Journal of Cellular Biochemistry 61:216–229 (1996)

Role of Hsp70 Synthesis in the Fate of the Insulin–Receptor Complex After Heat Shock in Cultured Fetal Hepatocytes

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Abstract The influence of a mild heat shock on the fate of the insulin–receptor complex was studied in cultured fetal rat hepatocytes whose insulin glycogenic response is sensitive to heat [Zachayus and Plas (1995): J Cell Physiol 162:330–340]. After exposure from 15 min to 2 hr at 42.5°C, the amount of ^{125}I-insulin associated with cells at 37°C was progressively decreased (by 35% after 1 hr), while the release of ^{125}I-insulin degradation products into the medium was also inhibited (by 75%), more than expected from the decrease in insulin binding. Heat shock did not affect the insulin-induced internalization of cell surface insulin receptors but progressively suppressed the recycling at 37°C of receptors previously internalized at 42.5°C in the presence of insulin. When compared to the inhibitory effects of chloroquine on insulin degradation and insulin receptor recycling, which were immediate (within 15 min), those of heat shock developed within 1 hr of heating. The protein level of insulin receptors was not modified after heat shock and during recovery at 37°C, while that of Hsp72/73 exhibited a transitory accumulation inversely correlated with variations in insulin binding, as assayed by Western immunoblotting from whole cell extracts. Coimmunoprecipitation experiments revealed a heat shock–stimulated association of Hsp72/73 with the insulin receptor. Affinity labeling showed an interaction between ^{125}I-insulin and Hsp72/73 in control cells, which was inhibited by heat shock. These results suggest that increased Hsp72/73 synthesis interfered with insulin degradation and prevented the recycling of the insulin receptor and its further thermal damage via a possible chaperone-like action in fetal hepatocytes submitted to heat stress.

Key words: insulin receptor, insulin degradation, fetal hepatocyte, heat shock, Hsp72/73, chloroquine

It has been postulated that plasma membrane proteins may be some of the first targets affected by heat shock [Calderwood and Hahn, 1983]. Susceptibility to loss of binding ability of plasma membrane receptors like EGF and insulin receptors has been shown in different cell lines [Magun and Fennie, 1981; Calderwood and Hahn, 1983] and in primary cultures of fetal hepatocytes [Zachayus and Plas, 1995]. Also, the binding ability of glucocorticoid receptors is affected by heat treatment [Ali and Vedeckis, 1989; Anderson et al., 1991]. It is not clear in these situations, in the case of glucocorticoid receptors for instance, whether the loss of the receptor binding property is linked to accelerated receptor degradation [Ali and Vedeckis, 1990] or to a transitory and reversible non-binding state in which the undegraded receptors may be protected from further thermal damage [Anderson et al., 1991; Benatmane and Plas, in press]. Heat shock proteins (Hsps) play an important role under normal conditions acting as molecular chaperones in facilitating protein folding and assembly, a function that must be amplified in cells experiencing heat stress which exhibit a highly increased Hsp synthesis [Welch, 1992]. Steroid receptors are characterized by their association with different Hsps, among them Hsp90 and Hsp56, and multiple roles for such association in normal as well as in stressed cells have been evoked [for review, see Pratt, 1993]. To our knowledge, no specific association of plasma membrane receptors with Hsps has been evidenced when compared to other proteins.

It is known that cultured fetal hepatocytes are among cellular models highly responsive to insu-

Received May 30, 1995; accepted November 3, 1995.

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lin [Plas and Nunez, 1976], which besides are particularly responsive to heat shock. When exposed to a mild heat shock, these cells reveal an inhibition of the insulin glycogenic response and a decrease in cell surface insulin receptor level whose time course is associated with the stimulation of Hsp72/73 synthesis. The rapid recovery of cell surface insulin receptors when cells are returned to 37°C has suggested that the insulin receptors are not degraded [Zachayus and Plas, 1995]. The cellular fate of insulin–receptor complexes in cultured fetal hepatocytes is characterized by an insulin degradation predominantly mediated via the endocytosis of insulin–receptor complexes and by an efficient cellular cycle of insulin receptors induced by hormone exposure [Plas and Desbuquois, 1982; Soubigou et al., 1986]. It was thus interesting to study the modifications of such processes during heat shock in relation to a possible role of Hsp72/73 synthesis. The results showed that insulin degradation was strikingly inhibited correlatively to the inhibition of the recycling of insulin receptors at a time when Hsp72/73 synthesis was stimulated. Association of insulin as well as that of its receptor with Hsp72/73 was regulated by heat shock, suggesting a role of cognate and heat-induced Hsp70 in insulin degradation and in the protection of the insulin receptor from thermal damage.

MATERIAL AND METHODS

Materials

Pig insulin was a gift from Lilly Laboratories. ^{35}S-methionine and Western blotting detection system (ECL) were purchased from Amersham. Nitrocellulose membranes (BA 85) were from Schleicher and Schuell. Horseradish peroxidase–labeled goat anti-mouse and anti-rabbit IgG were obtained from Southern Biotechnology and Sigma, respectively. Monoclonal antibody from mouse clone 3a3 directed against both the constitutive form (Hsc73) and the heat-inducible form (Hsp72) of Hsp70 was from Affinity BioReagents. Anti-Hsp72/73 monoclonal antibody from mouse clone W27 and Protein G PLUS/Protein A-Agarose were obtained from Oncogene Science. Polyclonal antibody raised in rabbits against insulin receptor β-subunit C-terminal domain (last 15 amino acids) was a generous gift from Dr. C.R. Kahn. Bovine brain Hsp72/73, consisting of 90–95% Hsc73 and 5–10% Hsp72, was from StressGen. Insulin and bovine brain Hsp72/73 were iodinated by a modification of the chloramine-T method [Plas and Desbuquois, 1982]. The sources of other materials have been specified previously [Plas and Nunez, 1976; Soubigou et al., 1986].

Culture Procedure

Primary cultures of hepatocytes were obtained from 18-day-old rat fetuses (Sprague–Dawley) as described previously [Plas et al., 1973]. After mild trypsin treatment, the isolated cells were plated on a collagen substratum to which only the hepatocytes adhered, and after 4 hr the nonadhering hematopoietic cells were removed. At this step, the culture medium (1 ml/well) was replaced; it was again replaced after 18 hr of culture. The culture medium consisted of NCTC 109 medium [Evans et al., 1964] supplemented with 10% fetal calf serum and 10 μM cortisol. All the experiments were performed after 2 days of culture in the presence of glucocorticoids, when the glycogenic response to insulin is fully expressed [Plas and Nunez, 1976].

Cell Heating and Recovery Studies

After 2 days of culture, heat shock was applied in conditioned medium, i.e., the medium in which cells had grown for the last 24 hr. The culture dishes were rapidly warmed in a water bath maintained at 42.5°C and then placed in the incubator at 42.5°C during the required time. The same protocol was used for returning cells to 37°C, the medium being replaced by a conditioned one maintained at 37°C. Incubation under these conditions allowed comparison of studied parameters between heat shocked and never heated (control) cultures, conditioned medium being taken from parallel control cultures.

Insulin Binding and Degradation Studies at 37°C

Cell-associated radioactivity at 37°C corresponded to ^{125}I-insulin either still bound to its receptor or dissociated in intact or degraded forms, while ^{125}I-insulin degradation concerned the total trichloroacetic acid–soluble products released into the medium. Incubations of 1 nM ^{125}I-insulin with fetal hepatocytes grown in monolayers (about 0.7×10^6 cells/dish) were performed for 60 min at 37°C and pH 7.4, in 0.4 ml of conditioned medium after heat treatment at 42.5°C for up to 120 min. Chloroquine at 70 μM or its solvent (H_2O) was introduced together with labeled hormone. At the end of incubation,

samples of medium were taken for measuring insulin degradation and the cells washed with 5 × 1 ml of ice-cold Hanks solution. This washing step was achieved within 12 sec. The cell-associated radioactivity was then extracted in 0.5 N NaOH. The total labeled material extracted represented the "total binding." Corrections were made for the nonspecific association of ^{125}I-insulin with cells by means of parallel incubations in the presence of 10 μM native hormone. Insulin-specific binding is expressed as fmol ^{125}I-insulin bound per culture. The rate of insulin degradation was determined by measuring the release of degradation products into the culture medium after 60 min using the trichloroacetic acid precipitation method [Plas and Desbuquois, 1982]. Results are expressed as fmoles of ^{125}I-insulin degraded/hr/culture. The rate of ^{125}I-insulin degradation is also expressed as a function of the amount of ^{125}I-insulin associated to cells.

Insulin Receptor Cycle Studies

Measurements of the variations in ^{125}I-insulin binding levels at the cell surface after a transitory exposure to the native hormone have allowed us to investigate the cellular insulin receptor cycle [Soubigou et al., 1986]. This approach was used to study heat shock effects with the following modifications. Cultured fetal hepatocytes were first exposed to 10 nM native insulin or its solvent in culture medium at 42.5°C or at 37°C for times from 15 to 120 min. Cell surface insulin binding was determined following heat treatment or after cell return to 37°C for 15 min in culture medium devoid or not of 10 nM insulin. Cell surface insulin binding was measured at 4°C as follows: the medium was first replaced by a cold NCTC 109 medium, then cultures were washed once with binding medium containing 10 nM ^{125}I-insulin and incubated for 3 hr in the same medium. Binding medium consisted of glucose-free NCTC 109 medium + 1 mM KCN, pH 7.8. At the end of incubation, insulin-specific binding was determined as described for binding studies at 37°C.

^{35}S-Methionine Labeling of Cellular Proteins and Induction of Hsp72/73 Synthesis

In a first set of experiments, cells were incubated either at 42.5°C or 37°C for various times up to 2 hr before being pulse labeled in conditioned medium containing ^{35}S-methionine (40 μCi/ml; specific activity >1,000 Ci/mmol) for 15 min at 37°C. In a second set of experiments, incubation with ^{35}S-methionine (40 μCi/ml) was performed for 12 hr at 37°C, then cells were heated for 2 hr at 42.5°C or maintained at 37°C in conditioned medium containing ^{35}S-methionine (40 μCi/ml). At the end of incubation, the medium was removed and cells were washed twice with ice-cold Hanks solution. Then, cell material was processed for immunoprecipitation using the W27 anti-Hsp72/73 antibody as described below.

Nondenaturing Immunoprecipitation Procedure

Immunoprecipitation was performed by a method allowing us to minimize nonspecific coprecipitation [Firestone and Winguth, 1990]. Cell material was extracted at 4°C in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing 1 mM PMSF, 1 mM benzamidine, and 1 μg/ml leupeptine. Then, insoluble material was sedimented by centrifugation at 20,000 *g* for 20 min at 4°C. The lysate supernatant was incubated with Protein G PLUS/Protein A-Agarose (50% w/v) for 2 hr at 4°C. Agarose beads were pelleted and the supernatant fraction corresponding to preadsorbed cellular material was supplemented with 1 mg/ml BSA and incubated overnight at 4°C with either the W27 anti-Hsp72/73 monoclonal antibody or the anti-insulin receptor β-subunit polyclonal antibody. Protein G PLUS/Protein A-Agarose, previously preadsorbed for 2 hr at 4°C with RIPA cell extracts, was added to the immunoprecipitation reaction mixture which was incubated thereafter for 2 hr at 4°C. Agarose beads were washed four times with RIPA buffer. The immunoprecipitated proteins were solubilized in concentrated Laemmli sample buffer before SDS-PAGE analysis under reducing conditions [Laemmli, 1970].

In Vitro Association Between ^{125}I-Hsp72/73 and Insulin Receptor

WGA-purified insulin receptor from 20-day-old fetal rat liver prepared as previously described [Zachayus et al., 1994] was incubated in PBS with ^{125}I-Hsp72/73 from bovine brain for 2 hr at 37°C or 43°C. Incubation mixture was then supplemented with RIPA buffer before performing immunoprecipitation with the anti-insulin receptor antibody.

Crosslinking Experiments After ^{125}I-Insulin Binding

After ^{125}I-insulin association with proteins under in situ or in vitro conditions, covalent crosslinking was achieved using the membrane-permeable bifunctional chemical reagent disuccinimidyl suberate (DSS) as previously described [Pilch and Czech, 1979]. In in situ experiments, cells were incubated for 2 hr either at 37°C or at 42.5°C in the presence of 20 nM ^{125}I-insulin. After washing four times with ice-cold Hanks solution, cells were incubated for 30 min at 4°C in this solution containing 0.2 mM DSS. The reaction was quenched for 5 min at 4°C with 50 mM Tris–HCl, pH 7.4. Cell material was either directly analyzed by SDS-PAGE under reducing conditions after extraction in Laemmli sample buffer or processed for immunoprecipitation using an antibody directed against Hsp72/73 or insulin receptor as described above. In in vitro experiments, bovine brain Hsp72/73 or WGA-purified insulin receptor from fetal rat liver was incubated in PBS with 30 nM ^{125}I-insulin. Incubation with Hsp72/73 was carried out for 2 hr at 37°C, 2 hr at 43°C, or 12 hr at 4°C. In the case of insulin receptor, incubation was performed for 12 hr at 4°C. Incubation mixture was then diluted 10-fold in 0.2 mM DSS-containing ice-cold Hanks solution. After 30 min at 4°C, the reaction was stopped by the addition of concentrated RIPA buffer before performing immunoprecipitation with the W27 anti-Hsp72/73 antibody and the anti-insulin receptor antibody.

Western Immunoblotting

Cells were first exposed at 42.5°C for 2 hr before being returned to 37°C for various times up to 8 hr, whereas control cultures were maintained at 37°C. After washing twice with ice-cold Hanks' solution, cells were dissolved in Laemmli sample buffer or processed for immunoprecipitation using antibody directed against insulin receptor and Hsp72/73 before SDS-PAGE analysis under reducing conditions. Resolved proteins were then transferred to nitrocellulose membranes for 4 hr at 60 V in buffer containing 25 mM Tris, pH 8.35, 193 mM glycine, 0.1% SDS, and 20% methanol. Blots were probed as described previously [Babajko et al., 1993] using either the 3a3 anti-Hsp72/73 monoclonal antibody or the anti-insulin receptor β-subunit polyclonal antibody. Horseradish peroxidase conjugate anti-mouse or anti-rabbit IgG was bound to the immunocomplexes, and visualization of immunobands was carried out by addition of chemiluminescence reagent.

Presentation of Results

Each protocol involved at least three separate experiments performed on different cell culture preparations. The results are expressed per culture or per mg protein. The cell population of a culture dish (3 cm diameter) is of the order of 0.7×10^6 hepatocytes, which corresponds to 270 μg of protein and to 1.90 mg of wet weight of liver.

RESULTS

Effect of Heat Shock on Cell-Associated ^{125}I-Insulin and Its Degradation

As it has been shown that the stimulation of glycogenesis by insulin is inhibited by heat shock in cultured fetal rat hepatocytes [Zachayus and Plas, 1995], the effect of such treatment was investigated regarding the fate of the insulin–receptor complexes under physiological conditions. After applying a mild heat shock from 30 min to 2 hr at 42.5°C, the association of ^{125}I-insulin with cultured fetal hepatocytes and its degradation were studied at 37°C. For this purpose, after various times of heat treatment, cells were incubated for 1 hr with 1 nM ^{125}I-insulin. Cell-associated ^{125}I-insulin at 37°C and ^{125}I-insulin degradation in the medium were determined as described in Materials and Methods. The amount of cell-associated ^{125}I-insulin (12.5 fmoles/hr/culture in control cultures) decreased progressively with heating time (Fig. 1A). It was partially affected after 30 min of heat shock to reach a steady state after 1 hr, which represented 60% of the initial value. In parallel cultures, the rate of ^{125}I-insulin degradation product release (57.6 fmoles of ^{125}I-insulin degraded/hr/culture in control cells) was decreased after 30 min of heat shock to reach after 1 hr a constant value corresponding to 25% of the control one (Fig. 1B). The amount of ^{125}I-insulin degradation was also represented as a function of the amount of ^{125}I-insulin associated with cells (Fig. 1C). A linear relationship was observed when considering unheated cells preincubated with increasing doses of tunicamycine up to 1 μg/ml for 20 hr, a situation in which the pool of insulin receptors was decreased progressively [Pringault and Plas, 1983]. By contrast,

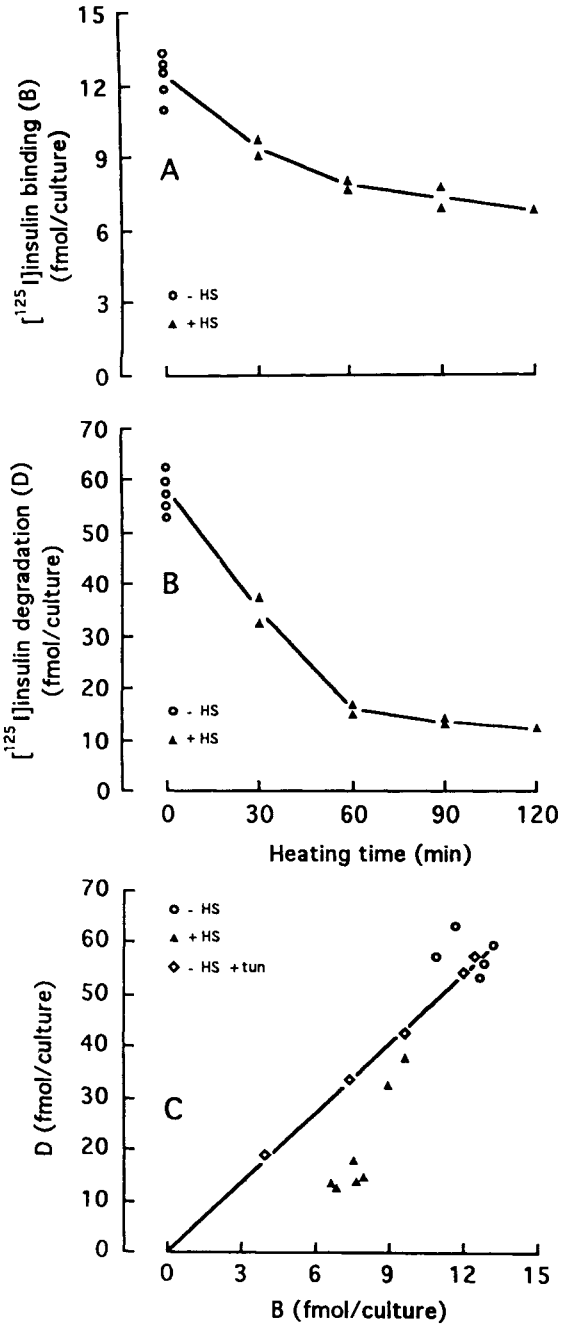

Fig. 1. Effect of heat shock on cell-associated ^{125}I-insulin and on ^{125}I-insulin degradation measured at 37°C in cultured 18-day-old fetal hepatocytes. At day 2, cells were either submitted to heat shock (+HS) at 42.5°C or maintained at 37°C (−HS) for various times up to 2 hr. Then, cells were incubated at 37°C in conditioned medium containing 1 nM ^{125}I-insulin for 1 hr. At the end of incubation, cell-associated ^{125}I-insulin as well as ^{125}I-insulin degradation in the medium were measured. Cell-associated ^{125}I-insulin (**A**), ^{125}I-insulin degradation (**B**), and ^{125}I-insulin degradation as a function of the amount of cell-associated ^{125}I-insulin for the same cultures (**C**) are shown. Represented in C are the corresponding values from cells which were preincubated with tunicamycine for 20 hr at 37°C (−HS + tun) before measurement of ^{125}I-insulin binding and degradation whose values were progressively depressed by increasing concentrations of tunicamycine (0.1, 0.2, 0.4, 0.6, and 1 μg/ml). Each symbol corresponds to a different culture dish from the representative experiment shown.

the values corresponding to heated cells were all situated under the straight line showing that the insulin degradation was more inhibited than expected from the decrease in insulin binding.

It is known that acidotropic drugs inhibit insulin degradation in cultured fetal hepatocytes while increasing the amount of cell-associated radioactivity [Plas and Desbuquois, 1982]. The presence of 70 μM chloroquine during ^{125}I-insulin binding in control cells increased cell-associated radioactivity by a factor of 2.5 (Fig. 2A). Heat treatment progressively abolished the chloroquine-stimulated effect, so that the amount of cell-associated radioactivity after 2 hr of heating represented only 25% of the initial value, thus returning to a situation close to that obtained in the absence of chloroquine. In control cultures, chloroquine produced a strong inhibition of the release rate of ^{125}I-insulin degradation products which was even more pronounced in heated cultures (15.4 vs. 57.6 fmoles/hr/culture) (Fig. 2B). This showed a cumulative effect of heat shock and chloroquine tending to abolish cellular insulin degradation.

Heat Shock and Insulin-Induced Variations of the Level of Insulin Binding Sites at the Cell Surface

A reversible translocation of insulin receptors from the cell surface to an internal compartment occurring after a transitory exposure to the hormone has evidenced a cellular cycle of insulin receptors in cultured fetal hepatocytes [Soubigou and Plas, 1986]. As heat treatment affected the amount of ^{125}I-insulin associated with cultured fetal hepatocytes at 37°C (Fig. 1), it was interesting to investigate the effect of heat shock on the internalization and externalization phases of the insulin receptor cycle induced by a short exposure to native insulin. In a first set of experiments after cell preheating for various times in the absence of insulin which progressively decreased the cell surface ^{125}I-insulin binding up to 30% after 2 hr, cells were returned to 37°C for 15 min in the presence of 10 nM insulin. In cells preheated for 15 min as well as in nonheated cells, exposure to insulin

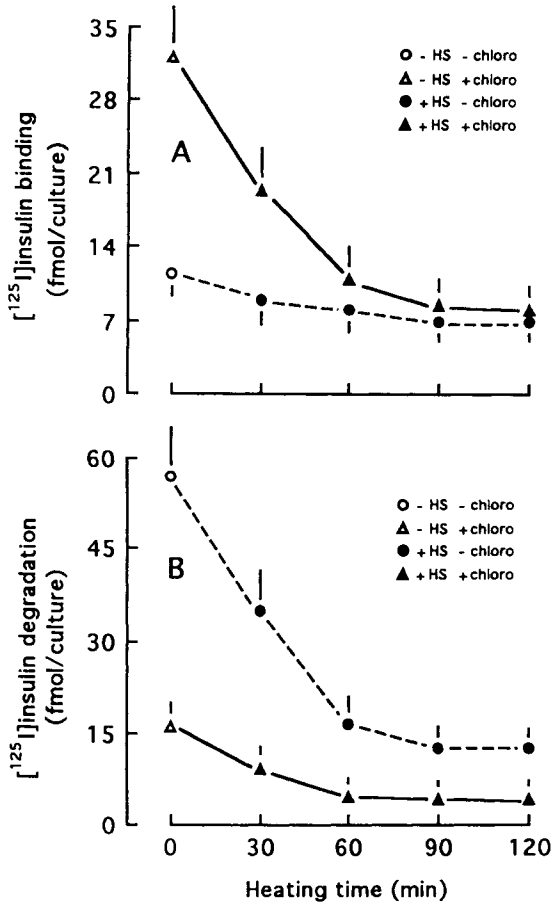

Fig. 2. Combined effect of heat shock and chloroquine on both cell-associated ^{125}I-insulin and ^{125}I-insulin degradation at 37°C. At day 2, cells were either subjected to heat shock (+HS) at 42.5°C or maintained at 37°C (−HS) for various times up to 2 hr. Then, cells were incubated in the presence or absence of 70 μM chloroquine (±chloro) at 37°C in conditioned medium containing 1 nM ^{125}I-insulin for 1 hr in order to determine cell-associated ^{125}I-insulin (**A**) and ^{125}I-insulin degradation (**B**). Results are presented as means ±SD for measurements of triplicate cultures from the representative experiment shown.

produced a sharp decrease in cell surface binding which represented 35% when compared to initial binding, such decrease corresponding to insulin–receptor complex internalization (Fig. 3A). The internalization rate was maintained until 1 h of heating to begin to be slightly altered for more prolonged preheating times, if compared to what was observed in nonheated cultures. In a second set of experiments after cell preheating for various times in the presence of 10 nM insulin, cells were returned to 37°C for 15 min in insulin-free medium. After 15 min of heating, removal of insulin was accompanied by the restoration of cell surface ^{125}I-insulin binding to a value close to that obtained in cultures heated in the absence of insulin (about 80%), while after 30 min of heating the restoration was only partial (45%) (Fig. 3B). For longer heating times, the restoration effect markedly decreased, tending to disappear after 2 hr of heating. In corresponding control cultures maintained at 37°C, the removal of insulin after 2 hr induced a complete restoration of insulin binding (results not shown). Thus, when compared to a similar study carried out on cells incubated at 37°C [Soubigou et al., 1986], heat shock did not produce any difference in the internalization phase of the insulin receptors, yet it affected the

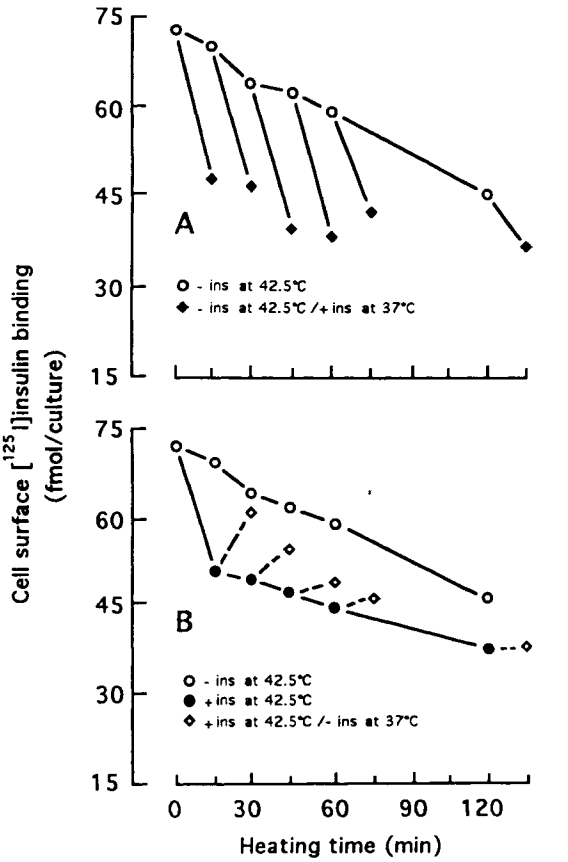

Fig. 3. Effects of heat shock on variations of cell surface insulin binding induced by a transitory exposure to the hormone. At day 2, after preheating for various times up to 2 hr at 42.5°C in the absence (○) or presence of 10 nM insulin (●), fetal hepatocytes were returned for 15 min to 37°C in a medium supplemented with 10 nM insulin (◆) or not supplemented (◇), respectively. At the end of incubation, cell surface insulin binding was measured for 3 hr at 4°C in NCTC 109 medium containing 10 nM ^{125}I-insulin. In **A** the insulin-induced decrease in cell surface ^{125}I-insulin and in **B** the degree of restoration of cell surface insulin binding following hormone removal are represented. A representative experiment is shown where the SD values for measurements of triplicate cultures were in all cases less than 10%.

subsequent recycling of the internalized insulin receptors following removal of the hormone.

The effect of heat shock on insulin receptor cycle was also tested in combination with chloroquine, which has been shown to inhibit the recycling of internalized insulin receptors in nonheated cells [Soubigou et al., 1987]. When fetal hepatocytes were heated in the presence of insulin together with 70 μM chloroquine, the internalization phase was more pronounced, reaching 40% of the control value. The restoration of cell surface binding at 37°C was only partial after insulin removal when the drug was still present (25%), and was improved by its simultaneous removal (60%) (results not shown). This situation was comparable to that previously described at 37°C [Soubigou et al., 1987]. Heat shock and chloroquine had in common the ability to inhibit insulin receptor recycling, yet the effect of chloroquine was immediate while that of heat shock developed slower.

Protein Level of Insulin Receptor and Hsp72/73 After Heat Shock and During Recovery at 37°C

As heat-induced insulin binding decrease has been previously correlated with the synthesis rate of Hsc73 and Hsp72 in cultured fetal hepatocytes [Zachayus and Plas, 1995], it was interesting to investigate the heat shock effect on protein levels of insulin receptor and Hsp72/73. These protein levels were determined after heat treatment for 2 hr and during recovery at 37°C up to 8 hr with concomitant measurement of cell surface insulin binding. Whole cell extracts were analyzed by SDS-PAGE and subsequent Western immunoblotting using either a polyclonal antibody raised against a C-terminal peptide of the insulin receptor β-subunit or the monoclonal 3a3 anti-Hsp72/73 antibody. Immunodetection with the anti-receptor antibody revealed a 95 kDa band which corresponded to insulin receptor β-subunit (Fig. 4A). The intensity of this band was not modified by heat treatment and remained unchanged during the recovery period at 37°C up to 8 hr, as compared to control cultures. The use of the anti-Hsp72/73 antibody allowed detection of an immunoreactive 70 kDa region which corresponded to both Hsc73 and Hsp72, the two Hsp70 family members being not distinguished under the Western immunoblotting conditions used. The 70 kDa region intensity was augmented strikingly after 2 hr at 42.5°C, decreased continuously during the recovery phase at 37°C, and tended to return to

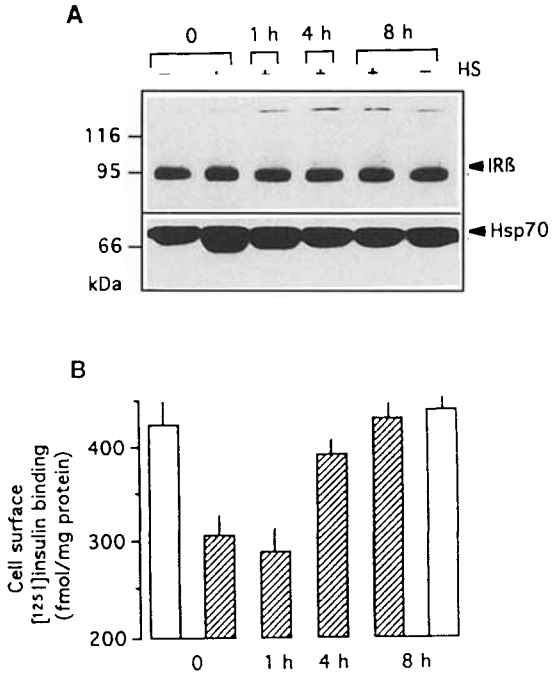

Fig. 4. Insulin receptor and Hsp72/73 protein levels and cell surface ^{125}I-insulin binding after heat treatment and during recovery at 37°C. At day 2, cells were preheated at 42.5°C for 2 hr before being returned to 37°C for various times up to 8 hr, whereas control cultures were maintained at 37°C. Then, protein level of insulin receptor and Hsp72/73 (**A**) and cell surface ^{125}I-insulin binding (**B**) were measured. **A:** Whole cell extracts obtained after cell lysis in Laemmli sample buffer were submitted to 7.5% SDS-PAGE under reducing conditions and Western immunoblotting. Equal quantities of cellular proteins (close to 440 μg) were applied to the gel, which corresponded approximately to the protein material quantities that actually run per lane. Upper and lower parts of blots, corresponding to cell proteins with a molecular mass superior to and inferior to 80 kDa, respectively, were probed separately with anti-insulin receptor β-subunit antibody (*upper part*) and 3a3 anti-Hsp72/73 antibody (*lower part*). Blots from cells submitted or not to heat shock (±HS) after the recovery times stated are represented, the 95 kDa immunoreactive band/insulin receptor β-subunit (IRβ) and the 70 kDa region corresponding to both Hsp72 and Hsc73 (Hsp70) being indicated. **B:** Cultures were incubated with 10 nM ^{125}I-insulin for 4 hr at 4°C to determine cell surface insulin binding. Preheated cells (*hatched bars*), which were returned to 37°C for the indicated times, and corresponding nonheated cells (*empty bars*) are represented. Results are presented as means ±SD for measurements of triplicate cultures from the representative experiment shown.

control values after 8 hr. In rat embryo fibroblasts, Hsp72/73 after stimulation by heat treatment possesses a half-life on the order of 48 hr [Mizzen and Welch, 1988]. The fast decrease in Hsp72/73 level over the course of the recovery period observed in the present study might be due to the proliferative features of cultured fetal hepatocytes [Plas et al., 1973], since an acceler-

ated turnover of the Hsp has been postulated to occur in proliferating cells [Marunouchi and Hosoya, 1993]. Cell surface insulin binding measured in parallel cultures was diminished after heat treatment to represent 72% of the control level (Fig. 4B). During the following recovery period at 37°C, it showed a two-thirds restoration within 4 hr, the return to full capacity being attained after 8 hr. Thus, inverse variations were seen between cell surface insulin binding and Hsp72/73 level in heat-treated cells while the insulin receptor protein level remained unchanged.

Association of Hsp72/73 With Cellular Proteins and Insulin Receptor During Heat Shock

The involvement of a direct protein–protein interaction between Hsp72/73 and insulin receptor in the heat shock effect on insulin binding was also investigated, since mature proteins which begin to unfold upon denaturation have been reported to become stably bound to Hsp72/73 [Beckmann et al., 1992]. The pattern of Hsp72/73-associated proteins after heat treatment was first characterized using short- and long-term labeling in the presence of ^{35}S-methionine followed by nondenaturing immunoprecipitation with the W27 anti-Hsp72/73 antibody and SDS-PAGE analysis. When assessed by 15 min ^{35}S-methionine pulse labeling, the synthesis rate of both Hsc73 and Hsp72 was enhanced as early as after 15 min of heat treatment, and continued to augment until 2 hr (Fig. 5A). No appreciable amounts of newly synthesized proteins were found coprecipitating with Hsp72/73 from unheated cells, which contrasted with the situation reported in HeLa cells [Beckmann et al., 1990]. However, several newly synthesized proteins appeared in a complex with Hsp72/73 from 30 min of heat treatment, among them 170, 58, and 56 kDa proteins which were detected in increasing amounts after 2 hr of heating. Another protein of 46 kDa present in constant quantity whatever the heating time could represent nonspecific coprecipitation. In cultures incubated with ^{35}S-methionine for 12 hr and a further 2 hr period at 42.5°C, numerous proteins coprecipitated with Hsp72/73 (Fig. 5B). Incubation of cell extracts with apyrase to degrade endogenous ATP did not alter the pattern of proteins coprecipitating with Hsp72/73 (results not shown), although such treatment has been reported to favor preservation of Hsp72/73–cellular protein interactions [Beckmann et al., 1990]. In the present study, some of the proteins detected in immunopurified Hsp72/73 complexes were present in both control and heated cells, such as the 170 kDa protein described above and a protein of 90 kDa. Yet most proteins coprecipitating with Hsp72/73 were revealed exclusively under heat stress, for instance the 58 and 56 kDa proteins already mentioned. Also, proteins with molecular masses of 135 and 95 kDa corresponding to those of insulin receptor α- and β-subunits could be observed only after heat treatment.

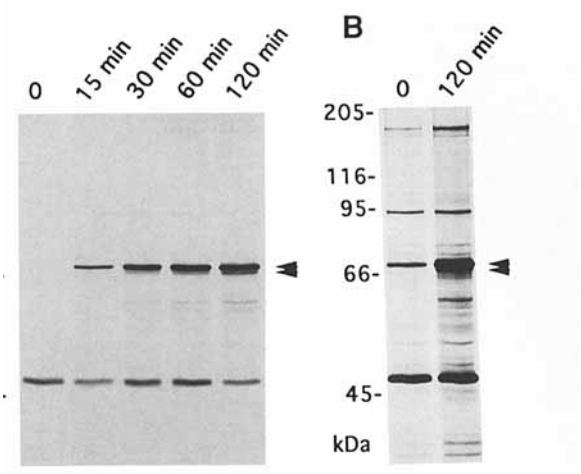

Fig. 5. Synthesis rate of Hsp72/73 and its association with cellular proteins after heat treatment. Cells were incubated with ^{35}S-methionine (40 μCi/ml) at 37°C either for 15 min at day 2 after heat treatment at 42.5°C for up to 2 hr (**A**) or for 12 hr from day 1 to day 2 before applying a 2 hr heat treatment still carried out in the presence of ^{35}S-methionine (40 μCi/ml) (**B**). RIPA-extracted material, which corresponded to 220 μg protein in A and 880 μg protein in B, was submitted to nondenaturing immunoprecipitation using an anti-Hsp72/73 antibody. The resulting immunoprecipitates were analyzed by SDS-PAGE under reducing conditions and autoradiography. Autoradiographs from control cultures or cultures heated for the indicated times are represented. Labeled bands corresponding to Hsp72 and Hsc73 are marked by *arrows*.

Immunoprecipitates obtained using antibodies directed against insulin receptor or Hsp72/73 were further examined by Western immunoblotting to determine coprecipitation of Hsp72/73 and insulin receptor, respectively. In immunopurified insulin–receptor complexes, detection with anti-insulin receptor antibody revealed two major bands of 85 and 95 kDa, together with a smear of immunoreactive material which could correspond to multiple insulin receptor crosslinked oligomeric forms (Fig. 6A, A', top). The 95 kDa band displayed a lessened intensity in immunoprecipitates from cells heated for 2 hr at 42.5°C as compared to control cells. The 95

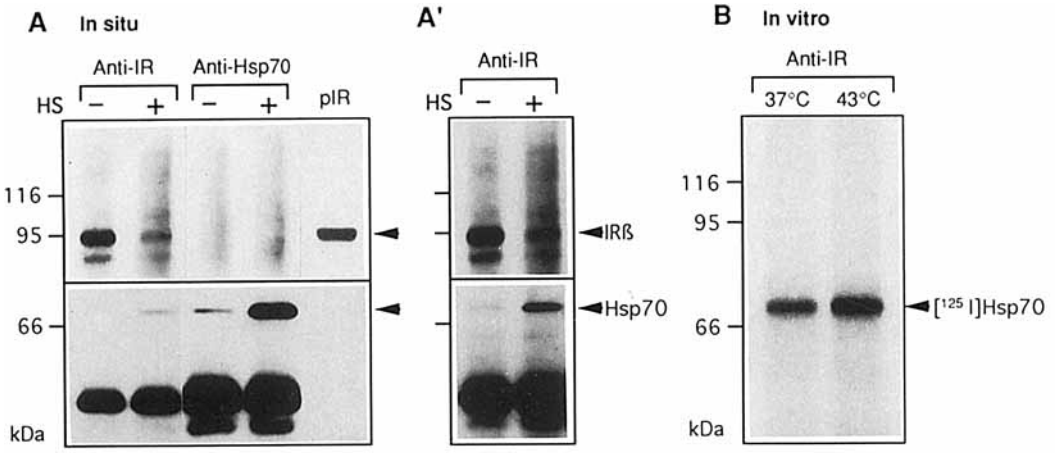

Fig. 6. Coimmunoprecipitation of Hsp72/73 with insulin receptor. **A** and **A'**: In situ. At day 2, cells were either heated at 42.5°C or maintained at 37°C for 2 hr. Immunoprecipitation using either an anti-insulin receptor β-subunit antibody or the W27 anti-Hsp72/73 antibody was performed on RIPA-extracted cellular material which corresponded to 3.6 mg and 1.8 mg protein for insulin receptor and Hsp72/73 immunoprecipitation, respectively. The resulting immunoprecipitates were analyzed by 7.5% SDS-PAGE under reducing conditions and Western immunoblotting. Upper and lower parts of blots were probed separately as described in Figure 4. Chemiluminescence visualization of immunobands was carried out for 30 sec (A) or 10 min (A'). Blots are represented for immunoprecipitates obtained from cells subjected or not to heat shock (±HS) with anti-insulin receptor (anti-IR) and anti-Hsp72/73 (anti-Hsp70) antibody, immunoreactive bands corresponding to insulin receptor β-subunit (IRβ) and Hsp72/73 (Hsp70) being indicated. WGA-purified insulin receptor (pIR) (4 μg protein) from 20-day-old fetal liver is also represented. **B:** In vitro. WGA-purified insulin receptor from fetal rat liver (4 μg protein) was incubated in PBS with ^{125}I-Hsp72/73 from bovine brain (0.2 nmoles, 200,000 cpm) for 2 hr at 37°C or 43°C as indicated prior to immunoprecipitation with anti-insulin receptor β-subunit antibody (anti-IR). The resulting immunoprecipitates were analyzed by SDS-PAGE under reducing conditions and autoradiography. Labeled band corresponding to ^{125}I-Hsp72/73 (^{125}I-Hsp70) is indicated.

and 85 kDa bands were not clearly detectable in the case of immunopurified Hsp72/73 complexes. The former band was the only one observed when analyzing WGA-purified fetal liver insulin receptor, which confirmed its identity as insulin receptor β-subunit. The slighter intensity of the insulin receptor β-subunit band in heated cells may reflect heat-provoked masking of epitopes within the β-subunit during immunoprecipitation, since insulin receptor protein level directly assessed in whole cell extracts was not affected by heating (Fig. 4A). Alternatively, the reduced amount of insulin receptor precipitated after heat shock treatment could be due to some loss of the receptor during cell lysate clarification by centrifugation since heat shock is known to provoke partition into the detergent-insoluble phase of mature proteins [Beckmann et al., 1992]. Immunodetection with anti-Hsp72/73 antibody showed in immunopurified insulin–receptor complexes from control cells a 73 kDa band which was found in higher amounts in heated cells (Fig. 6A, A', bottom). This was accompanied by the expected heat-enhanced Hsp72/73 level. The immunoreactive bands seen in each lane at 50 kDa corresponded to the heavy chains of antibodies used during immunoprecipitation.

The possible direct interaction between insulin receptor and Hsp72/73 was also investigated in in vitro experiments. Purified fetal liver insulin receptor was incubated in the presence of ^{125}I-Hsp72/73 for 2 hr at 37°C or 43°C, and immunoprecipitates obtained with the anti-insulin receptor antibody were analyzed by SDS-PAGE. A 73 kDa labeled band corresponding to ^{125}I-Hsp72/73 was coprecipitated with the purified insulin receptor (Fig. 6B). It appeared in higher amounts in immunopurified insulin–receptor complexes after incubation at 43°C.

Effect of Heat Shock on Affinity Labeling of Hsp72/73 With ^{125}I-Insulin

As heat shock interfered with insulin degradation concomitantly with an increased Hsp72/73 synthesis, it was interesting to determine whether Hsp72/73 interacted with insulin. After cell incubation for 2 hr at either 37°C or 42.5°C in the presence of 20 nM ^{125}I-insulin, protein complexes labeled with ^{125}I-insulin were stabilized by covalent crosslinking. When submitting whole cell extracts to SDS-PAGE analysis, a major band of 135 kDa as well as several minor affinity-labeled components of 175, 75, and 43 kDa were observed in both control and

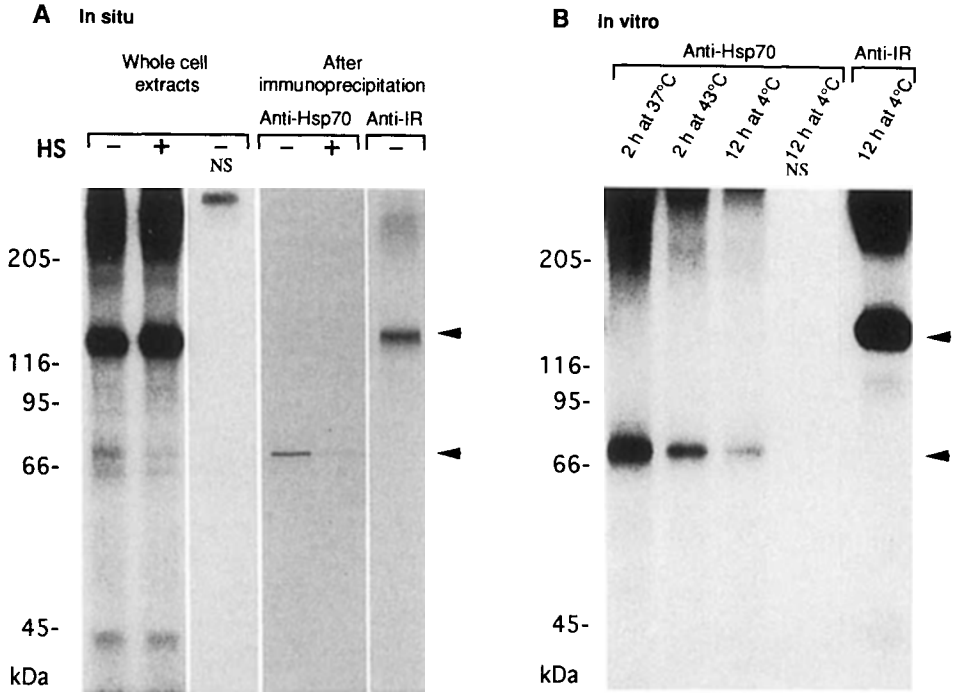

Fig. 7. Affinity labeling of Hsp72/73 with ^{125}I-insulin. **A:** In situ. At day 2, cells were incubated for 2 hr at either 37°C or 42.5°C in the presence of 20 nM ^{125}I-insulin. Then, crosslinking was performed for 30 min at 4°C in the presence of 0.2 mM DSS. Whole cell extracts obtained after cell lysis in Laemmli sample buffer were directly analyzed by 7.5% SDS-PAGE under reducing conditions, equal quantities of cellular proteins (220 μg protein) being applied to the gel. Also, RIPA-extracted material (880 μg protein) was submitted to immunoprecipitation using antibody directed against either Hsp72/73 (anti-Hsp70) or insulin receptor β-subunit (anti-IR) prior to 7.5% SDS-PAGE analysis. Cells submitted or not to heat shock (±HS) are represented. **B:** In vitro. Hsp72/73 from bovine brain (3 μg protein) or WGA-purified insulin receptor from fetal rat liver (4 μg protein) was incubated in PBS with 30 nM ^{125}I-insulin under the conditions indicated prior to crosslinking and subsequent immunoprecipitation with anti-Hsp72/73 (anti-Hsp70) and anti-insulin receptor β-subunit (anti-IR) antibody, respectively. The 135 kDa insulin receptor α-subunit and 75 kDa affinity-labeled bands are marked by arrows. Corresponding ^{125}I-insulin incubations in the presence of an excess of unlabeled insulin are also shown (NS).

heated cells, yet with a lessened intensity in heated cells for the 75 kDa band (Fig. 7A). A poorly resolved band of 300 kDa was also found. All the bands represented insulin-specific binding components since their labeling could be prevented by a simultaneous incubation with an excess of native insulin. Whole cell extracts were also processed for immunoprecipitation with an antibody directed against either Hsp72/73 or insulin receptor β-subunit prior to SDS-PAGE analysis. When considering Hsp72/73 immunoprecipitates, a single component of 75 kDa was revealed whose affinity labeling appeared reduced for heat-treated cultures as compared to unheated cells. This component might represent the 75 kDa band observed in whole cell extracts. As expected, the major 135 kDa band together with a 300 kDa labeled protein were immunoprecipitated by the anti-insulin receptor antibody, the former representing insulin receptor α-subunit and the latter likely corresponding to the α-β-subunit of the insulin receptor in a crosslinked form. It must be noted that no affinity-labeled insulin receptor species were found using the anti-Hsp72/73 antibody. This might be due, for instance, to a regulation by insulin of the possible Hsp72/73–insulin receptor association (seen in Fig. 6). Further work will be necessary to ascertain this hypothesis.

The 75 kDa labeled band immunoprecipitated by the anti-Hsp72/73 antibody, given its molecular mass, could originate from the affinity labeling of Hsp72/73 with ^{125}I-insulin. To ascertain this hypothesis, in vitro crosslinking experiments were performed. Bovine brain Hsp72/73 or insulin receptor purified from fetal rat liver was allowed to associate with 30 nM ^{125}I-insulin at different temperatures prior to crosslinking and immunoprecipitation with anti-Hsp72/73 and anti-insulin receptor antibody, respectively. Incubation of Hsp72/73 with ^{125}I-insulin revealed a 75 kDa band whose labeling was more

efficient after 2 hr at 37°C when compared with the other binding conditions tested, i.e., 2 hr at 43°C and 12 hr at 4°C (Fig. 7B). This band, which was impaired in the presence of an excess of native insulin, appeared to reproduce in vitro the affinity-labeled 75 kDa component observed in cell extracts. In the case of insulin receptor, after an incubation of 12 hr at 4°C with ^{125}I-insulin, the affinity-labeled insulin receptor pattern showed the 135 kDa insulin receptor α-subunit major band together with 110 and 43 kDa components.

DISCUSSION

In cultured fetal hepatocytes in which the development of the glycogenic response is sensitive to heat [Zachayus and Plas, 1995], mild heat treatment induced a time-dependent decrease in the ability of cells to bind as well as to degrade ^{125}I-insulin when measured at 37°C under physiological conditions. It has been reported that the amount of ^{125}I-insulin bound at steady state and the ^{125}I-insulin degradation rate are depressed to the same extent in isolated adult hepatocytes after trypsin or chymotrypsin treatment [Terris and Steiner, 1975]. Similar results have been obtained in cultured fetal rat hepatocytes whose insulin receptor level was depressed after preincubation with tunicamycine [Pringault and Plas, 1983]. In the present study, insulin degradation was also proportional to insulin binding in nonheated cells pretreated or not with tunicamycine, whereas in cells heated at 42.5°C insulin degradation was more inhibited than expected from the decrease in insulin binding. This is at variance with the situation found for HA-1 CHO cells, where heat shock induces an inhibition of insulin binding with no modification of insulin degradation [Calderwood and Hahn, 1983]. Besides, in Rat L1 cells, heat treatment causes a decrease in EGF binding and strongly inhibits the degradation of internalized EGF [Magun and Fennie, 1981]. Given the results obtained in cultured fetal hepatocytes, heat shock seemed able to exert a proper effect on insulin degradation.

Acidotropic agents, such as chloroquine, which are known to inhibit the endosomal compartment acidification process [De Duve, 1983], have been shown to inhibit insulin cellular degradation in cultured fetal hepatocytes [Plas and Desbuquois, 1982]. In our experiments, chloroquine strongly increased insulin binding at 37°C and markedly impaired the release of insulin degradation products into the medium, while heat shock depressed both insulin binding and insulin degradation. The combined effects of chloroquine and heat shock tended to prevent the stimulation of insulin binding by chloroquine and to abolish insulin degradation. The enhanced binding caused by chloroquine in cultured fetal hepatocytes reflects the accumulation of undegraded insulin within the cells [Plas and Desbuquois, 1982]. After injection of ^{125}I-insulin into chloroquine-treated rats, the amount of the insulin degradation products recovered in isolated liver endosomes was decreased by drug treatment [Clot et al., 1990]. Besides, ATP-dependent acidification of isolated endosomes which have concentrated ^{125}I-insulin administered in vivo is required for optimum insulin degradation either by promoting the dissociation of insulin–receptor complexes or by facilitating the activity of an endosomal protease [Desbuquois et al., 1990; Authier et al., 1994]. In cultured fetal hepatocytes, heat shock and chloroquine had in common the inhibition of insulin degradation, yet as they exerted opposite effects on insulin binding this suggested that the dissociation of the insulin–receptor complexes likely occurred in heated cells.

A rapid decrease in cell surface insulin binding after short exposure of cultured fetal hepatocytes to insulin, which has been shown to represent translocation of insulin receptors from the cell surface to an internal compartment [Soubigou et al., 1986], was observed in both unheated and in heat shocked cells. Then, insulin removal triggered the recovery of cell surface binding in nonheated cells, due to the reversible translocation toward the cell surface of insulin receptors previously internalized in the presence of insulin [Soubigou et al., 1986]. By contrast, the recovery of cell surface binding after insulin removal did not take place in cells heated for longer than 30 min at 42.5°C. Contrary to heat shock, it should be noted that chloroquine inhibited the recycling of internalized receptors immediately [Soubigou et al., 1987; this paper]. The decrease in insulin receptor binding which occurred in heated cells could not be attributed to an accelerated insulin receptor degradation since receptor protein level remained unchanged. Thus, the insulin binding decrease could rather correspond to a modification in the cellular distribution or in the state of insulin receptors which prevented their recycling. The importance of the endosome hepatic compartment in

the trafficking of plasma membrane receptors providing a passageway between the cell surface and the lysosomes and controlling receptor recycling has been emphasized [Evans et al., 1986]. In the case of cultured fetal hepatocytes, it has been postulated that in the presence of chloroquine insulin–receptor complexes are led to a compartment whence it is impossible for insulin receptors to be recycled to the cell surface [Soubigou et al., 1987]. The lag time observed in the inhibitory effect of heat shock on insulin receptor recycling likely corresponded to the onset of a heat shock–dependent event. In Fao hepatoma cells, some internalization and recycling of insulin receptors occurs independently of receptor occupancy by insulin, thus revealing a constitutive receptor cycle [Chvatchko et al., 1984]. An insulin-independent continual insulin receptor cycle likely occurred in the same way in cultured fetal hepatocytes. Such a constitutive cycle possibly affected by heat shock could account for a progressive and reversible sequestration of insulin receptors.

Interaction between Hsp72/73 and cellular proteins, among which are 90 and 56 kDa proteins and also insulin receptor, was revealed by coimmunoprecipitation studies. The extent of such associations was strikingly augmented after heat stress, especially in the case of insulin receptor. Also, in vitro binding experiments allowed a coprecipitation of ^{125}I-Hsp72/73 with purified fetal liver insulin receptor. In unheated cells, Hsp72/73 has been reported to be a molecular chaperone which interacts with newly synthesized proteins and facilitates their folding and assembly [Welch, 1992]. Besides, Hsp72/73, within a heterocomplex also comprising Hsp90 and Hsp56, can be linked to the glucocorticoid receptor, which regulates the ligand and DNA binding receptor functions [Diehl and Schmidt, 1993; Pratt, 1993]. Hsp72/73 has also been reported to be associated with other cell proteins, among which are keratin polypeptides [Liao et al., 1995], immunophilins [Nadler et al., 1992], tumor suppressor gene products [see Nihei et al., 1993], and the peroxisome proliferator-activated receptor [Huang et al., 1994]. In cells experiencing heat stress, mature proteins which begin to denature or unfold become new targets for Hsp72/73 [Beckmann et al., 1992]. Notably, heat-induced reorganization of intermediate filaments is accompanied by a transient vimentin–Hsp72/73 association [Cheng and Lai, 1994]. Heat shock also promotes an interaction between Hsp72/73 and nuclear topoisomerase I, which may limit thermal denaturation of the enzyme and favor restoration of its function during recovery [Ciavarra et al., 1994]. The fact that insulin receptor likely formed in unstressed fetal hepatocytes a complex with Hsp72/73 suggested that the Hsp might participate in post-translational modifications of the insulin receptor and/or in the trafficking of the receptor. Under heat stress, the physiological significance of the enhancement of Hsp72/73–insulin receptor association could be to protect the receptor from irreversible thermal damage by its possible involvement in the postulated heat-induced insulin receptor sequestration.

In cultured fetal hepatocytes, a 75 kDa component affinity labeled with ^{125}I-insulin appeared immunoprecipitated by an anti-Hsp72/73 antibody. This might reflect an interaction between the hormone and Hsp72/73. Indeed, a similar crosslinked 75 kDa component was visualized after in vitro binding between ^{125}I-insulin and purified Hsp72/73. The cognate form of Hsp70, i.e., Hsc73, has been shown to bind insulin A-chain in vitro presumably at its amino terminal sequence (GIVEQ) [Terlecky et al., 1992]. This is related to the ability of Hsc73 to recognize KFERQ-like sequence motifs within proteins that are destined for lysosomal degradation [Terlecky et al., 1992]. Hsc73 is supposed to alter the conformation of these KFERQ-containing proteins into a transport-competent state facilitating their import into lysosomes, a process which recalls the Hsp72/73 proposed role in the translocation of proteins into microsomes, mitochondria, nucleus, and peroxisomes [see Walton et al., 1994]. The latter organelles correspond to the main subcellular localization of insulin-degrading enzyme, a thiol-metalloendopeptidase postulated to play a crucial role in cellular insulin degradation [Duckworth, 1988; Authier et al., 1994]. In cultured fetal hepatocytes, Hsp72/73 might recognize insulin after hormone–receptor complex dissociation, which would lead to possible regulation of insulin processing resulting from the Hsp interaction with the hormone in the cytosol compartment. In heat-treated fetal hepatocytes where insulin degradation was inhibited, the postulated affinity labeling of Hsp72/73 with ^{125}I-insulin was lessened despite an increase in Hsp72/73 synthesis. In cells experiencing heat stress, Hsp72/73 is known to be recruited to perform a number of emergency tasks and is thus diverted from its

constitutive function [Welch, 1992]. In heated fetal hepatocytes, Hsp72/73 may no longer assume its putative role in insulin metabolism, which would account for the heat shock–induced inhibition of hormone degradation. The postulated association of both insulin and its receptor with Hsp72/73 suggests an involvement of the Hsp in the insulin–receptor complex fate, a role which should be modified in cultured fetal hepatocytes experiencing heat stress where Hsp72/73 may exert a possible chaperone-like action on insulin receptor as a means of protecting it from thermal damage.

ACKNOWLEDGMENTS

B.S. and J.L.Z., whose contributions to this work were equivalent, should be considered equally as first authors. This investigation is a part of Doctorat d'Université that B.S. will be submitting to the University of Paris 7, France. It was supported by Institut National de la Santé et de la Recherche Médicale (Grant CRE 910715) and by the "Association pour la Recherche sur le Cancer." We are grateful to Dr. M. Ali for his helpful discussions. We thank Mr. E. Marie-Rose for skilful secretarial services.

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