Mechanistic Implications of the Induction of Thermotolerance in Chinese Hamster Cells by Organic Solvents

George M. Hahn, Esther C. Shiu, Brian West, Larry Goldstein, and Gloria C. Li

Department of Radiology, Stanford University School of Medicine, Stanford, California 94305 [G. M. H., E. C. S., B. W., L. G.], and Department of Radiation Oncology, University of California, San Francisco, California 94143 [G. C. L.]

ABSTRACT

Several organic solvents were shown to induce thermotolerance. These included: aliphatic alcohols (ethanol to octanol); local anesthetics; dimethyl sulfoxide; and dimethyl formamide. The minimum concentrations of the various alcohols required to induce tolerance were similar to the threshold doses for cytotoxicity. When only intramembrane concentrations were considered (by multiplying by the appropriate membrane:buffer partition coefficient), then the alcohols' threshold dose to induce tolerance was only mildly a function of carbon number. The efficiency increased almost linearly with a membrane dose from propanol to pentanol, but was slightly reduced for octanol. All alcohols induced tolerance rapidly (within a few hours after 30-min exposure), but the thermotolerance ratio decreased with increasing carbon number.

Of the three anesthetics examined, lidocaine was a highly efficient inducer, procaine less so, and tetracaine did not induce tolerance even at a concentration mildly cytotoxic. Dimethyl sulfoxide and dimethyl formamide induced tolerance without cytotoxicity, but the kinetics of doing so was different from that seen with the other solvents. Only cells exposed to these water-soluble agents for 18 h or longer showed evidence of induced heat resistance.

Our results indicate that membranes likely are the site for the initiation of solvent-induced cell killing and thermotolerance. The minimum dose of induction may be related to the degree of disorder induced by the individual alcohol, but the amount of tolerance induced is inversely related to this. The data on the effects of local anesthetics show that neither their common functional mode of inhibiting nerve transmission nor their similar level of cytotoxicity insures similar efficiency as tolerance inducers. Results with dimethyl sulfoxide and dimethyl formamide suggest that tolerance can be induced by more than one mechanism, or alternately, that a multiple-step mechanism can be activated at different points along this pathway.

We also measured heat shock protein synthesis after several treatments; efficient inducers of thermotolerance also induced these proteins.

INTRODUCTION

The transient resistance to heat induced by heat itself has been called thermotolerance (1). It has been shown to occur in culture systems (2–4) and in vivo in both normal tissue and tumors (5–8). Thermotolerance can even by induced to protect animals during whole-body exposure (9, 10). Furthermore, it can be induced, not just by heat itself, but also by various drugs; ethanol (11), sodium arsenite, and cadmium (12) have all been shown to induce resistance to heat. Curiously these latter agents, with the exception of ethanol, do not induce protection against their own toxicity (12). Little is known about the molecular events involved either in the induction of tolerance, nor in the events responsible for the protection against heat, although considerable evidence has accumulated that suggests the involvement of a certain class of proteins (usually termed heat shock proteins) (13, 14). It is, however, clear that at least two independent processes are involved in the induction of tolerance. These are (a) an initial event that occurs at all temperatures and (b) the expression of resistance, a process limited in most cell lines to temperatures of about 42.5°C or lower. Li and Hahn (15) have called these “triggering” and “development.”

Because so little is known about the molecular mechanisms relating to thermotolerance, we initiated a study designed to accumulate data on various organic compounds capable of inducing the phenomenon. Our goal was to investigate those agents that might throw light on the biochemical or biophysical events involved in the induction of tolerance. As part of this study, we examined the question of whether tolerance was necessarily associated with increased rates of heat shock protein synthesis (16).

Agents Examined

Alcohols. Based on the finding that ethanol induces tolerance (11), we decided to examine a range of alcohols with linear, increasing carbon numbers for their ability to do the same. There were two reasons for doing so. The first related to the question of where in the cell the initiating event occurs. It is well known that the octanol:water (or membrane:buffer) partition coefficient for alcohols changes rapidly with increasing carbon length (17). The alcohols having longer carbon chains are extremely hydrophobic and are therefore confined almost exclusively to lipid regions. We therefore thought that the concentration of alcohols required to induce tolerance would tell us whether the site of the initiating event is in the lipid-rich membranes or in the cytoplasm. If it were in the former then the molar dose of alcohol required for induction of tolerance should decrease appreciably as the carbon number increases. (This would be expected because the partition coefficient increases rapidly with increasing carbon number.) The reverse should be the case if the site is in the cytoplasm. As we will demonstrate, the molar concentration in the medium that is required to initiate tolerance is reduced as a function of alcohol length, but this reduction can be almost eliminated by normalizing concentrations by the appropriate partition coefficient (so that only the alcohol concentration within the lipids is considered). We therefore implicate membrane(s) as the site of the generation of the signals that lead to the development of
thermotolerance. The second reason for the use of aliphatic alcohols was that we thought it possible to determine the possible role of membrane disorder in initiating the trigger for thermotolerance. To examine this, we followed a suggestion that, although the amount of disorder introduced by aliphatic alcohols into membranes depends on the carbon number, the order parameter is not expected to increase monotonically. Beginning with propanol, as the alcohols' length increases, more disorder (per alcohol molecule) should be induced until the alcohols become so long that they would have a tendency to line up parallel to the phospholipids' carbon tails. Once that started to occur, the amount of disorder induced should then decline with additional length of the alcohol. Thus, if the amount of disorder were the key variable in determining tolerance induction, then the molar efficiency (within the lipid bilayer) for doing so should initially increase with carbon number, reach a maximum, and then decline. Here our data show that, while this hypothesis is tenable for cell killing, it does not hold for the induction of tolerance efficiency. The latter effect decreases monotonically with carbon number.

**Local Anesthetics.** Some drugs, such as the local anesthetics, interact in a specific way with heat (when cell killing is used as the assay). They appear to mimic heat in the sense that it is possible to equate quantitatively a certain heat "dose" with a dose of the anesthetic (18). We wanted to see if this equivalence could be extended to the induction of tolerance. Our answer appears to be negative. Of the three agents that we examined, only lidocaine induced tolerance efficiently. Procaine and particularly tetracaine were only mildly effective in doing so, and the kinetics of induction appeared to be different. To further test the drug-heat equality, we also examined whether heat could induce tolerance to the local anesthetics.

**Other Solvents.** We also looked at the ability of either DMSO or DMF to induce tolerance. Here we were asking whether a water-soluble solvent was as efficient in inducing tolerance as the far more lipophilic alcohols and anesthetics. We show that these two solvents are relatively slow inducers of tolerance; the kinetics of induction is much slower than either with heat or with alcohols.

### MATERIALS AND METHODS

**Cells and Culture Conditions**

Chinese hamster cells (HA-1) were maintained in Eagle's basic medium (GIBCO, Santa Clara, CA) supplemented with 15% fetal calf serum and antibiotics (penicillin and streptomycin). Three days before testing, approximately 100,000 cells were seeded in each 60-mm dish (Falcon) and kept in a humidified 37°C incubator with a mixture of 95% air and 5% CO₂. For alcohol studies, cells were plated in Corning T-25 flasks at the same density, and experiments were also performed on the fourth day.

In all experiments, medium was changed just before exposure of cells to either heat or drug. After each drug treatment, the monolayer of cells was rinsed at least twice with phosphate-buffered saline (GIBCO). The assay for cell survival was the cloning technique of Puck and Marcus (19). Each experiment was performed at least twice, and the results shown are the mean of all the trials.

**Heating and Thermotolerance Experiments**

The cells were heat treated in hot water baths, and pH was maintained with a mixture of 95% air and 5% CO₂ (20, 21). The actual temperature in the heat bath varied from the preset value by no more than ±0.1°C (20).

For heat-induced drug tolerance experiments, cells were pretreated at 45°C for 15 min and returned to a 37°C incubator for various lengths of time, followed by drug treatment at 37°C at the appropriate time. The sequence was reversed when drug-induced thermotolerance was studied. Cells were initially treated with drug at 37°C, and after adequate rinsing, they were returned to a 37°C incubator. At the appropriate time after 37°C incubation, cells were heated at 45°C, usually for 45 min.

**Drug Exposure**

**Alcohols.** Four alcohols were used: 1-propanol (J. T. Baker Chemical Co., Phillipsburg, NJ); 1-pentanol (Aldrich Chemical Company, Milwaukee, WI); 1-hexanol (J. T. Baker Chemical); 1-octanol (Aldrich Chemical Company). (From Ref. 17.)

### Table 1

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Threshold alcohol concentration in medium</th>
<th>Partition coefficient (membrane:buffer)</th>
<th>Threshold alcohol concentrations (m) in membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Propanol</td>
<td>7 × 10⁻⁴</td>
<td>0.45</td>
<td>3.2 × 10⁻⁴</td>
</tr>
<tr>
<td>1-Pentanol</td>
<td>5 × 10⁻²</td>
<td>3.6</td>
<td>1.8 × 10⁻¹</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>9 × 10⁻⁴</td>
<td>13.0</td>
<td>1.2 × 10⁻¹</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>1.6 × 10⁻³</td>
<td>151.8</td>
<td>2.4 × 10⁻¹</td>
</tr>
</tbody>
</table>

* From Ref. 17.

† Obtained by multiplying threshold concentration by partition coefficient.

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**INDUCTION OF THERMOTOLERANCE BY ORGANIC SOLVENTS**

**Chart 1.** The cytotoxicity of various alcohols at 37°C: 1-propanol (C₃); 1-pentanol (C₅); 1-hexanol (C₆); and 1-octanol (C₈). A, surviving fractions after 30-min exposure to graded doses of alcohols plotted against alcohol concentrations in medium. B, survival fractions after 30-min exposures plotted against alcohol concentrations in lipids. The membrane concentrations were obtained by multiplying the concentrations in A by the appropriate membrane:buffer partition coefficients (17).
The alcohol concentrations were chosen to yield survival values above 40%. Alcohol concentrations: 1-propanol, $6.8 \times 10^{-2}$ M; 1-pentanol, $5.2 \times 10^{-2}$ M; 1-hexanol, $8.4 \times 10^{-2}$ M; and 1-octanol, $1.8 \times 10^{-2}$ M.

Survival assays were then performed. Cells were returned to 37°C incubators. At the appropriate time, cells were heated at 45°C for 45 min only. Survival assays were then performed.

Chart 2. Thermotolerance induced by various alcohols. Cells were initially exposed to the indicated dose of alcohol at 37°C for 30 min. After adequate rinsing, the alcohol was removed. Experiments were performed on the same day. At the beginning of the experiment, medium was replaced with fresh medium containing DMF (or DMSO). The cells were then incubated at 37°C for various lengths of time. At the appropriate time, the medium containing DMF (or DMSO) was removed. Cells were rinsed and heated at 45°C for 30 min in Eagle’s medium supplemented with 15% fetal calf serum. Survival assays were then performed.

Radiolabeling and Gel Electrophoresis

Cells were labeled with 30 μCi of $[^{35}S]$methionine (specific activity, 1200 Ci/mmol; Amersham, Arlington Heights, IL) for 2 h at 37°C in minimal essential medium containing 1:100 normal levels of methionine. At the end of this period, cells were rinsed 3 times with cold phosphate-buffered saline and lysed with boiling sample buffer (22). Extracts were further boiled for 5 min, and equal numbers of cells were analyzed on a 10% polyacrylamide gel in the buffer system of Laemmli (23). Following electrophoresis, gels were fixed in 30% trichloroacetic acid, stained with Coomassie Blue G-250 in 3.5% perchloric acid, dried, and exposed directly to Kodak SB-5 X-ray film. Autoradiographs were developed by standard procedures. The levels of HSP synthesized during the 2-h exposure to $[^{35}S]$methionine were quantitated by comparing peak height on the radiograph to that of actin which remained unchanged by these treatments in our cells.

RESULTS AND DISCUSSION

The cytotoxicity at 37°C of various alcohols is shown in Chart 1A. The same set of data is redrawn in Chart 1B. There the cytotoxicity is plotted as a function of molar concentration within the cell (i.e., we have multiplied the concentrations in the medium by the appropriate partition coefficient). Clearly the molar concentrations of alcohols in the membranes necessary to cause dramatic killing (i.e., the “breaking” point of the survival curves) are only mildly a function of the number of carbon atoms in the alcohol chains (Table 1). Data for butanol and ethanol are not shown here; however, concentrations of butanol fall well within the appropriate range, but those for ethanol do not. Ethanol is metabolized intracellularly and, because of its solubility, is involved in many reactions. Thus it is not realistic to compare ethanol with the other alcohols.

The ability of these alcohols to induce tolerance is shown in...
INDUCTION OF THERMOTOLERANCE BY ORGANIC SOLVENTS

Chart 3. Thermotolerance induced by local anesthetics. Cells were initially treated with various anesthetics at the indicated concentrations for 1 h at 37°C, followed by 37°C incubation for various lengths of time in the absence of anesthetics. Cells were then exposed to 45°C for 45 min. Closed symbols (A, •, □), drug controls (no heat); ®, 45°C, 45 min only; open symbols, experimental points.

Chart 4. Heat-induced tolerance to anesthetics at 37°C. Cells were heated at 45°C for 15 min, followed by 37°C incubation. At appropriate time after incubation, cells were exposed to graded doses of the anesthetics for 1 h at 37°C and then plated for colony formation. Closed symbols (A, •, □), drug control (no heat); ®, 45°C, 15 min only; open symbols, experimental points.

Chart 2. While the rates of tolerance induction are similar, the magnitudes of tolerance, as measured by the ratio of additive to maximum survival, vary appreciably (Table 2). In these experiments, the concentrations of various alcohols to induce tolerance were chosen so that survival was at least 40% after the alcohol exposure at 37°C. These concentrations were the lowest that resulted in induction of maximum tolerance. As the carbon number increased from C3 to C8, the molar concentration required to initiate maximum thermotolerance was reduced from 0.67 M for propanol to 1.8 × 10^-3 M for octanol. This reduction can be almost eliminated when medium concentrations are normalized by the appropriate membrane:buffer partition coefficients. This result suggests strongly that the alcohol concentration in the lipid region is the key factor for both the induction of tolerance as well as for cell killing (Chart 1; Table 1). It would further implicate membrane(s) as the site of the triggering signal for thermotolerance, at least for these agents.

We also examined the relationships between alcohol concentration in the membrane, magnitude of thermotolerance, and degree of thermal sensitization. This is also shown in Table 2. When membrane concentrations are examined, cell killing efficiency and sensitization are inversely correlated; e.g., hexanol is the most efficient cell killer (Table 1) but the least efficient sensitizer, while propanol is the most efficient sensitizer and the least efficient agent for cell killing. For both of these effects, hexanol represents an extreme. The magnitude of the thermotolerance ratio correlates with neither of the two, falling monotonically from propanol to octanol. These results show that thermotolerance induction is not just a reflection of either cell killing or thermal sensitization.

Next we examined the possibility that local anesthetics induce thermotolerance. The results are shown in Chart 3. Of the three
INDUCTION OF THERMOTOLERANCE BY organic solvents

[Chart 5: Densitometer tracing from the autoradiograph of a sodium dodecyl sulfate:polyacrylamide slab gel of [3S]methionine-labeled proteins from HA-1 cells after an initial exposure to procaine (31 mw) and lidocaine (14 mw). Molecular weights (x10^-3) are shown at the bottom. Actin (M, 43,000) is indicated as A. Heat shock proteins with molecular weights of 70,000, 87,000, and 110,000 are indicated by arrows.]

MOLECULAR WEIGHT (kD)

Table 3: Rate of heat shock protein synthesis

<table>
<thead>
<tr>
<th>HSP (M)</th>
<th>HSP:actin&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lidoceine&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>70,000</td>
<td>0.28</td>
</tr>
<tr>
<td>87,000</td>
<td>0.39</td>
</tr>
<tr>
<td>110,000</td>
<td>0.31</td>
</tr>
<tr>
<td>Propanol&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Octanol</td>
</tr>
<tr>
<td>70,000</td>
<td>0.53</td>
</tr>
<tr>
<td>87,000</td>
<td>0.62</td>
</tr>
<tr>
<td>110,000</td>
<td>0.44</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are obtained by dividing magnitude of HSP peak by magnitude of actin peak on densitometer tracings obtained from autoradiographs of sodium dodecyl sulfate gels. Labelling was for 2 h beginning at the indicated time.

<sup>b</sup> The concentrations of anesthetics were the same as those used in Chart 5.

<sup>c</sup> The alcohol concentrations were the same as those used in Chart 2.

Overall the results of our study show the following. Cell killing and induction of thermotolerance by alcohols appear to be membrane-related phenomena. The degree of drug-induced membrane disorder may be important in determining whether tolerance is induced. The amount of tolerance, however, decreases monotonically with increasing carbon number and thus may not be related to disorder. Although all anesthetics show cell killing characteristics similar to those of heat and interact with heat to achieve "temperature shifts," the three anesthetics examined are not similar in their ability to induce either thermotolerance or HSPs. Lidocaine induces thermotolerance and HSPs effectively and rapidly, procaine induces tolerance only at the highest dose usable, and tetracaine does not. On the other hand, anesthetics we studied, all sensitized to the heat exposure (18), but only lidocaine unequivocally induced tolerance at all the concentrations used. In the case of procaine, development of tolerance was seen only at doses higher than 30 mw. No thermotolerance was induced by tetracaine over the dose range studied. The three doses were again chosen because they induced similar cell killing (see control values in Chart 3). Thus the data show a segregation of the efficiency of these drugs for cell killing versus tolerance induction.

Chart 4 shows the results of an experiment designed to measure heat-induced resistance to anesthetics (i.e., the reverse of the experiment depicted in Chart 3). Lidocaine tolerance was seen after a pretreatment of 15 min at 45°C, followed by 37°C incubation in excess of 8 h, although only at the higher test doses. Tolerance to procaine, on the other hand, was seen only at the highest dose. There was only a hint of tolerance to tetracaine at the highest dose usable. These data show that not only do the local anesthetics induce heat tolerance but that heat induces tolerance against at least two of the anesthetics. This finding suggests that the same mechanism is at least partially responsible for cell killing by heat and by local anesthetics, again very likely at the level of a membrane, and confirms an earlier finding of Yau (24).

We also performed experiments to examine synthesis of HSP after exposure of cells to local anesthetics and to alcohols (Chart 5; Table 3). We only examined the cells exposed to anesthetics at one time point (0 h). At that time, lidocaine had induced HSPs while procaine had not. Additionally propanol induced increased synthesis of these proteins, both immediately and 4 h after exposure; octanol did so also, but at reduced levels. Thus the agents that more efficiently protected cells against heat also induced HSPs more efficiently. Octanol, a weaker inducer of tolerance, induced a lesser amount of HSP synthesis, while procaine had induced neither tolerance nor HSPs at the time point we studied (2 h after drug exposure).

The ability of DMSO and DMF to induce tolerance is shown in Chart 6. In these experiments the solvent was present continuously until the heat exposure. Solvent exposures shorter than 8 h were ineffective in inducing tolerance. Chart 6 shows that the cells exposed to DMSO or DMF for about 10 h or longer did develop thermotolerance at both concentrations tested. The protection against heat by exposures to solvents is too great to be attributable to cell cycle effects; furthermore thermotolerance persists even after proliferation is resumed. The solvents are slow inducers of thermotolerance, and this tolerance induction occurs in the absence of cell killing (drug toxicity has a survival fraction above 80%).

Overall the results of our study show the following. Cell killing and induction of thermotolerance by alcohols appear to be membrane-related phenomena. The degree of drug-induced membrane disorder may be important in determining whether tolerance is induced. The amount of tolerance, however, decreases monotonically with increasing carbon number and thus may not be related to disorder. Although all anesthetics show cell killing characteristics similar to those of heat and interact with heat to achieve "temperature shifts," the three anesthetics examined are not similar in their ability to induce either thermotolerance or HSPs. Lidocaine induces thermotolerance and HSPs effectively and rapidly, procaine induces tolerance only at the highest dose usable, and tetracaine does not. On the other hand,
heat induces tolerance both against itself and against at least two of the anesthetics. We have no suggestion as to why the anesthetics behave so differently from each other. These findings suggest that cell killing and the process of induction of thermotolerance do not necessarily involve similar "targets," although they do not rule out such a possibility. The data involving the water-soluble DMSO and DMF show that the kinetics of tolerance induction varies greatly, depending on the inducing agent. Obviously some DMSO or DMF also enters the membrane, so we can't claim that these solvents act necessarily via cytoplasmic action. Nevertheless this last finding raises the possibility that the "trigger" leading to induction of tolerance (or perhaps tolerance itself) has associated with it more than one mechanism of action, depending upon the particular inducing agent. This latter finding is consistent with data that show that, at physiologically relevant concentrations, steroid hormones whose receptors are located in the cytoplasm are also capable of inducing thermotolerance.4

ACKNOWLEDGMENTS

We thank Dr. J. Trudell for suggesting the relationship between order parameter and carbon number and Ine Van Kersen for the use of DMSO and DMF data.

REFERENCES

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