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THERMAL SENSITIVITY AND RESISTANCE OF INSULIN-RECEPTOR BINDING IN THERMOTOLERANT CELLS

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Cells may be made extremely resistant to elevated temperatures (thermotolerant) by a mild heat shock a few hours prior to more rigorous heating. In the present report, we show that a single cellular process – insulin binding to its receptor (in HA-1 Chinese hamster ovary cells) – may be made similarly heat-resistant. Heat resistance, whether expressed as cell survival or insulin binding, had similar dose-response characteristics, showing maximum resistance after 30 min at 43°C. The processes had similar induction kinetics (2–6 h) and decayed over a similar time-course (100 h) after 43°C, 30 min preheating. Thermal resistance of insulin binding was induced only when residual receptor loss (due to heating) occurred. Also, decay of resistance was closely correlated with recovery of insulin binding capacity. There thus appeared to be an inverse relationship between receptor number and the degree of heat resistance of both receptors and whole cells. (Scatchard analysis indicated that decreased insulin binding was due to receptor loss, not affinity decrease.) Whether the insulin receptor has a direct role in the mediation of cell killing or whether it passively reflects the state of the whole cell is not clear. However, identification of the receptor as an entity specifically protected in the thermotolerant cells may permit examination of the expression of thermotolerance at the molecular level.

Introduction

The use of heat (temperatures of 41–50°C) in the treatment of cancer is an increasingly valuable new approach [1,2]. One limit to the potential of the modality is the acquisition of heat resistance by tumor cells [1,3,4]. Further advances in tumor therapy by heat would thus clearly benefit from a knowledge of the mechanisms of thermal cell killing and protection from such killing in resistant cells.

Many studies point to membranes as likely candidates for the target of thermal cell killing [1,5–9]. Most evidence appears to exclude gross changes in membrane lipid fluidity [9] in mammalian cells subject to hyperthermia. The studies of Lepock and co-workers [11–13] indicate no lipid phase transition in cell membranes over the 37–50°C range. Most evidence points to changes in membrane proteins in this temperature zone [7,14–16]. In the present study, we have investigated the effects of heat on a membrane protein function. We have chosen to study the thermal sensitivity of the insulin receptor, using hormone binding as an assay for receptor integrity. The receptor was selected because of its importance in the metabolism of a wide spectrum of cell types and for the ease with which binding could be studied. Our earlier work [17] indicated that the effect of heat on insulin-receptor binding paralleled the effects on whole cells in a number of ways. The inhibition of binding was time- and temperature-dependent and exhibited a break in thermal behavior between 42 and 43°C which reflected the behavior of whole cells. (Below 42°C, cell killing was extremely low and inhibition of

insulin binding was not observed.) When the slopes of the binding inhibition curves were subjected to Arrhenius analysis, a figure of 164 kcal/mol was obtained for the activation energy of thermal inhibition of insulin binding. This figure is similar to that obtained for the activation energy of cell killing in this cell line [1]. Thus, denaturation of some protein may be involved at some stage in inhibition of insulin binding and in cell killing. The preliminary investigation also indicated that induction of heat resistance (thermotolerance) in these cells led to thermal resistance of insulin binding. Thus, heat-induced changes in insulin binding reflect closely the ultimate fate of the cell. For the present study, we have further analyzed thermal resistance of the binding activity in thermotolerant cells. The data indicate that insulin binding resistance and thermotolerance have similar induction kinetics, and dose-response characteristics and that the decay of heat resistance occurs over a time-course of like duration whether assayed as cell survival or insulin binding.

Materials and Methods

Cells and culture conditions. Chinese hamster ovary (HA-1) fibroblasts were grown in Eagle's minimal essential medium supplemented with 15% fetal calf serum, penicillin and streptomycin. The cultures were maintained in a humidified incubator with a mixture of 95% air and 5% CO_2 and routinely checked for mycoplasma. Confluent monolayers of cells, fed daily with fresh medium, were used for experiment on day 3 after reaching confluence. At this time, cultures contained $(2.4–2.6) \cdot 10^7$ cells.

Heat treatment. Monolayers of cells on plastic Petri dishes were heated in a hot-water bath located inside an incubator with an atmosphere maintained at a ratio of 95% air and 5% CO_2. This gas mixture maintained the pH of the culture medium between 7.2 and 7.4 in all the heating conditions (pH was monitored before and after heating). Water-bath temperature was controlled to within ± 0.1°C. The time required to reach a steady temperature was about 3 min and is included in the heating times reported later. Immediately preceding and following each heat treatment, the cultures were overlaid with 4 ml of fresh medium.

Cell survival. The techniques for measurement of cell survival used in the present study have been described in detail previously [9]. In brief, at the conclusion of treatment, cells were trypsinized and counted by hemocytometer and then diluted to yield approx. 100–200 colonies per 60-mm Petri dish. Trypsinization has been shown previously to have minimal effect on survival in heated or unheated cultures [9]. After 10 days incubation at 37°C, colonies were fixed, stained and counted; surviving fraction was determined by comparison with untreated control cultures. All experiments were performed on a minimum of two occasions.

^{125}I-labelled insulin. Bovine monocomponent insulin was iodinated by the chloramine-T method [18–20] and separated from free ^{125}I by gel filtration using Sephadex G-25. The integrity of ^{125}I-labeled insulin synthesized by this technique was tested by trichloracetic acid precipitation: only preparations which exhibited ^{125}I precipitability values of 98% or over were used. After iodination, aliquots of insulin samples were introduced into tubes and stored at −20°C for periods up to 4 weeks in Dulbecco's phosphate-buffered saline containing 2.5% bovine serum albumin.

Insulin-binding assay. Binding studies were carried out on confluent monolayers of HA-1 cells $(2.5 \cdot 10^7$ cells per dish).

Cells were incubated for 150 min on ice (0–1°C) in 2 ml phosphate-buffered saline + 0.1% albumin + 0.1 mM bacitracin containing 20 ng ^{125}I-labeled insulin. Under these conditions, binding was confined almost exclusively to the cell surface, and internalization and degradation of the label was minimal. At the end of the incubation period, monolayers were washed four times in 5 ml ice-cold phosphate-buffered saline + 0.1% albumin (pH 7.4). Cells were then digested in 2.0 ml 0.5 M NaOH (overnight at room temperature) and the viscous digest transferred to a plastic counting vial. The dish was washed with 1 ml NaOH, and this washing combined with the 2 ml digest was counted in a Beckman γ-spectrometer. All assays were carried out in replicate dishes, at least three for each data point. All data were expressed as specific binding; this parameter is obtained after subtraction of the nonspecific fraction from total insulin binding. Nonspecific binding was determined as the amount of ^{125}I-labeled insulin

uptake in the presence of a large excess (10 μg/ml) of unlabeled insulin. This concentration appeared to give an accurate estimate of nonspecific binding; higher concentrations (100 μg/ml) led to an underestimation of nonspecific binding as described previously for insulin by Cuatrecasas [20]. Binding of ^{125}I-labeled insulin to cell-free culture dishes was both minimal (100–200 cpm) and nonspecific; and binding to the dishes was thus accounted for by the nonspecific binding control.

The insulin-receptor binding equilibrium was studied at a range of receptor occupancies using the Scatchard plot [20–24]. Binding was carried out with insulin concentrations ranging from 0.02 to 2000 pM. For each concentration, it was necessary to do a separate control for nonspecific binding. Data were plotted as the ratio of bound insulin to free insulin.

Control experiments were also carried out to ensure that the heating and binding assay conditions used did not lead to loss of cells from the monolayers (and thereby underestimation of the degree of insulin binding). Control experiments employing the most severe heating conditions used (160 min, 43°C; 80 min, 44°C; 40 min, 45°C) indicated no such loss. Cell loss was, however, routinely checked for in each experiment by measuring the protein content of the NaOH digest used for ^{125}I counting. Protein concentrations were measured by the method of Lowry et al. [25].

Results

The time-course of induction and decay of thermal tolerance in plateau phase HA-1 cells is shown in Fig. 1. The degree of resistance is indicated by the magnitude of the survival values shown on the ordinate of Fig. 1. The process is characterized by rapid induction (within at most 6 h), an apparent steady state and finally a gradual decay in the order of 4–5 d. The rate of induction of tolerance was apparently independent of dose at the two temperatures employed in the study (30 min and 80 min at 43°C). In contrast to this, the decay of tolerance was dose-dependent, resistance decaying more rapidly after the lower preheating dose; thermal tolerance decayed to trace levels in approx. 100 h at the lower preheating dose (30 min, 43°C) and in 144 h at the higher dose (80 min, 43°C).

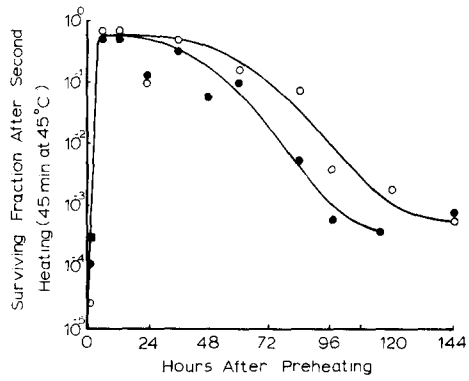

Fig. 1. Effect of preheating cells at 43°C for periods of 30 min (●) and 80 min (○) on subsequent sensitivity to a standard heat dose (45 min at 45°C). Survival of non-pretreated cells given the same heating dose (■) is also shown. Culture medium was replaced with fresh medium immediately preceding and subsequent to each heat treatment.

Full survival curves were constructed at selected time points after the induction of thermotolerance, viz. 12, 60, 100 and 144 h (Fig. 2). This was done in order to test whether heat resistance shown in Fig. 1, using a single heating period to assay for cell survival, was due to alterations in the shoulder region or in the exponential part of the cell survival curve. The effects observed appeared to be due to a decrease in exponential cell killing. The curves provide information essentially similar to that obtained from Fig. 1. Both preheating conditions made cells thermotolerant to a similar degree up to 60 h (Fig. 2A). Tolerance had largely decayed in the 30 min, 43°C preheated cells by 100 h, while cultures given 80in, 43°C still retained considerable heat resistance (Fig. 2C). After 144 h, cells preheated at 43°C also regained normal thermal sensitivity (Fig. 2D).

Response to heating of insulin receptors in control and thermotolerant cells is indicated in the Scatchard curves of Fig. 3. The curves indicate that heat had no major effect on the affinity constants for insulin binding; moreover, binding affinity of insulin to both heated and unheated thermotolerant cells was similar to that of untreated controls. Heat (80 min, 43°C) decreased receptor number in non-thermotolerant cells by approx. 50% (legend, Fig. 3). Thermotolerant cells had 25% reduction in receptor number; heating these cells (80 min, 43°C) caused only a slight

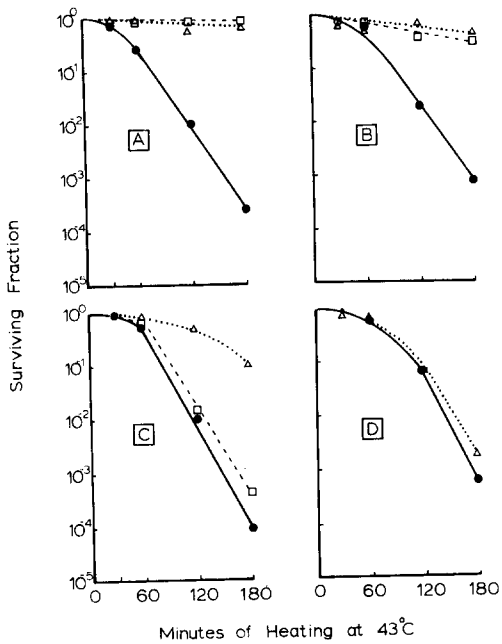

Fig. 2. Cell survival at 43°C of cultures preheated at 43°C for periods of 30 min (□) and 80 min (△) and of non-pretreated cultures (●). Experiments were carried out 12 h (A), 60 h (B), 100 h (C) and 144 h (D) after the pretreatments. The survival values shown are normalized to 100% plating efficiency at the commencement of the test heating period. In experiments carried out at 12 h (A) and 60 h (B), plating efficiencies were below 100% in preheated cultures. This was not observed at 100 and 144 h, presumably due to repair of potentially lethal heat damage at these later time points. Repair of potentially lethal damage has been observed in this cell line previously [17]. The values shown are means of triplicate assays carried out in one large single experiment. Variation between replicates (standard deviation) was less than 20% of the means at all time points. The experiment was repeated in entirity once and gave similar findings.

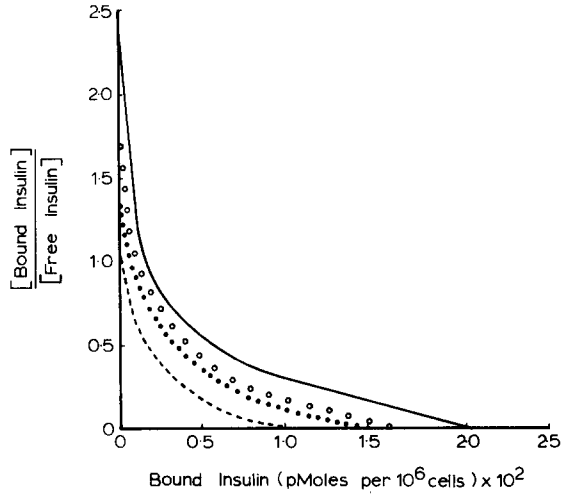

Fig. 3. Scatchard plots of insulin-binding data in heated and unheated HA-1 cells (both thermotolerant and normally heat-sensitive). The curves are hand-fitted to the data points. Scatchard curves were plotted using control (———), heated (— — —), thermotolerant (○○○) and thermotolerant/heated (●●●) cells; actual datum points are omitted for the sake of clarity. Affinity constants and receptor numbers derived from the curves are shown below.

Cell treatment	Derived binding parameters		
	Binding capacity (sites/cell)	$\bar{K}_e$ (M^{-1})	$\bar{K}_f$ (M^{-1})
Unheated	12940	$1 \cdot 10^{-9}$	$2.6 \cdot 10^{-7}$
80 min, 43°C	6500	$8.2 \cdot 10^{-9}$	$2.6 \cdot 10^{-7}$
Tolerant (12 h after 30 min, 43°C)	9000	$9.1 \cdot 10^{-8}$	$2.3 \cdot 10^{-7}$
Tolerant + 80 min, 43°C	8250	$9.0 \cdot 10^{-8}$	$2.3 \cdot 10^{-7}$

$\bar{K}_e$ = affinity constant of empty receptor; $\bar{K}_f$ = affinity constant of filled receptor.

further reduction in this value, from 9000 to 8250 binding sites per cell (legend, Fig. 3).

The dose-response characteristics of the thermal induction of heat resistance in the insulin receptor are shown in Fig. 4. Pretreatment time at 43°C is plotted against heat resistance of insulin binding (expressed as percentage of a non-pretreated control); data are shown for two second 'test' heating doses, 80 min at 43°C and 160 min at 43°C. The two curves are essentially similar; there appears to be a threshold preheating dose (5 min) up to which period no resistance is observed. Subsequently, resistance develops rapidly, reaching a maximum by 30 min; increasing the preheating period beyond 30 min produces no further increase in resistance. It may be of significance to note that the induction of insulin binding resistance occurred concurrently in all cases, with some residual decrease in insulin binding (data shown inset in Fig. 4). This decreased binding was observed in cultures preheated at the times shown on the abscissa but given no further test heating. As decreased binding was shown in all cases studied to be the result of receptor loss (Fig. 3), the data inset in Fig. 4 would appear to indicate that heat-induced decrease in receptor number is a prerequisite for

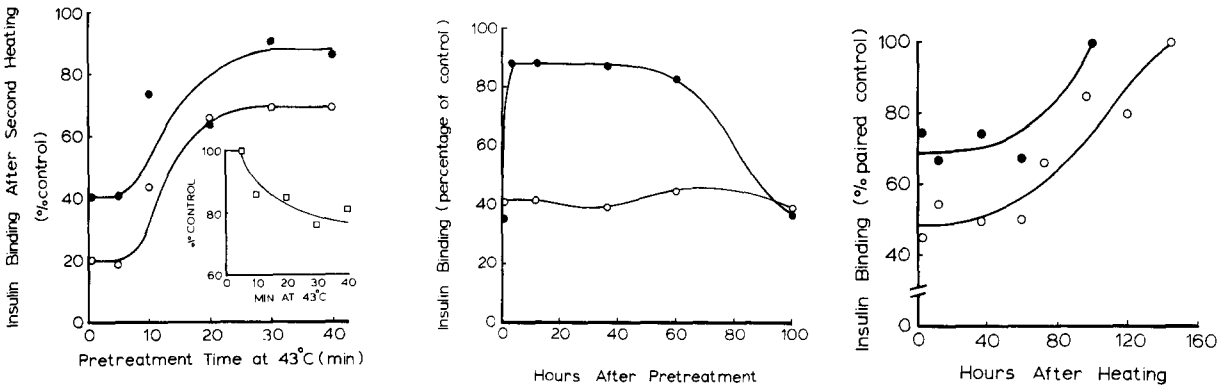

Fig. 4. Dose-response aspects of heat-induced thermal resistance of insulin binding. Insulin binding after heat-shock (80 min (●) or 160 min (○)) is plotted against the dose of heat used to induce thermal tolerance 12 h prior to experiment. Each point is expressed as the percentage of appropriate control. In controls, binding was measured in cultures given the heat pretreatment but not the second test heating. Although this value was decreased compared to the binding value in non-pretreated controls, increased binding observed in pretreated cultures was not due to amplification by the lowered baseline; even when non-pretreated control values were used to calculate percentage binding, insulin uptake was enhanced in pretreated cells (10–40 min at 43°C) compared to non-pretreated cells. Inset in the figure is shown the decreased binding in preheated control cultures 12 h after the pretreatments. Datum points are means of triplicate assays of specific insulin binding. Each experiment was repeated at least once giving consistent values.

Fig. 5. Decay of heat resistance in insulin binding at 37°C. Binding was measured in normally sensitive (○) and thermotolerant cells (●) heated at 43°C for 80 min at period of up to 100 h after the pretreatment. Binding is expressed as percentage of appropriate unheated, paired control. Datum points (means of triplicate assays) are expressed as specific insulin binding. The experiment was performed on three separate occasions and the curve shown is representative of the series.

Fig. 6. Recovery of insulin binding to control values after initial heat shock at 43°C, for 30 min (●) and for 80 min (○). Binding is expressed as percentage of binding in paired, unheated control cultures. As before, datum points are means of triplicate assays of specific insulin binding. The experiment was repeated once and gave similar shaped surves and indicated similar linetics for the recovery of binding after heat shock.

subsequent resistance in the remaining receptors.

The duration of heat resistance in insulin binding following a preheating at 43°C for 30 min (Fig. 5) closely paralleled the duration of thermotolerance induced by this treatment (Fig. 1). Resistance was present for at least 60 h, decaying to control values by 100 h. The duration of resistance in insulin binding after the 30 min, 43°C pretreatment corresponded almost entirely to the period needed for binding to recover after that heat shock. This is shown in one of the recovery curves in Fig. 6. Binding was inhibited by 25% immediately after heating and remained depressed at approximately this level for 60 h, recovering to control values by 100 h. This finding is in accord with the data in Fig. 4, which indicate a close correlation between decreased binding (receptor number) after heating and heat resistance of surviving receptors. Fig. 6 also shows binding recovery after 80 min at 43°C; binding recovers more gradually after these conditions, reaching control levels by 144 h.

TABLE I

THERMAL RESISTANCE OF INSULIN BINDING IN CELLS MADE THERMOTOLERANT BY A RANGE OF PREHEATING TEMPERATURES (43, 44, 45°C) AND SUBSEQUENTLY HEATED OVER A SIMILAR SPECTRUM OF TEMPERATURES (43, 44, 45°C)

Results are expressed as percentage binding in non-preheated control cultures receiving the same test heating dose as the tolerant cells. Values shown in the table are means derived from replicate assays on six cell cultures. Variation was less than 8% of the mean in all cases.

Preheating temperature (°C)	Test temperature (°C)		
	43 (80 min)	44 (40 min)	45 (20 min)
43 (30 min)	257	189	233
44 (15 min)	249	215	220
45 (7.5 min)	380	177	254

Discussion

Thermotolerance has now been studied in a range of cell types, both in vitro and in vivo [1,3,27–31]. This transient type of heat resistance seems to be composed of a number of phases which most studies show to have kinetic characteristics similar to those shown in Figs. 1 and 2; rapid induction of tolerance after the initial heat shock (taking a few hours) precedes a plateau period which is followed by a gradual decay of thermal resistance. The plateau and decay portions of the curve are generally of relatively longer duration, persisting for several days after heat shock. Heat resistance appears to be expressed in terms of insulin receptor function in a manner similar to resistance measured as increased clonogenic capacity (Figs. 1, 2, 4–6). Heat pretreatment inhibited both the rate of exponential cell killing (Fig. 2A) and the rate of decrease in insulin binding [17]. The dose response of initial heat treatment to subsequently induced heat resistance was almost identical whether measured in terms of insulin binding (Fig. 4) or cell survival [27]. The rates of induction of binding resistance (Fig. 5) and thermotolerance [27] were also similar, as were the rates of decay of these parameters (Fig. 5). These findings are in close agreement with those reported previously [17], which indicated that the fate of the insulin receptor mirrors intimately the ultimate destiny of the cell after heat treatment.

A consistent finding in the present study, which may be of significance to investigations of thermotolerance induction was that resistance was accompanied in each case by prolonged decreases in insulin-receptor binding (Figs. 4–16). As is shown in Fig. 3, decreased binding after heat reflected a reduced receptor number; affinity constants were not altered by the heat (legend, Fig. 3). Thus, binding resistance and thermal tolerance both appeared concomitantly with the loss of insulin receptors. Cells had a reduced complement of receptors which had become heat-resistant. The persistence of tolerance closely mirrored the period over which the receptor number remained depressed (Figs. 5, 6); decay of resistance and thermotolerance (Fig. 1) followed precisely the same kinetics as the recovery in receptor number. Thus, binding resistance appeared to be inversely related to receptor number over a certain range of treatments (the system appeared saturated after a 30 min, 43°C pretreatment; further heating, although causing more receptor loss, did not produce increased resistance). It is not clear why these effects are related; it is easy to envisage how receptor loss could provide a signal to the cell for the induction of thermotolerance when the role of the receptor in transmembrane signaling is considered [32,33]. It is more difficult to assign a direct role for the receptor in the maintenance of the thermotolerant state. A more likely explanation is that the receptor is protected by whatever mechanism is responsible for protection of the whole cell. However, a direct role for the receptor in maintenance of heat resistance remains a possibility.

The precise mechanism of protection of receptors from heat in thermotolerant cells is not clear from the current study. Moreover, the mechanisms of thermotolerance are, in general, not well understood. Two current hypotheses suggest either lipid alterations [28] or a role for a class of heat-induced proteins (heat shock proteins [34,35]) in thermotolerance. Alteration in the microenvironment of the receptor might be expected to cause allosteric effects and lead to changes in its equilibrium binding properties. However, the affinity constants of thermotolerant cells were similar to those of nonpretreated cells (legend, Fig. 3). Affinity changes are induced in the insulin receptor by alterations in membrane lipid composition [36] or cross-linking with lectins [37]. These affinity changes may be related to alteration in subunit structure of the receptor which may exist as monomers or polymers. Ginsberg et al. [36] suggested that when a high proportion of receptors exist as monomers (such as in conditions of increased membrane fluidity), this is reflected in binding properties as increased binding-site number (R_0) with decreased affinity ($\bar{K}_a$). Polymerization (perhaps due to decreased fluidity or to cross-linking) seems to correspond with decreased R_0, increased $\bar{K}_a$. Agents causing thermal protection might be expected to increase the rigidity of heat-resistant structures; such an effect might be predicted from the model of Ginsberg to cause increased $\bar{K}_a$. Absence of any effect of thermotolerance on $\bar{K}_a$ indicates that whatever agent is responsible for resistance exerts a subtle influence on the insulin

receptor which does not affect affinity properties. The delayed replacement of insulin receptors ($t \approx$ 100 h; Fig. 6) which was inversely correlated with decay of resistance implies complex changes in cellular metabolism in thermotolerant cells. Inhibition of receptor synthesis, glycosylation or insertion into membranes might be involved.

In summary, therefore, the study indicates that the behavior of a discrete membrane protein, the insulin receptor, mimics the heat sensitivity of cells as a whole and develops thermal resistance after mild heat shock in a way similar to thermotolerance in whole cells. It is not clear whether the relationship between thermal effects on receptor number and cell killing is a causal one. Any alteration in the thermotolerant receptor appears to be subtle and causes no change in receptor-binding affinity. Whatever alterations do occur in the thermotolerant insulin receptor or in its milieu to cause heat resistance have no effect on binding affinity; this seems to argue against major changes in the protein-lipid environment of thermotolerant cells and to contraindicate cross-linking reactions involving perhaps heat-shock proteins and the receptor. The study appears to indicate some kind of inverse correlation between insulin-receptor number and heat resistance. We are currently investigating whether this correlation is or is not a causal one. The major value of the current study may be the finding of a discrete chemical entity showing thermotolerance which may be used in the investigation of the basic biochemical mechanisms of thermotolerance.

Acknowledgement

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