Influence of Rate of Heating on Thermosensitivity of L1210 Leukemia: Membrane Lipids and Mr 70,000 Heat Shock Protein

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ABSTRACT

We examined the effect of rate of temperature rise on the thermosensitivity of a murine lymphoblastic leukemia. L1210 cells suspended in RPMI 1640 medium:5% fetal bovine serum at pH 7.4 were heated from 37°C-42°C, 43°C, or 44°C over variable times (immediately, 30, 60, 120, 180 min) in a circulating water bath controlled by an electronic temperature programmer. Survival of the cells using a soft agar clonogenic assay was plotted against the time at final temperature so that a \( D_0 \) (min of heat required to reduce survival by 63% on the exponential portion of the survival curve) could be calculated as an estimate of thermosensitivity. Cells heated from 37°C-42°C over a time period of 30 min (10°C/h) were less thermosensitive (\( D_0 62.7 ± 12.5 \) min) as compared to those exposed immediately to 42°C (\( D_0 38.5 ± 2.2 \) min). Cells heated over a period of 180 min (1.6°C/h) showed almost no death even after 4 h at 42°C. Thermosensitivity of cells heated to several other high temperatures was also a function of rate of heating. This relative thermal resistance induced by slow heating was not a result of a change in membrane cholesterol content or fatty acid composition. Similarly, there was no difference between cells heated at slow and fast rates in cell cycle distribution or in cellular protein concentration. The major heat shock protein of \( M_r 70,000 \), which was induced by immediate heating, was not synthesized at the same high rate 1-12 h after heating treatment by the cells made thermotolerant with slow heating. We conclude that the thermosensitivity of this neoplastic cell can be altered considerably by the rate of heating. This alteration is not due to a change in membrane lipids. Furthermore, the heat shock protein at \( M_r 70,000 \) which was synthesized after immediate heating could not be demonstrated in the gradually heated L1210 leukemia cells.

INTRODUCTION

The sensitivity of mammalian cells to hyperthermia can be decreased by changes in many factors. These include higher pH of the environment (1, 2), position in \( G_1 \) or \( G_2 \) phase of the cell cycle (3, 4), exponential growth phase (4, 5), presence of serum (5), and decrease in proportions of cellular polyunsaturated fatty acids (6, 7).

Sensitivity to heat can also be decreased by prior heat conditioning. The effect of this prior heat that is a function of its scheduling and fractionation (8). In this regard there are several ways of inducing thermoderterotolerance. First, thermoderterotolerance can be induced by continuous heating at temperatures below 43°C and usually appears after about 3 h of heating (2, 9). Second, cells which have been acutely conditioned by heating to temperatures greater than 43°C and then incubated at temperatures nearer to 37°C are resistant to subsequent hyperthermia between 43.5°C and 45°C (10, 11). In spite of considerable study, the mechanism of thermoderterotolerance is unknown. It has been speculated that a change in membrane fluidity is an early event in the development of thermoderterotolerance (12). The subject of thermoderterotolerance has been reviewed (8, 12).

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Herman et al. (13) reported that the heat sensitivity of Chinese hamster ovary cells and a fibroblast line was a function of the rate of delivery of the heat. By delivering the heat at a slow rate, a thermoderterotolerant population of cells could be developed. This method of bringing about thermoderterotolerance differs from the other techniques and merits further study, because of its pertinence to the manner by which heat is delivered clinically. Since it is important to know how generalized it is, we studied this method of inducing thermoderterotolerance. For these studies we chose a neoplastic cell, since original studies were on cell lines derived from normal tissues. We explored the possibility that a change in membrane lipids is the mechanism of this type of heat resistance. In this regard it is well known that poikilotherms adjust their membrane lipids as the temperature increases in order to prevent lipid fluidity from increasing to a level incompatible with membrane function (14, 15). Finally, we examined whether HSPs develop synchronously with thermoderterotolerance induced by a slow heating gradient. Data from other investigators (16, 17) had demonstrated a temporal relationship between the development of thermoderterotolerance and HSP.

MATERIALS AND METHODS

Cell Culture. L1210 murine leukemia cells in suspension culture were grown at 37°C in medium consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY), 5% FBS (KC Biological, Inc., Lenexa, KS), and gentamicin sulfate (40 \( \mu \)g/ml; Gibco) in a humid atmosphere of 5% CO2:95% air. Cells were reseeded into fresh medium every 3 days.

Hyperthermia and Survival Determination. Exponentially growing cells were resuspended at a concentration of 2.5 \( \times 10^6 \) cells/ml in flasks containing 10 ml of heating medium consisting of RPMI 1630 and 5% FBS. pH was adjusted to 7.4. The flasks with tightened caps were completely submerged in a water bath at the specified temperature. Cells which were in suspension were heated from 37°C-42°C, 43°C, or 44°C over times which varied from immediately to 3 h. Cells were then maintained at hyperthermic temperature for various experimental times, after which they were quickly transferred to a 37°C water bath to terminate the hyperthermia exposure. Aliquots were taken immediately for cloning, lipid analysis, or flow cytometry studies. Immediately heated cells attained a temperature of 42°C in 5.7 ± 0.3 (SE) min (n = 4). A circulating water bath (Neslab Instruments, Inc., Portsmouth, NH), which is capable of maintaining the desired temperature within ±0.2°C, was used in all experiments. Rate of temperature change was controlled by an electronic temperature programmer (Model ETP-3; Neslab Instruments). Temperatures were measured using a thermometer calibrated in tenths of a degree against a National Bureau of Standards-certified thermometer (Fisher Scientific Co., Pittsburgh, PA).

The fraction of cells surviving the heating interval was determined using a soft-agar clonogenic assay, as described previously (7). Büchers, cells were suspended in medium consisting of RPMI 1640, 20% horse serum, gentamicin sulfate (40 \( \mu \)g/ml), and Bacitracin agar (0.3 g/100 ml; Difco Laboratories, Detroit, MI) and plated onto 10- x 35-mm tissue
culture dishes. After the agar was solidified, dishes were incubated at 37°C for 1 wk at which time the number of colonies consisting of at least 50 cells was counted using an inverted microscope. In this assay, a linear relationship exists between number of cells plated and number of colonies formed. The cloning efficiency of unheated cells was 62%.

Lipid Analysis and Cellular Fatty Acid Modification. Cells were washed 3 times in phosphate-buffered saline and extracted using CHCl₃;CH₃OH (2:1, v/v) (18). Neutral lipids and phospholipids were separated using silicic acid chromatography (19). Each fraction was then saponified for 60 min at 56°C in 1.2 N KOH and 80% ethanol (20). Fatty acids in the saponifiable fraction were methylated for 10 min at 95°C with 14% BF₃;CH₃OH (21), and the methyl esters were separated by gas chromatography using a 1.8-m column packed with 10% SP2330 on 100/200 mesh Chromosorb (Supelco, Inc., Bellefonte, PA). Peak areas were quantitated and identified by comparison of retention times to those of standards obtained from Supelco.

Membranes were isolated using a modification of previously reported methods (22). Aliquots of 10⁶ cells were disrupted in 10 ml of an isotonic homogenizing medium containing 0.5 M hexylene glycol, 1.5 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM Na₃PO₄ by forcing 4-ml aliquots through a 25-gauge needle (1.6 cm long). Following centrifugation at 10,000 x g for 1 min to remove nuclei and unbroken cells, the supernatant was placed on a discontinuous sucrose gradient (30 and 45%, v/v) and centrifuged at 24,000 x g for 30 min, using a Beckman L3-40 ultracentrifuge with the 10-30% interface was collected, diluted with saline, and centrifuged at 45%, v/v) and centrifuged at 17,500 x g for 30 min. The fraction at 24,000 x g for 30 min, using a Beckman L3-40 ultracentrifuge with the 10-30% interface was collected, diluted with saline, and centrifuged at 17,500 x g for 30 min. The fraction at the 10–30% interface was collected, diluted with saline, and centrifuged at 24,000 x g for 30 min, using a Beckman L3-40 ultracentrifuge with a SW-27 swinging bucket rotor to yield a crude plasma membrane preparation. Phospholipid (23), cholesterol (24), and protein (25) content were determined. The cellular fatty acids of LI210 cells were modified by growing the cells for 2 days in RPMI 1640 containing 5% FBS supplemented with 32 /¿Ci/mmol; 25 ¿iCi/ml; New England Nuclear Corp., Boston, MA) Li²¹₀ cells were heated from 37°C-42°C at various rates in a precision water bath with programmable temperature control. Survival after 3 h at 42°C was measured in a soft agar clonogenic assay. Points, mean of at least 4 determinations; bars, SE.

We next determined whether the relationship of thermosensitivity to rate of heating in LI210 cells occurs when cells are heated to higher temperatures. Fig. 2 shows survival curves of cells heated to 43°C either immediately or over a period of 3 h. While less dramatic than those at 42°C, differences in cell

To evaluate the effect of rate of heating on thermosensitivity at 42°C, LI210 cells were heated to 42°C at various rates (Fig. 1). When cells were heated immediately to 42°C, less than 1% of the cells survived a subsequent 4-h exposure at 42°C. In contrast, there was almost no cell death after 4 h at 42°C when cells were heated slowly (1.6°C/h) over a 3-h period. Cells heated to 42°C at an intermediate rate (10°C/h) were intermediate in thermosensitivity. Table 1 shows the effect of various rates of heating from 37°C–42°C on thermosensitivity of the cells after 3 h at 42°C. There was a progressive decrease in thermosensitivity as compared to immediately heated cells at each of the four slower rates of heating.

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Survival was measured using a clonogenic assay. The exponential portion of the survival curve, was higher for the cells heated at slower rates to test temperatures at 42°C and 43°C, the time to 90% death is longer for those cell cultures heated more slowly. At a test temperature of 44°C, the difference was no longer evident.

We next explored the mechanism of the relationship of rate of heating to thermosensitivity. Since an inverse relationship of cell cholesterol content and heat sensitivity has been reported in a series of mammalian cell lines (28), we examined cholesterol in the isolated plasma membranes of L1210 cells heated immediately or slowly to 42°C (Table 3). The molar ratio of cholesterol to phospholipid in the plasma membrane of cells heated immediately and cells heated over a period of 3 h was not significantly different either at the time of reaching test temperature or for up to 2 h thereafter.

We next examined the fatty acid composition of the phospholipid fraction of cells which had undergone various heat treatments (Table 4). It is known that modification in the fatty acid composition of the L1210 cell changes its heat sensitivity (7), and therefore an acute change in the proportions of polyunsaturated and saturated fatty acids could be the mechanism for thermotolerance. The major fatty acids of the L1210 cells were 18:1, stearate (18:0), and palmitate (16:0). This distribution is similar to that found in our earlier studies (7). The percentage of palmitoleate (16:1) showed a small but significant increase in all heated cells compared to unheated cells, and the rate of heating or duration of exposure did not affect this increase. Otherwise heating itself had no effect. There was no change in all heated cells compared to unheated cells, and the rate of heating or duration of exposure did not affect this increase. Otherwise heating itself had no effect. There was no change in all heated cells compared to unheated cells, and the rate of heating or duration of exposure did not affect this increase. Otherwise heating itself had no effect. There was no change in all heated cells compared to unheated cells, and the rate of heating or duration of exposure did not affect this increase. Otherwise heating itself had no effect. There was no change in all heated cells compared to unheated cells, and the rate of heating or duration of exposure did not affect this increase. Otherwise heating itself had no effect. There was no change in all heated cells compared to unheated cells, and the rate of heating or duration of exposure did not affect this increase. Otherwise heating itself had no effect. There was no change in all heated cells compared to unheated cells, and the rate of heating or duration of exposure did not affect this increase. Otherwise heating itself had no effect. There was no change in all heated cells compared to unheated cells, and the rate of heating or duration of exposure did not affect this increase. Otherwise heating itself had no effect.
difference in the relative proportion of the fatty acids of the L1210 phospholipids after slow heating as compared to immediate heating (Table 4). Therefore, the development of thermal resistance of this type is not mediated by a change in fatty acid composition.

We desired to further define the role of fatty acids in this process. If membranes are involved in the development of thermotolerance, it seemed possible that prior modification of a major structural feature of the membranes would have an effect on the ability to adapt to heat exposure. We have previously demonstrated that enrichment of the phospholipids of the L1210 leukemia is associated with a marked increase in sensitivity to hyperthermia temperatures (7). Therefore, we modified the fatty acid composition of the cellular lipids of L1210 leukemia cells by growing the cultures in medium supplemented with either 22:6 or 18:1 (7). This results in a 30% or greater enrichment in 22:6 or 18:1. The enriched cells were then heated gradually (30 min) from 37°C-42°C and tested for subsequent thermosensitivity. As seen in Table 5, cells enriched with either fatty acid developed thermotolerance. Furthermore, there was no difference in cells enriched with the polyunsaturated fatty acid 22:6, which we have previously shown markedly increases heat sensitivity in nonthermotolerant cells, and those enriched with 18:1.

Whole cell protein contents of the L1210 cells which were heated immediately to 42°C versus cells heated slowly to 42°C (1.6°C/h) were not significantly different after a subsequent 1 h at that temperature [100 ± 1 (SE) μg/10⁶ cells versus 94 ± 7 μg/10⁶ cells; n = 3-4; P = not significant]. Similarly, the protein content was not different in the slowly versus immediately heated cells which were maintained at 42°C for shorter or longer periods of time.

We also examined the effects of slow and fast heating on the distribution of cells in the cell cycle. Since cells in G₁ or G₂ phase of the cell cycle are less sensitive to heat, a shift in the phase distribution could explain the change in heat sensitivity manifested by the cells which were heated at a slow rate. There was no difference in the percentage of cells in G₁ (immediately heated cells, 51 ± 1, versus gradually heated cells, 52 ± 4), S (42 ± 2 versus 45 ± 4), or G₂-M (7 ± <1 versus 4 ± 1).

We next examined how long the thermotolerance induced by gradual heating persists. For these studies L1210 cells were heated from 37°C-42°C over 180 min to induce thermotolerance, and then thermosensitivity was determined at multiple times from 0-110 min during subsequent incubation at 37°C. Thermosensitivity at each time point was tested by heating immediately to 42°C for 2 h and measuring survival in a clonogenic assay. For a control, the clonogenic survival of nonheated control cells (Lane A), immediately heated (Lane B), or slowly heated (Lane C) cells. Arrow indicates the position of the M, 70,000 HSP.
Table 6  Quantitation of new protein synthesis following heat treatment

Following heat treatment, L1210 cells were kept at 37°C for 2 h prior to the
addition of [35S]methionine for 1 h in a methionine-free RPMI 1640 medium.
Proteins were extracted, analyzed by electrophoresis using a gradient
SDS/polyacrylamide slab gel, and visualized by fluorography. Bands were quan-
tititated by densitometry scan. For comparison with the HSP (M, 70,000), we
chose the nearest peak on densitometry tracing (M, 73,100) and a typical distant
one.

<table>
<thead>
<tr>
<th>Heating technique</th>
<th>Time to 42°C (h)</th>
<th>Protein synthesis (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time at 42°C (h)</td>
<td>Mf</td>
</tr>
<tr>
<td>Immediate</td>
<td>1</td>
<td>3.9 ± 0.9b</td>
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<tr>
<td></td>
<td>3</td>
<td>2.5 ± 0.2</td>
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<tr>
<td></td>
<td>3</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>Unheated control</td>
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<td>2.6 ± 0.1</td>
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a Percentage of total protein synthesis rate.
b Mean ± SE of 3–4 separate determinations of the rate of protein synthesis as a percentage of total rate.
c —, not detected.

HSP at the same high rate. Enhanced synthesis failed to appear at
multiple time intervals at 37°C up to 24 h after heating. Two other proteins were chosen for comparison. We selected the
closest major peak on the densitometry tracing (M, 73,100) and
an arbitrary distant peak. The synthesis of neither of these non-
HSP was affected by the different heating techniques (Table 6). Studies of the synthesis of HSP by cells heated from 37°C–
43°C at various rates were similar to the 42°C studies. These
data indicate that there was little synthesis of HSP at the times
and conditions studied in the thermotolerant as compared to
thermosensitive cells.

DISCUSSION

The dependency of thermosensitivity on the rate of tempera-
ture change is an important biophysical relationship. Our studies demonstrate this important relationship in the L1210
lymphoblastic leukemia, a neoplastic cell line which is fre-
quently used as a model for cancer experimental therapeutic
studies. A better understanding of the cellular defenses against
hyperthermia should yield important clues to the mechanism
and site of heat damage. Rate of heating has a major effect on
extent of heat damage. Therefore, we studied the mechanism
by which the rate of heating affects thermal sensitivity. Our
studies examined several possibilities to explain this relation-
ship. Membrane cholesterol content is a major determinant of
membrane fluidity (29, 30). In studies of Chinese hamster ovary
cells and fibroblasts, it was reported that cells heated rapidly to
hyperthermic temperatures had a phasic rise and fall in whole
cell cholesterol as compared to cells heated slowly which had
no change in cholesterol (13). However, in that study total cell
cholesterol was measured. Membrane cholesterol is likely to be
the determinant of cellular physical properties and response to
heat. Therefore, we performed subcellular fractionation and
determined the cholesterol:phospholipid ratio of membrane
preparations. There was no difference in membrane cholesterol
of cells heated slowly or rapidly to temperature. Likewise, there
was no change in the fatty acid composition of the cellular
phospholipids in this study which is another major determinant
of membrane physical properties and sensitivity to heat (7, 31).
Perhaps most importantly, prior fatty acid modification,
which is known to alter the physical properties of cellular
membranes, had no effect on the induction of thermotolerance.
Taken together, these observations indicate that changes in
membrane lipids are not likely to be the mechanism of the
relationship of rate of heating and thermal sensitivity. Studies

of the membrane lipid composition of thermotolerant Chinese
hamster ovary fibroblasts or Ehrlich ascites cells showed no
alterations of cholesterol or fatty acids to explain decreased
heat sensitivity in those cells (32, 33). However, it should be
noted that in these studies the cells were made thermotolerant by techniques other than slow heating rates.

The thermal resistance which appears after slow heating has
been studied in another neoplastic cell. MTC rat mammary
adenocarcinoma cells heated slowly (180 min) from 37°C–42°C
were only slightly more resistant than immediately heated cells
upon reaching target temperature, and this difference was mainly
due to an effect on the shoulder of the survival curve (34). After a subsequent 3–4 h, the Do after slow heating was
about twice that of the immediately heated cells. The thermal
resistance induced in the L1210 cells in our studies is greater
in comparison to immediately heated cells (Do larger by a factor
of 10) and occurs earlier.

The rate of heating had a particularly strong effect on heat
sensitivity at 42°C, which is the temperature used in previous
studies (13, 34). We have shown that the relationship of heat
sensitivity to rate of delivery of heat persists at even higher
temperatures but disappears at 44°C, at which temperature
90% of the cells are dead after 30 min regardless of the rate of
heating. It seems likely that the extent of damage caused by
continued exposure to 44°C may limit the cell in mounting a
protective response.

There has been considerable interest in the group of specific
cellular proteins whose enhanced synthesis is stimulated by
heat, chemical, or physical stress (35, 36). These HSP have
afforded a model for the study of transcriptional or translational
events; however, their biological function remains obscure.
There exists one clue to their function; they are often closely
associated with the development of thermotolerance (16, 37–
39). Landry et al. (16, 40) have shown a temporal relationship
between the appearance and decay of thermotolerance and HSP
in Morris hepatoma cells. This correlation holds for various
temperatures, time of exposure, and sequences of heating (40).
Li et al. have shown a similar correlation of induced thermal
tolerance and HSP synthesis in Chinese hamster ovary cells
(17), fibroblasts (38), and in vitro murine tumor models. Based
on this type of study, it has been suggested that HSP may
represent protective molecules for heat exposure. Using data
from studies of tissue culture cells, rat normal tissues, and
murine tumors, Li has suggested that the levels of M, 70,000
HSP can be used as an assay to determine thermal sensitivity
during fractional hyperthermia (41, 42).

Because of the importance of this question of the relationship
of thermotolerance and HSP to thermobiology, we undertook
studies of the expression of HSP by a neoplastic murine cell
line which manifests a marked thermotolerance with gradual
heat delivery. We found that L1210 cells which were made
thermotolerant by gradual heating failed to manifest to any
great extent the M, 70,000 HSP which was expressed after heat
shock of nonthermotolerant cells. Other investigators have had
similar findings. Landry and Chretien (40) reported that sodium
arsenite induced HSP, but not thermotolerance, and conversely
that cyclohexamide induced thermotolerance but inhibited HSP
synthesis in Morris hepatoma cells. Widelitz et al. (43) reported
that in rodent fibroblasts the M, 68,000 HSP, in contrast to
those with molecular weights of 70,000 and 89,000, was not
synthesized in thermotolerant cells. In studies of a rat mamma-
mary carcinoma, Tomasovic et al. (34) demonstrated HSP synthesis
during the expression of thermotolerance brought about by
procedures utilizing a rapid temperature transient. In contrast,
when thermotolerance was induced using a slow temperature rise, the synthesis of several HSP was less 2 h after reaching 42°C than by cells heated rapidly.

We have shown that gradually heated and thermotolerant L1210 cells have no appreciable HSP synthesis at times that rapidly heated cells synthesized a M, 70,000 protein (1–12 h after the end of heat treatment). This might suggest that the synthesis of the most obvious HSP is not a necessary condition for the development of heat tolerance. However, our experimental design did not include measurements of HSP synthesis at early time points, such as during the gradual heating process itself. In this regard, Tomasovic et al. (44) found that slowly heated MTC cells had higher rates of synthesis of M, 112,000 and 90,000 HSP over the first 2 h after reaching 42°C as compared to rapidly heated cells. At later time points, rapidly heated cells had equal or higher rates of synthesis of most HSP. Therefore, it is possible that we failed to detect a HSP synthesized briefly during the time of gradual heating. In any case, these studies provide information on the similarities and differences in membrane lipids and HSP synthesis between thermotolerant and thermosensitive neoplastic lymphoid cells.

REFERENCES


RATe OF HEATING AND THERMOSENSITIVITY
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