Induction of Thermotolerance and Heat Shock Protein Synthesis in Normal and Respiration-deficient Chick Embryo Fibroblasts

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ABSTRACT

Normal and transformed chick embryo cells and their respective ethidium bromide-treated derivatives that are devoid of a functional respiratory chain were comparatively evaluated for their responses to hyperthermia treatment. No significant difference was found between the control and the respiration-deficient cells. The cells have a similar intrinsic thermosensitivity as judged by their capacity to form colonies after treatment at supraoptimal temperatures, and heat triggers in both cases an equal production of heat shock proteins and a strong induction of thermotolerance. In addition, sodium arsenite, carbonyl cyanide m-chlorophenylhydrazone, oligomycin, and antimycin A induce a similar heat shock protein response in the control and the treated cells. Based on these results, it is concluded that the inhibition by heat of the mitochondrial energy production does not constitute an obligatory rate-limiting event in hyperthermic cell killing and that the intracellular signal triggering development of thermotolerance or heat shock protein production does not obligatorily originate from damages to the respiratory chain. Moreover, the results indicate that the modifications responsible for the increased heat resistance of thermotolerant cells may not, or do not necessarily, involve a stabilization of the mitochondrial energy production.

INTRODUCTION

Cells from various organisms have been shown to develop a transient thermal resistance in vitro as well as in vivo after exposure to an initial treatment at supraoptimal temperatures (5, 9, 27, 29, 31, 44). In mammalian cells, this phenomenon is called thermotolerance and is recognized as a potentially important factor influencing the tumor response to hyperthermia and thus the effectiveness of thermotherapy. Despite an extensive characterization of the time and temperature dependence for the induction, development, and decay of thermotolerance (9, 19, 23), there is very little known concerning the nature of the signal triggering this cellular response nor concerning the particular biochemical or structural modifications responsible for the transformation of a cell from a normal state of thermosensitivity to a state of thermotolerance. Recently, the HSP, a family of proteins induced in response to a variety of chemical or environmental stresses (1, 39, 44), have been proposed to play an active role in this process (reviewed in Ref. 17). This is mainly based on the observations of a temporal correlation between the accumulation kinetics of some of these newly synthesized proteins and the development kinetics of thermotolerance (18-20, 24, 26, 29, 42, 43) and of a close relationship between the kinetics of degradation of some HSP and the kinetics of decay of thermotolerance (18, 29). Other similarities in the temporal and temperature dependence of the 2 phenomena were presented (19, 25, 27), and Minton et al. (30) proposed a general mechanism by which cellular molecules and structures might be protected against heat denaturation by HSP.

Several experimental results favor the idea that the mitochondria are the primary targets from which originates the intracellular signal generated by heat and responsible for HSP induction (1). Indeed, a number of chemicals and experimental conditions which disturb the respiratory metabolism have been reported to induce HSP. Leenders et al. (21) proposed that the HSP are part of the nuclear-mitochondrial interaction mechanisms controlling the respiratory metabolism. More direct evidence was presented by Sin (41), who reported that an extract from heat-treated mitochondria, but not from control mitochondria, induced HSP gene activity when injected intracellularly.

Mitochondria have also been suggested as a probable target of hyperthermia cell killing and for thermotolerance development. Inhibition of respiration (3, 32) and direct effect on mitochondrial oxidative phosphorylation (2) have been shown to be an early event in cells exposed to elevated temperature. Moreover, 5-thio-D-glucose (13) or deprivation in glucose (14, 40) potentiates hyperthermia cell killing. Also, Li and Werb (26) and Sciandra and Subjeck (40) demonstrated that cells recover from hypoxia or glucose deprivation with an increased resistance to heat. Have- man and Hahn (8) presented data suggesting that the availability of energy might be an important factor in determining the ability of cells to maintain viability at elevated temperatures. Their study further suggested that thermotolerant cells can sustain glucose deprivation longer than control cells, perhaps indicating that cells in the thermotolerant state use energy in a more economical way. In addition, Lunec and Cresswell (28) reported recently an increased ability of thermotolerant cells to maintain their intracellular ATP level when challenged with hyperthermia treatment.

In the work reported in this paper, we examined in a comparative manner the hyperthermia responses of normal and respiration-deficient cells with the aim of evaluating the participation of functional mitochondria in the induction of HSP and thermotolerance. The results revealed no significant difference in the heat response of the cells, therefore bringing into question the idea that these phenomena are induced in response to respiratory stress or required a functional respiratory chain to occur.

MATERIALS AND METHODS

Cell and Culture Conditions. CEF cells were prepared by trypsin treatments of 8- to 9-day-old White Leghorn embryos (35) and were

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2 Scholar of the Medical Research Council of Canada. To whom requests for reprints should be addressed, at Centre de Recherche sur le Cancer, Hôtel-Dieu de Québec; 11, côte du Palais, Québec, Canada G1R 2J6.
3 The abbreviations used are: HSP, heat shock protein; CEF, chick embryo fibroblasts(s); EB, ethidium bromide; SDS, sodium dodecyl sulfate; CCCP, carbonyl cyanide m-chlorophenylhydrazone.

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cells, a clone originating from a transformed cell line that spontaneously arose from a culture of CEF cells (11), were obtained from Dr. O-R. Kaaden, Hanover, Germany. These cells were maintained in conditions similar to the CEF cells except that 15% fetal bovine serum was used in place of the standard serum complement described above.

Preparation of the Respiration-deficient Cells. CEF and H32 cells were rendered respiration deficient by a chronic exposure to EB (0.4 µg/ml) following the procedure described by Morais and coworkers (33, 35, 36). CEF cells were incubated in the presence of EB for a minimum of 3 weeks starting at the time of the first subculture. This resulted in the irreversible loss, down to less than 1 copy per cell, of mitochondrial DNA (34) and a consequential auxotrophy for pyrimidines, disappearance of cytochrome oxidase activity, 95% reduction in oxygen consumption, and complementary increase in the glycolytic metabolism (6, 33, 35, 36). It was concluded from these studies that the respiratory chain in the EB-treated cells had come to a halt and that the residual 5% oxygen consumption, which is also rotenone and antimycin A resistant, was related to cellular metabolic pathways utilizing molecular oxygen, like the mixed-function oxygenases. Furthermore, as shown in Chart 1 for one of the cell preparations used in this study, the EB-treated cells (Trace B) lack the usual absorption bands of the mitochondrially coded cytochromes b (563 nm) and aa3 (603 nm) and remain respiratory deficient after removal of EB from the growth medium (Ref. 35; Chart 1, Trace C). The respiration-deficient cells were returned to the standard EB-free medium 3 days before administration of hyperthermic treatments and left in this medium during colony formation. Respiration-deficient H32 cells were obtained following a similar procedure. In the present study, we used a clone (designated CF7) derived from a H32 cell culture grown in the presence of EB for 60 days. A detailed characterization of this clone has been performed. It is also devoid of mitochondrial DNA and possesses respiration-deficient characteristics similar to the EB-treated CEF cells. 4

Relative Cell Survival. Cell survival was determined by counting the relative number of individual cells capable of proliferating at 37°C into colonies greater than approximately 50 cells within 8 to 12 days. All survival data were corrected for the plating efficiency of the respective control nonheated cells (15 to 20%).

Hyperthermia Treatments. Heat treatments were administered by immersing the flasks (25 sq cm) containing the attached cells plus 5 ml of medium into a 30-liter water bath thermoregulated at ±0.05°C. Under these conditions, a temperature equilibrium between the medium and the water bath was reached within 5 min.

Analysis of HSP Synthesis. The newly synthesized proteins were labeled by incubating the cells in medium containing 25 µCi of [35S] methionine per ml (New England Nuclear; translation grade, 1000 Ci/ mmol). At the end of the incubation, the cells were washed 3 times in 0.85% NaCl solution (saline), removed from the dishes with a rubber policeman in the presence of 1% SDS in water, and passed through an 18-gauge syringe needle. The SDS extracts were boiled for 3 min at 100°C and then precipitated with 4 volumes of absolute ethanol at −20°C overnight. The ethanol-insoluble material was dissolved in a sampling buffer made of 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), and 0.01% bromophenol blue. Electrophoresis was carried out in 0.75-mm-thick, 10% polyacrylamide gel slabs of Laemmli (16). The gels were dried, and the labeled polypeptides were detected by autoradiography on X-Omat AR-5 films (Kodak). In all cases, constant amounts of proteins were loaded on the top of each gel track.

RESULTS

Survival and Thermotolerance Response of Normal versus Respiration-deficient Cells. The survival response of control CEF cells to treatments at temperatures of 45°C and 47°C is shown in Chart 2. CEF cells show a very high resistance to these treatments. At 47°C, a D0 value (time to reduce the survival in the exponential portion of the curve to 1/e) of 83 min is calculated, which corresponds to D0 values generally reported with mammalian cells for treatment temperatures in the range of 42–43°C. This thermal characteristic probably reflects the evolutionary adaptation of the chicken to a normal body temperature 4–5°C higher than the body temperature of mammals. Also shown in Chart 2 is the survival response of the respiration-deficient CEF cells to treatments at 45°C and 47°C. The results reveal no apparent differential thermosensitivity between the 2 cell types.

We next evaluated the ability of these cells to become thermotolerant in response to a mild heat treatment. The cells received a first treatment of 30 min at 47°C or 37°C; they were returned to 37°C for 5 h to allow for thermotolerance development and, then, reexposed to 47°C for graded periods of time (0 to 4 h). The results shown in Chart 3 indicate that the initial 30-min treatment at 47°C induced both in the control and in the respiration-deficient cells a marked increase in thermal resistance. The amplitude of the effect observed is similar to that reported in a variety of other cell lines (19, 23).

A similar comparative evaluation of survival responses was performed for the H32 versus the CF7 cells. Again, as shown in

* P. Desjardins and R. Morais, manuscript in preparation.

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THERMOTOLERANCE AND HSP IN RESPIRATION-DEFICIENT CELLS

Chart 2. Relative survival of normal (○, △) and respiration-deficient (O, ▽) CEF following treatments at 45 and 47°C.

Chart 4, the control and respiration-deficient cells show no major differential characteristics in terms of intrinsic thermosensitivity or thermotolerance induction.

**HSP Response of Normal versus Respiration-deficient Cells.** As shown in Fig. 1, heat treatments of 30 min at 45°, 46°, and 47°C induced in the respiration-deficient and in the control CEF cells a strong and identical pattern of HSP with M, of 89,000, 70,000, 35,000, and 29,000. Moreover, no major difference in the induction kinetics of HSP was observed between the 2 cell types. In both cases, the autoradiogram of protein labeled between 0 and 2.5 h after the end of treatment was apparently identical to that obtained when labeling was performed between 2.5 and 5 h. The pattern of HSP obtained here is identical to the one described before for CEF cells (10, 12, 39). No difference was observed either in the HSP pattern induced in H32 versus CF7 cells by a 47°C treatment (Chart 4).

We next used the respiration-deficient CEF cells to test for the specificity of various chemicals, known as inducers of HSP, the action of which is believed to be primarily at the level of the respiratory metabolism. Sodium arsenite, CCCP, antimycin A, and oligomycin were found to induce HSP in EB-treated as well as in control CEF cells (Fig. 2). Among these chemicals, arsenite is by far the most potent HSP inducer, inducing at 25 μM a HSP pattern similar to that induced by heat, while affecting very little the level of the general protein synthesis as judged from the relative intensity of the autoradiograms. An interesting feature observed in both cell types as the concentration of arsenite is increased up to 750 μM is the progressive loss of inductiveness of the HSP with M, of 35,000, concomitant with the appearance of a new species with M, of 42,000. CCCP at 100 μM also induced HSP. However, it completely inhibits general protein synthesis at 300 μM and failed to induce any species at 10 μM, a concentration already 2 times higher than that sufficient to completely disrupt the mitochondrial electrochemical gradient in these cells (data not shown). A similar situation is also observed with antimycin A, which starts to induce some HSP at 300 μM, whereas, in these cells, it inhibits respiration maximally already at 5 nM (33), and with oligomycin, which only weakly induces HSP at 300 μM and 20 ng/ml suffice to inhibit respiration in these cells (33). In the conditions tested here, sodium azide failed to induce any species at concentrations up to 30 μM (data not shown).

DISCUSSION

In this study, we comparatively analyzed the heat responses of normal and transformed CEF and their respective EB-treated derivatives that lack mitochondrial DNA and are devoid of a functional respiratory chain. The results revealed no major difference between control and respiration-deficient cells in their responses to hyperthermia treatments. The cells have a similar intrinsic thermal sensitivity, and moreover, heat triggers in all cell
THERMOTOLERANCE AND HSP IN RESPIRATION-DEFICIENT CELLS

Chart 4. Heat responses of the control and the respiration-deficient H32 cells. Left and center, survival responses. H32 (left) and H32-CF7 (center) cells were first exposed to a conditioning treatment of 30 min at 47°C (O) or 37°C (•) and then subjected to a test treatment at 47°C for the time indicated. Right, HSP induction. The cells were subjected to a 30-min treatment at 47° or 37°C and incubated for 5 h in the presence of [35S]methionine. Total cellular proteins were extracted and processed as described in "Materials and Methods" to obtain the autoradiogram. The arrowheads indicate the position of the HSP (see the legend of Fig. 1).

types a similar induction of HSP and thermotolerance. Three conclusions can be deduced from these findings.

The first conclusion concerns the role of the energy produced by the mitochondria in the maintenance of cell integrity after hyperthermia or, in other words, the importance of alterations induced at the respiratory energy production level in determining the intrinsic cellular thermal sensitivity. A few studies have looked before at the effect of hyperthermia on respiration in either whole cells or isolated mitochondria. Mondovi et al. (32) and Dickson and Oswald (3) demonstrated a rapid inhibition of respiration and loss of the Pasteur effect in hepatoma and breast adenocarcinoma cells upon treatment at 43° and 42°C, respectively. Apparently compensating for the inactivation of respiration, the aerobic glycolysis was strongly stimulated after hyperthermic treatments, whereas the anaerobic glycolysis was little modified. Christiansen and Kvamme (2) observed that isolated mitochondria were even more sensitive to hyperthermia than mitochondria heated in situ. Rapid inhibition of electron transport, loss of respiratory control, and uncoupling of phosphorylation were noted, and the authors concluded in agreement with Morris and King (37) that the cytochrome segment was probably the most heat-sensitive part of the respiratory chain. These studies suggested a role of central importance for respiration in the thermal inactivation of proliferative capacity but could not conclude as to whether cessation of respiration was the causative factor of cell death in heat-damaged cells. In the hypothesis, that the direct thermal inactivation of the energy-producing pathways is responsible for cell death after hyperthermia, the present comparative study between normal and respiration-deficient cells can allow the determination of whether respiration or glycolysis is the supporting energy-producing pathway after treatment. If glycolysis is the more sensitive pathway, then control cells should survive better being able to rely on respiration for their energy requirement after potentially lethal heat shocks. Conversely, if respiration is the more sensitive pathway, then normal and respiration-deficient cells should survive equally well, inactivation of glycolysis being in both cases the rate-limiting event. The results presented in Charts 2 and 4 revealed a similar thermo-sensitivity between control cells and cells lacking a functional respiratory chain. This indicates that, in these cells, the inhibition by heat of respiratory energy production does not constitute an obligatory rate-limiting event in hyperthermic cell killing, and it must be concluded that, in heated cells, the energy requirement can be met by either the basal cellular energy reserve or the energy produced by the more heat-resistant glycolysis pathway. However, it has not been proven that heated cells die owing to a direct thermal inactivation of their energy-producing pathways, and consequently, it cannot be concluded that inactivation of glycolysis is the rate-limiting event in hyperthermic cell killing. On the contrary, preliminary studies indicate that glycolysis-deficient cells are not more sensitive to heat than respiration-deficient or control cells, suggesting that neither pathway is very sensitive to direct thermal inactivation.

The second conclusion of this work concerns the possible role of the energy derived from the mitochondria in the development of elevated heat resistance in the cells which survive a first hyperthermic treatment. Heat-induced thermotolerance is believed to proceed via a triggering signal, resulting most likely from an initial sublethal damage or a metabolic perturbation, which in turn generates a series of metabolic processes that aim
THERMOTOLERANCE AND HSP IN RESPIRATION-DEFICIENT CELLS

at preparing the cells to eventually better sustain a subsequent heat treatment (19, 23). Our recent suggestion, that HSP induction might result from such a metabolic activation and might be involved in the induced protective mechanism, makes the mitochondria probable candidates for being the target from which originates the initial triggering signal (19). Indeed, in spite of the lack of a demonstration for a causal relationship (17, 19, 45), there is evidence that HSP are involved in the development of thermotolerance as well as a large body of studies suggesting that their induction originates from mitochondrial perturbations or in response to disturbances in the energy homeostasis (1, 7, 17–22, 24–27, 41–45, 47). The hypothesis, that the intracellular signal for thermotolerance development comes from damage induced to the mitochondria, becomes even more plausible with the above finding that cessation of respiration does not constitute a rate-limiting event in cell killing. Indeed, a rate-limiting event could not trigger such a process, because it occurs by definition only in dying cells. The nature of the intracellular target(s) which is protected during thermotolerance is also unknown. However, the stabilization of the mitochondrial structures or metabolisms is a possible way by which it may be hypothesized that cells increase their resistance to heat. Indeed, Haveman and Hahn (8) have presented data that suggested that the availability of energy plays an important role in the inactivation of cells after hyperthermia and that one reason cells die after being exposed to supraoptimal temperatures may be a lethal lack of energy. This is further supported by the study of Lunec and Cresswell (28), who demonstrated that thermotolerant cells have an increased ability to maintain their intracellular ATP level when challenged with hyperthermic treatments. If this hypothesis is true, it means that the stabilization of any of the energy-producing pathways would be sufficient to protect the cells. The results presented in Charts 3 and 4, indicating that thermotolerance can be induced in both control and respiration-deficient cells, clearly demonstrate, however, that cessation of respiration does not constitute in CEF the exclusive signal capable of triggering the development of thermotolerance. Moreover, thermotolerance is expressed at a similar level in both cell types, indicating that thermotolerance in these cells does not result from an induced heat-resistant, respiratory energy-producing machinery or depends necessarily on respiratory energy for development.

The final conclusion emerges from the data presented in Chart 4 and Fig. 1, revealing that hyperthermia yields a similar induction of HSP in the control or the respiration-deficient cell lines. This indicates that the proteins are not synthesized exclusively in response to a respiratory stress or to a shift from an aerobic to an anaerobic type of metabolism, as suggested previously (1, 7, 21), and that there exist in the cell other types of heat-inducible alterations that converge to the production of HSP. Other workers have reported previously a lack of correlation between a drop in the ATP level in treated cells and the induction of HSP (15, 46). For example, arsenite was found to increase the synthesis of HSP under conditions that caused no concomitant drop in ATP concentration (46). It has been suggested that arsenite, because it is not mitochondrion specific and because probably it also provokes a number of other alterations in cellular homeostasis by binding sulfhydryl groups, may induce HSP by a route other than inhibition of mitochondrial oxidation. The results presented in Fig. 2 confirm this suggestion and indicate that, similarly to heat, the action of arsenite in relation to HSP does not involve perturbations at the level of the production of mitochondrial energy. This seems also to be the case with CCCP, antimycin A, and oligomycin. (a) Similarly to arsenite and heat, these agents induce HSP in respiration-deficient as well as in control cells. (b) They induce HSP at a concentration much higher in all cases than that required to induce mitochondrial perturbations.

More studies are needed, however, before concluding that the mitochondria are not involved in thermal sensitivity, HSP induction, and thermotolerance development. Besides energy production, mitochondria accomplish a number of other essential cellular functions which may not be impaired in the respiration-deficient cells studied here. For example, it has been reported that mitochondria from the yeast rho+ mutants, which lack a functional respiratory chain, can still maintain an electrochemical gradient, a function that seems to be required for mitochondrial protein assembly (4, 38). Preliminary results, using cationic fluorochromes and flow cytometry, indicate that this function also exists in the respiration-deficient chick cells used here. As noted above, however, this particular mitochondrial function does not seem to be involved in HSP induction, since CCCP disrupts the electrochemical gradient at a concentration 20 times lower than that required to induce HSP.

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Fig. 1. Heat induction of HSP synthesis in CEF. Autoradiogram of the SDS-polyacrylamide slab gel of total proteins extracted from control (CONT) or respiration-deficient (EB) CEF. In A, the cells were incubated at 37°C for 5 h in the presence of [35S]methionine after a treatment of 30 min at the indicated temperature. In B, the cells were incubated for 2.5 h in the presence of the 35S precursor at 0 or 2.5 h following a 30-min treatment at 45°C or (Track C) for 2.5 h after a sham treatment at 37°C. The arrowheads indicate the position of the HSP. From top to bottom: M, 89,000; 70,000; 35,000; and 29,000.
Fig. 2. Effect of respiration-disturbing agents on HSP synthesis in control (A) and respiration-deficient (B) CEF. The cells were first incubated for 2 h in medium containing 25, 250, or 750 μM sodium arsenite (As); 10, 100, or 300 μM CCCP; 10, 100, or 300 μM antimycin A (ANTI A); or 5, 50, or 150 μg oligomycin (OLIGO) per ml. They were then incubated in the presence of [35S]methionine for 5 h at 37°C and processed for electrophoresis and autoradiography. The arrowheads indicate the position of the HSP (see legend of Fig. 1). →, an additional M, 42,000 polypeptide induced at a high concentration of arsenite.
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