellular lactate levels are not responsible for 2-CC toxicity.

On the other hand, 2-CC markedly inhibits pyruvate oxidation, and a role for this effect in enhancing toxicity must be considered. Recent studies on the relationship between energy generation and thermosensitivity indicate that, while nearly total inhibition of ATP synthesis by either complete nutrient deprivation (16) or by simultaneous hypoxia and glucose deprivation (17) will sensitize cells to killing, neither ATP levels (18) nor energy charge (16) correlates well with thermal killing in complete medium. Thermosensitivity of respiration-defective mutants is also not increased (19, 20), and oxidation of the major respiratory substrates is not inhibited by heat shock (21). Thus, even though pyruvate oxidation must be supplying considerable energy in the absence of glutamine and the consequences of restricted energy production in the face of increased demand at high temperature could be especially severe, the preponderance of evidence suggests that sensitzation by 2-CC does not depend on inhibition of pyruvate oxidation. Since carbon derived from intramitochondrial pyruvate is also used for lipid synthesis and to supply intermediates for a variety of pathways, functions of intramitochondrial pyruvate related to these processes cannot be excluded.

Stimulation of hsp synthesis by pyruvate and inhibition by 2-CC suggest that a function of pyruvate is also involved in hsp induction. As was the case for hyperthermic cytotoxicity, the data do not support a role for pyruvate in hsp induction related to lactate synthesis or energy production. However, inhibition of hsp induction by 2-CC is consistent with the suppression by glucose deprivation and enhancement by pyruvate previously observed (4), so further work on the mechanism of the pyruvate effect seems justified. The relationship of hsp synthesis to heat resistance has received much attention and is relevant to the present study because 2-CC inhibits induction in medium that lacks glutamine and pyruvate. Under other conditions, i.e., in complete medium, 2-CC did not inhibit induction, so the correlation between hsp synthesis and thermosensitivity is not strong. This conclusion is consistent with studies showing that neither hsp levels (22, 23) nor hsp inducibility (24, 25) correlates well with intrinsic heat resistance, but does not preclude a role for heat shock proteins in heat-induced thermotolerance.

REFERENCES


Fig. 3. Effect of 2-CC on heat shock protein synthesis. Cultures were maintained in either complete DMEM or DMEM lacking glutamine and pyruvate (glucose only) and heated at 42°C for 2 h with the indicated concentration of 2-CC (µM) followed by [35S]methionine labeling, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiography. Equal cpm were loaded per gel lane.
2-Cyanocinnamic Acid Sensitization of L929 Cells to Killing by Hyperthermia

Hui Wang and Karl W. Lanks


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/10/5349

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.