Induction of Heat Shock Proteins by Canavanine in *Tetrahymena*

NO CHANGE IN ATP LEVELS MEASURED IN VIVO BY NMR*

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During induction of the heat shock response by temperature jump in the protozoan *Tetrahymena*, a decrease in cellular ATP levels occurs within minutes and cells become thermotolerant. Treatment of *Tetrahymena* with the amino acid analog canavanine also induces synthesis of heat shock proteins, but more slowly than by temperature jump. No changes in cellular ATP levels were observed during the course of canavanine induction of heat shock protein synthesis measured in vivo by the technique of 31P NMR spectroscopy. *Tetrahymena* do not become thermotolerant following induction of heat shock protein synthesis with canavanine. However, *Tetrahymena* will develop thermotolerance in the presence of canavanine if they are first subjected to a nonlethal temperature jump before exposure to a normally lethal temperature.

Following exposure of cells to a rapid sublethal temperature jump, there are immediate changes in cellular physiology. These changes define the universal and well-conserved response, known as the heat shock response, which is remarkably similar in both eukaryotes and prokaryotes (1-4). During induction of the response, there is a rapid change in cellular transcription patterns which results in the synthesis of a specific set of proteins: the heat shock proteins (hsp's). There are also associated changes in protein synthesis patterns. Heat shock proteins range in molecular mass from over 100,000 to approximately 10,000 daltons, of which the most well-characterized is the 70,000-dalton hsp (hsp70). Synthesis of hsp is also induced following exposure of cells to a variety of other agents, including amino acid analogs, release from anoxia, some inhibitors of oxidative phosphorylation or electron transport, and in higher eukaryotes during some viral infections. This broad list of agents suggests that hsp are more properly designated stress proteins.

The exact functions of the heat shock response and the induced proteins have not been defined. The universal appearance of the response and induced hsp following heat stress suggests that they play a fundamental role in cellular homeostasis. It is possible that hsp may be involved in the phenomenon of acquired thermotolerance. This correlation is suggested by the observation that a mild temperature jump, sufficient to induce hsp synthesis, will protect cells for some period of time from a subsequent severe temperature jump that would otherwise be lethal (5, 6). While, in general, the acquisition of thermotolerance after heat shock is observed in all stages of development and in all tissues of an organism, there are exceptions. For example, in early development of *Drosophila* (7) and sea urchins (8), a temperature shock does not induce synthesis of hsp and these embryos die. In the ciliated protozoan *Tetrahymena*, a moderate temperature jump is lethal during the first hour following transfer to starvation media if the ionic strength of the starvation media is greater than 60 mM (9).

The cellular response to a sublethal temperature jump occurs within minutes, but the response to agents other than heat generally requires longer incubation periods. Most agents capable of inducing hsp, regardless of the kinetics involved, also cause cells to become thermotolerant (6, 10). Amino acid analogs represent an interesting exception. Treatment with analogs slowly induces production of hsp, but thermotolerance is not observed (6, 11). It has been presumed that the reason cells fail to become thermotolerant is that hsp synthesized in the presence of amino acid analogs are not functional (12). However, in *Tetrahymena* treatment with cycloheximide or emetine at low concentrations, which transiently inhibit protein synthesis, can confer thermotolerance under conditions in which hsp are not synthesized (13). At this time the basis of cellular thermotolerance is not known, and protein synthesis may not even be required (11, 14).

Using the noninvasive technique of 31P nuclear magnetic resonance, we have been studying, in vivo, the heat shock response in *Tetrahymena*. We have previously demonstrated that a major decrease in cellular ATP levels accompanies induction of the response and synthesis of hps (15). The change occurs within 3 min following heat shock, which is before synthesis of hps is observed. We have now investigated the effects on ATP levels in *Tetrahymena* treated with the arginine analog canavanine. Although hps are induced by this treatment, no significant changes in ATP levels were observed. In addition, cells do not develop acquired thermotolerance following induction of hps with canavanine, unless they are also given a nonlethal temperature shift.

**MATERIALS AND METHODS**

*Cells—* *Tetrahymena thermophila* (strain B7) was grown to midlog phase (2 X 10⁶ cells/ml) at 30 °C in 2% proteose peptone, 0.1% yeast extract, 0.2% glucose, and 0.003% sequestrin. Cells were harvested by gentle centrifugation and washed and resuspended at a concentration of 7 X 10⁶ cells/ml in 20 mM MES, 1.0 mM sodium phosphate, pH 6.0 (MES buffer) (15). Concentrated cultures (30 ml) were maintained for approximately 2 h at 30 °C with shaking at 250 rpm in 250-ml culture flasks before NMR measurements were made. Cell viability was monitored by microscopy.

*Canavanine Treatment—* Canavanine (Sigma) was added to con-
centrated cultures to a final concentration of 1 mM. In some cases, canavanine was removed by collecting the cells by gentle centrifuga-
tion, washing them in MES buffer, recentrifuging the cells, and resuspending them in MES buffer containing 1 mM arginine.

Proteins—Proteins were labeled with high specific activity H-amino-acids (Amersham Corp.) at 50–100 μCi/ml in MES buffer. Whole cell protein was prepared as described (15, 16). Equal volumes of lysates (approximately 1 × 10⁶ cells) were subjected to electropho-
resis on 10% SDS-polyacrylamide gels (17), and fluorographs were prepared using Autofluor (National Diagnostics).

NMR—NMR measurements were made in a Bruker WH-360-WB NMR spectrometer operating at 145.78 MHz in pulse-Fourier trans-
form mode. Spectra B were accumulated every 6 min as the sum of 300
free induction decays arising from 60 °C tipping pulses applied every 1.2 s. Samples of 14 ml of concentrated cell suspension were placed in a 20-mm NMR tube capped with an aeration manifold (18). A mixture of 95% O₂, 5% CO₂ was bubbled through the cells at a rate well above the Kₜₜ for O₂ uptake. The temperature of the probe housing the NMR tube was set at 30 °C. After 1 h of accumulation of control spectra, a sample of cells was removed for labeling with H-amino-acids. Canavanine was then added to the NMR tube to 1 mM, and NMR spectra were accumulated for an additional 2 h. A second sample of cells was removed from the NMR tube for labeling with H-amino-acids 15 min after canavanine was added. Cells were labeled under conditions similar to those used for spectra accumulation.

Thermotolerance—For thermotolerance experiments, Tetrahy-
mena were concentrated as described for NMR measurements. After resuspension at approximately 7 × 10⁶ cells/ml, the cultures were maintained at 30 °C for 2 h with shaking at 200–250 rpm. Samples of 2–4 ml were then placed in 50-ml culture flasks, shaken as before, and treated as described in Table I. For temperature shifts, flasks were placed in water bath shakers maintained at either 40, or 43–43.5 °C. Cells were examined by microscopy to determine their viabili-
ty. Swimming cells, or cells that recovered their ability to swim upon return to 30 °C, were considered viable (13).

RESULTS

Induction of Heat Shock Proteins by Canavanine—Following exposure of Tetrahymena cells to the arginine analog canavanine, synthesis of proteins with electrophoretic mobil-
ities similar to those induced by a rapid temperature jump was observed (Fig. 1). The proteins induced by either cana-
vanine or temperature jump are referred to as hsps. Treatment with 100 μM canavanine is sufficient to induce a low level of synthesis of hsps. At 1 mM canavanine, synthesis of hsps is detectable following 30 min of treatment, and after 1.5–2 h of exposure to the analog, hsp80 and hsp73 are the most prom-
inently labeled proteins in the cell. Synthesis of hsps declined after 3 h of treatment to levels that are similar to those observed at 1 h of treatment. Cells treated in the same way, but without addition of canavanine, do not show synthesis of hsps.

The general level of synthesis of non-heat shock proteins does not appear to be changed by treatment with canavanine (Fig. 1). The transient induction of hsp synthesis in response to canavanine is similar to the normal heat shock response in Tetrahymena, although longer in duration. In T. thermophila a temperature jump from 30 to 41 °C results in rapid synthesis of hsps with maximum synthesis occurring from 15 to 30 min after the shift. However, after 1 h at the elevated temperature, the protein synthesis pattern has returned essentially to pre-
heat shock conditions.

Canavanine Does Not Confer Thermotolerance—As shown in Table I (lines 2 and 3), following a 45-min exposure to a 40 °C temperature jump, greater than 95% of the cells sur-
vived a 60-min upshift to 43 °C. Less than 0.1% of the cells 
without a 40 °C pretreatment survived a similar exposure to 43 °C. If instead of a 40 °C heat shock, cells were pretreated with 1 mM canavanine for 110 min at 30 °C to produce maximum synthesis of hsps, a subsequent shift to 43 °C for 60 min was lethal. Continuous treatment with canavanine at 30 °C did not affect cell viability (Table I, lines 4 and 5).

The possibility that canavanine pretreatment can confer thermotolerance if cells are subsequently permitted to syn-
thesize functional heat shock proteins was then tested. Cells were treated with canavanine for either 65 min, or 110 min, at 30 °C, washed, and resuspended in 1 mM arginine prior to the shift to 43 °C. As shown in Table I (lines 6 and 7), this treatment did not increase the survival of cells. Only those cells shifted to 40 °C for 45 min prior to incubation at 43 °C survived the higher temperature (Table I, line 8). Pretreat-
ment with canavanine at 30 °C for 110 min before shifting the cells to 40 °C and subsequently to 43 °C also did not affect cell survival (Table I, line 10).

The alternate possibility that canavanine has no effect on the acquisition of thermotolerance was also examined. Cells maintained continuously in canavanine survived at 43 °C if pretreated at 40 °C, just as well as cells grown without cana-
vanine (Table I, line 9).

The rate at which cells die following a shift from 30 to 43 °C was also determined. Cells treated as described in Table I (lines 3, 5, 7) were examined by microscopy at 10-min inter-
vals following transfer to 43 °C. At 30–40 min following transfer, cells had ceased swimming and did not recover if returned to 30 °C. The presence or absence of canavanine had no effect on the rate of cell death in these three cultures. It is clear for Tetrahymena that, during the first 2 h of treatment with canavanine, neither the ability of cells to become ther-
mortolerant nor the rate at which they die if not thermostoler-
ant are affected.

ATP Levels during Canavanine Treatment—Intracellular ATP levels in Tetrahymena measured in vivo decrease im-
mediately following a rapid temperature jump and remain at the lower level for at least 1 h if the cells are maintained at the elevated temperature (15). Both the induction of hsp synthesis and the decrease in ATP occur within minutes after such a temperature jump. By comparison, synthesis of hsps

Fig. 1. Time course of induction of hsps by canavanine in Tetrahymena. Concentrated cell suspensions were treated with 1 mM canavanine in the presence of H-amino-acids. Samples were removed at the indicated times, and whole cell protein was prepared. Equal volumes of cell lysates were analyzed on 10% SDS-acrylamide gels. Cells were labeled with H-amino-acids under the following conditions: A and B, no canavanine: A, 30 °C, 1 h; B, SanC, 1 h, standard heat shock; C–H, 30 °C with 1 mM canavanine for: C, 0–15 min; D, 0–30 min; E, 0–60 min; F, 0–90 min; G, 0–120 min; H, 0–180 min; I, 0–180 min, without canavanine. Molecular masses of hsps are indicated.

2 R. L. Hallberg, personal communication.
ATP in Canavanine-treated Cells

TABLE I

Thermotolerance ± canavanine

Temperatures at which cells were tested (30, 40, or 43–43.5 °C) and the length of time they were kept at the temperatures are shown. The letters A or C next to the temperature indicate whether 1 mM arginine or 1 mM canavanine, respectively, was added to the culture.

| Treatment                      | Time (min) | Survivors
|-------------------------------|------------|----------------|
|                               | 0-65       | 66-110        | 111-170 | 171-230 | Survivors
<table>
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<tr>
<td>1 Control</td>
<td>30°C</td>
<td>30°C</td>
<td>30°C</td>
<td>30°C</td>
<td>100%</td>
</tr>
<tr>
<td>2 Acquired thermotolerance</td>
<td>30°C</td>
<td>40°C</td>
<td>43°C</td>
<td>30°C</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>3 Thermotolerance</td>
<td>30°C</td>
<td>30°C</td>
<td>43°C</td>
<td>30°C</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>4 Control + canavanine</td>
<td>30°C</td>
<td>30°C</td>
<td>30°C</td>
<td>30°C</td>
<td>100%</td>
</tr>
<tr>
<td>5 Thermotolerance + canavanine</td>
<td>30°C</td>
<td>30°C</td>
<td>43°C</td>
<td>30°C</td>
<td>&lt;0.1%</td>
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<tr>
<td>6 Thermotolerance ± canavanine</td>
<td>30°C</td>
<td>30°C</td>
<td>43°C</td>
<td>30°C</td>
<td>&gt;95%</td>
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<tr>
<td>7 Thermotolerance ± canavanine</td>
<td>30°C</td>
<td>40°C</td>
<td>43°C</td>
<td>30°C</td>
<td>&gt;95%</td>
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<tr>
<td>8 Acquired thermotolerance ± canavanine</td>
<td>30°C</td>
<td>30°C</td>
<td>43°C</td>
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<td>&gt;95%</td>
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* Cultures were examined by microscopy, and survivors (swimming cells) were estimated immediately after return to 30 °C and again at several later times. No differences in survivors between these times were noted. In addition to showing no movement, cells that did not survive had a distinctly different appearance than survivors including a rounder shape. Cell lysis also occurred in these cultures.

When thermotolerance is examined at the high cell densities used to obtain NMR spectra (7 × 10⁶ cells/ml), greater than 95% survival is observed. At lower cell densities (2 × 10⁶ cells/ml), cells did not survive this treatment. The reason for this difference in survival is not known, but may be related to the differences in cell densities relative to the canavanine concentration.

Fig. 2. Protein synthesis and ³¹P NMR spectra in control and canavanine-treated Tetrahymena. Spectra were accumulated for 1 h (control), canavanine was added to 1 mM and spectra were accumulated for an additional 1 h (canavanine). P₃, inorganic phosphate; PA, phosphoarginine; SP, sugar phosphates. Portions of cell samples used to obtain NMR spectra were removed before and 15 min after initiating canavanine treatment and were labeled with ³H-amino-acids. Whole cell protein was extracted and analyzed on SDS-acrylamide gels. A, heat shock reference, 40 °C, 1 h; B, cells treated with 1 mM canavanine, 1 h at 30 °C; C, control cells, 1.25 h, 30 °C.

in Tetrahymena following exposure of cells to canavanine follows a more gradual time course (Fig. 1). We have measured in vivo ATP levels in Tetrahymena during the course of canavanine induction. In contrast to induction by temperature jump, we find no significant change in ATP levels during canavanine treatment.

Representative spectra corresponding to 1 h of accumulation for control and canavanine-treated cells are shown in Fig. 2. The spectra demonstrate the relative intensities of the phosphate resonances of ATP in the cells under these conditions and show no appreciable changes during canavanine treatment. That the canavanine treatment did, in fact, induce hsp synthesis in cells used to obtain NMR spectra was determined by removing a small portion of cells from the NMR tube and labeling them with ³H-amino-acids at the same time and under the same conditions that were used to acquire spectra. As expected, only treatment with canavanine induced synthesis of hsps (Fig. 2).

Although spectra representing 1 h of accumulation showed no change, it was possible that a transient decrease in ATP levels occurred, followed by recovery to pre-heat shock levels. The data suggest that this did not occur. In Fig. 3 the relative intensities of the α, β, and γ peaks of ATP are presented as a function of time for both untreated cells and cells exposed to

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ATP in Canavanine-treated Cells

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1 mM canavanine. Compared to the intensities of the ATP peak in control cells, there are no differences in the intensities of these three peaks during the course of canavanine treatment. Of the three, the intensity of the resonance peak corresponding to the β-phosphate of ATP is the most indicative, because it is unique to the nucleoside triphosphates and, as such, is the most accurate for measuring intracellular ATP levels (19). Its intensity clearly remains unchanged during incubation with canavanine relative to control cells. Both the α and γ peaks also contain contributions from other metabolites (e.g. ADP and NADPH) that are not resolved under these conditions of measurement (19). However, these peaks also show no appreciable variation during this treatment. In contrast, the intensity (peak height) of the β-phosphate of ATP dropped 55% at 30 min after a temperature jump from 30 to 40 °C (15).

The data do indicate that there is a slight decrease in cellular ATP levels in both control and canavanine-treated cells during the 2-h period examined. As these cells had been shifted from logarithmic growth phase in complete medium to starvation medium 2 h before we began measuring ATP levels, this continued slight decrease in ATP is probably related to the overall lower metabolic activity found in starved cells (20). Although there are slight differences in the ATP levels in canavanine-treated cells compared to control cells, we do not think the differences are significant. The data shown for control and canavanine-treated cells were accumulated on different preparations of cells and not successively on the same culture. The variations shown here between control and canavanine-treated cells lie in the same range as observed between successive measurements of ATP levels in either control or experimental cells.

**DISCUSSION**

In *Tetrahymena*, a sublethal heat shock causes transient synthesis of hsp's and reduction of total protein synthesis.
Total protein synthesis begins to return to pre-heat shock patterns after about 1 h. Induction by canavanine is also transient in nature, and hsp synthesis appears to be imposed over the normal pattern of protein synthesis, reaching a maximum level after 1.5–2 h. By 3 h synthesis of hsps is declining, and synthesis of non-heat shock proteins continues apparently unchanged. In this respect, the transient response to canavanine in *Tetrahymena* is unlike the response observed in mammalian cells in which synthesis of hsps continues for at least 24 h in the presence of amino acid analogs [3, 21].

The data presented here suggest that induction of hsps by canavanine has no effect on the development of acquired thermotolerance in *Tetrahymena*. In this study, *Tetrahymena* demonstrate thermotolerance only if they are first subjected to a moderate temperature jump before exposure to a normally lethal temperature. If during the 40 °C pretreatment cells are incubated in 1 mM canavanine, there is no subsequent effect on survival following a shift to 43 °C. This demonstrates that thermotolerance can be induced even when presumably aberrant hsps are being synthesized and suggests that protein synthesis may not be necessary for the development of acquired thermotolerance. Similar observations have previously been reported for yeast [11] and *Tetrahymena* [13], using both amino acid analogs and cycloheximide treatments, although failure to develop thermotolerance in yeast following cycloheximide treatments has also been described [22].

Hsps displaying aberrant properties [12] and the failure of cells to become thermotolerant are restricted to induction by amino acid analogs. For this reason, it has been presumed that hsps become nonfunctional following incorporation of the analogs and, consequently, cells fail to become thermotolerant. The data presented here and that from previous reports [11, 13] suggest that the synthesis of functional hsps and the acquisition of thermotolerance by cells may not be coupled. The role that hsp70 cognate proteins [14, 23] play in the response and the effects of analogs on the cognates are not known.

Following imposition of a sublethal temperature jump on *Tetrahymena*, ATP levels decrease within 3 min and then remain at the new steady state level while the cells are maintained at the elevated temperature [15]. In contrast, we found no change in cellular ATP levels in *Tetrahymena* during the course of canavanine induction of hspsynthesis. As the decrease in ATP following a temperature jump occurs before new hsps are synthesized, the lack of change in intracellular ATP observed following canavanine treatment cannot result from synthesis of nonfunctional hsps. This further suggests that the process of amino acid analog induction of hspsynthesis differs from induction by temperature jump.

Our data demonstrate that neither transient fluctuations nor stable changes in ATP levels occur following induction of hspsynthesis by canavanine. It is, of course, possible that during the gradual induction of hsps by canavanine the rate of ATP turnover is slightly increased, but that under these conditions cells are able to accommodate this new rate by increased synthesis, resulting in no net change in ATP levels. Such a change would not be observed by our measurements.

It is possible that changes in ATP levels are a fundamental part of the pathway leading to a functional heat shock response in which cells acquire thermotolerant properties. The absence of a change in ATP levels following canavanine treatment may be an additional indication that incorporation of amino acid analogs results in an aberrant response. It is also possible that decreases in ATP levels reflect undefined secondary effects that follow induction of a heat shock response. ATP metabolism during treatment with other slow inducers of the response that also confer thermotolerance on cells, such as ethanol, has not yet been examined. The role cellular energetics and ATP levels play in the pathway leading to a functional heat shock response remains unclear.

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REFERENCES