CMLS, Cell. Mol. Life Sci. 55 (1999) 1473–1481
1420-682X/99/111473-09 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

The effect of temperature and protein synthesis on the renaturation of firefly luciferase in intact H9c2 cells

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Received 19 May 1999; received after revision 18 June 1999; accepted 21 June 1999

Abstract. A mild increase in temperature that does not exert an effect on tolerance development or synthesis of heat shock proteins (Hsps) in control cells can stimulate these processes when applied to cells that have previously been heat shocked. To study the underlying mechanism of this effect, H9c2 cells were stably transfected with the gene encoding firefly luciferase (Luc). Heat-shock-induced inactivation of Luc and its subsequent reactivation is frequently used as a model for cellular protein denaturation and renaturation. Luc reactivation was determined following a damaging heat shock (43 or 44 °C for 30 min) in cells that were subsequently exposed to either control temperatures (37 °C) or various mild hyperthermic conditions (from 38.5 to 41.5 °C for 1 h). To prevent changes in Luc activity consequent to new synthesis of Luc, Luc reactivation was monitored in the presence of cycloheximide, an inhibitor of protein synthesis. The results showed that reactivation of Luc was inhibited when heat-treated cells were post-treated under mild hyperthermic conditions. The observed increase in Hsp synthesis under mild hyperthermic post-heat shock conditions therefore appears to be the result of an increase in the period during which denatured proteins are present. In addition, we studied Luc reactivation in the absence of protein synthesis inhibitors. This condition led to much higher Luc activity. By estimating half-life times of Luc, the contribution of new Luc synthesis in this recovery could be determined, and only partially explained the observed increase in Luc reactivation after heat shock. Thus the synthesis of other proteins must be important for the renaturation of heat-damaged proteins.

Key words. Heat shock; heat shock protein; luciferase; H9c2 myoblasts; luminescence.

Introduction

A brief and moderate heat shock causes a rapid increase in the synthesis of heat shock proteins (Hsps) and initiates development of thermotolerance, resulting in an increased ability to survive exposure to otherwise lethal temperatures [1–3]. Hsps are known to fulfil crucial roles in cellular protection and repair by binding to denatured proteins, reshaping damaged proteins and dissociation of protein aggregates [4–6]. Furthermore, it has been suggested that Hsps are involved in the development of a thermotolerant state [2, 7–9].

Immediately after stress exposure and prior to the development of tolerance, a short period of increased stressor sensitivity has been observed [7, 10–12]. Our recent studies have shown interesting consequences of this period of increased sensitivity for Hsp synthesis and development of tolerance. When low doses of physical or chemical stressors are applied during this sensitive period following a more vigorous stress, survival capacity and the synthesis of heat shock proteins were enhanced [12–16]. This stimulation was observed at low

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doses of stress conditions that alone could not induce Hsp synthesis.

The aim of the present study was to determine the underlying mechanism of this stimulatory action. According to the generally accepted model of Hsp induction, heat-denatured proteins are the molecular signal for the induction of heat shock gene transcription [2, 17, 18]. An increase in denatured proteins activates the heat shock factor (HSF) which binds to the regulatory element of heat shock genes, initiating their transcription followed by Hsp mRNA production and subsequent synthesis of Hsps. As chaperones, Hsps can bind to (partly) denatured proteins and assist in their renaturation, thereby decreasing the amount of denatured proteins and terminating the signal for Hsp induction. We recently developed a mathematical model of this feedback process [19]. Based on simulations using this model, we propose that interference with the process of renaturation might result in a prolonged binding of Hsps to denatured proteins, continuation of the activated state of HSF and continuation in the synthesis of Hsps. Mild hyperthermic conditions have previously been demonstrated to lead to prolonged existence of an activated form of HSF in cells that were previously heat-shocked [20]. In the work described here, we investigated whether protein renaturation is inhibited when heat-shocked cells are post-incubated under mild hyperthermic conditions.

In mammalian cells, firefly luciferase (Luc) provides one of the best non-toxic and sensitive enzymes to study characteristics of the processes of denaturation and renaturation of thermolabile proteins in the same temperature range as the onset of heat-induced cellular protein denaturation [21, 22]. Heat inactivation and subsequent reactivation of Luc is usually studied in cell lysates. Recently, changes in Luc activity following a heat shock were detected in suspensions of living plant cells [23] and mammalian cell cultures [24]. We showed that Luc activity can be continuously monitored inside the cell in a quantitative, non-invasive and highly sensitive way, allowing the inactivation and reactivation kinetics of Luc in monolayer cultures of mammalian cells to be monitored in detail [24].

With the use of Luc as a reporter enzyme we examined whether a slight increase in incubation temperature following a more vigorous heat shock modulates the reactivation kinetics of heat-inactivated Luc. Rat myoblast H9c2 cells were stably transfected with a modified firefly luciferase gene whose product is expressed in the cytoplasm. It could be demonstrated that reactivation of Luc was inhibited when heat-treated cells were post-treated under mild hyperthermic conditions. Furthermore, the contribution of overall protein synthesis to reactivation of Luc was evaluated by comparing reactivation rates in the presence or absence of protein synthesis inhibitors.

Materials and methods

Chemicals. Cell culture media were purchased from Gibco/Life Technologies (Alphen a/d Rijn, The Netherlands). ATP assay mix, firefly luciferase and ATP were obtained from Sigma (St. Louis, USA) and luciferin from Applichem GmbH (Darmstadt, Germany).

Cell culture. The embryonic rat heart-derived cell line H9c2 was obtained from the American Type Culture Collection (CRL1446). The cells were propagated as monolayer cultures with L15 medium supplemented with potassium penicillin G (100 units/ml), streptomycin sulphate (100 μg/ml), and 10% foetal calf serum (Gibco/Life Technologies). The H9c2 cells were stably transfected with the plasmid pGL3 luciferase reporter vector (Promega) comprising a modified luciferase gene integrated under the control of the SV40 promoter with the SV40 late poly(A) signal and the SV enhancer sequence. Along with other changes (see technical manual pGL3 of Promega), the code for the C-terminal tripeptide has been removed to eliminate peroxisome targeting of the expressed protein and allow constitutive expression of enzyme activity in the cytoplasm. Transfection was carried out with the pGL3 vector with a neomycin resistance gene insert by the standard calcium phosphate precipitation method. Stably transfected cells were selected by incubation of the cells in culture medium containing geneticin (Gibco/Life Technologies). For experiments, cells were grown as monolayers in 8-cm^2 dishes.

Protein synthesis, gel electrophoresis and analysis of labelled proteins. Protein synthesis was measured by incorporation of [^{35}S]-methionine and [^{35}S]-cysteine (specific activity of both amino acids: 1300 Ci/mmol; Amersham, Bristol, UK). For incorporation studies, L15 medium without methionine and cysteine was used to which 5 μCi of the radioactive tracers were added/ml medium. Labelling was carried out for various time intervals (0–2, 2–4, 4–6 and 6–8 h). Cells were then lysed and solubilized in sample buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 10% β-mercaptoethanol, 15% glycerol). Radioactivity incorporated into protein was determined as the radioactive label in the hot trichloroacetic-acid-precipitable material, measured as counts per minute in the liquid scintillation counter. Proteins from samples containing equal amounts of radioactive protein were separated by polyacrylamide gel electrophoresis (acrylamide 10%, bisacrylamide 0.27%) according to Laemmli [25]. Autoradiography was performed using Hyperfilm-MP (Amersham). The labelled proteins were quantified using a laserscan (Enhanced Laser Densitometer, Ultrascan XL, LKB Bromma, Pharmacia, Woerden, The Netherlands). For valid comparisons of the values ob-

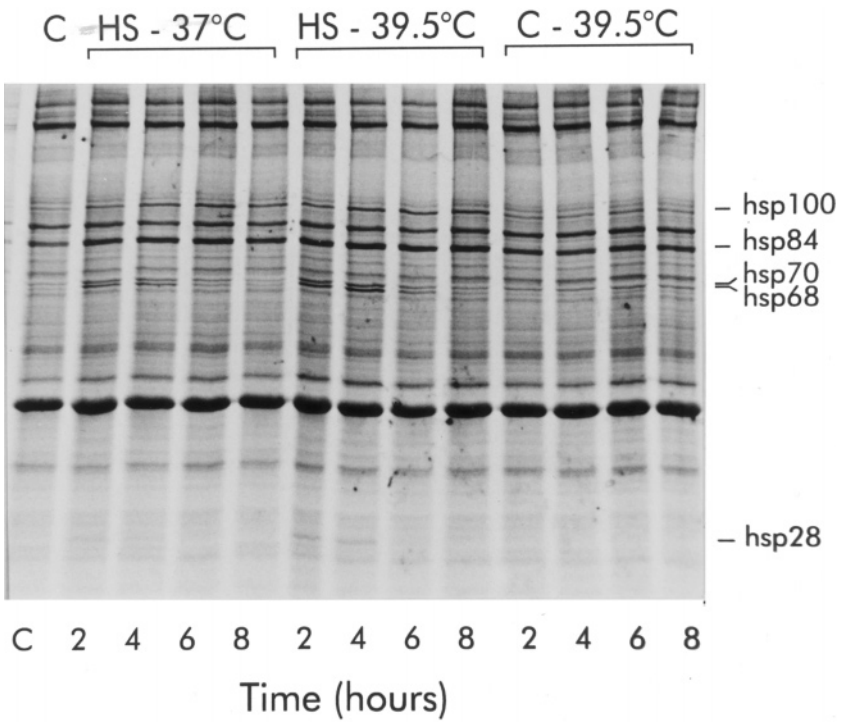

Figure 1. Kinetics of induction of Hsp synthesis in H9c2 cell cultures pre-treated with a heat shock at 43 °C for 30 min followed by a continuous treatment at either control temperature (HS-37 °C) or at a mild hyperthermic temperature (HS-39.5 °C) for 10 h. The effect of incubation at 39.5 °C without a previous heat treatment (C-39.5 °C) is also shown. At various times after pre-treatment, cultures were incubated with [^{35}S]-labelled amino acids for 2 h. Samples were subsequently prepared for gel electrophoresis. Equal amounts of radioactivity were used in each lane. The different Hsps are indicated by their molecular weight (kDa). The first lane C represents proteins from control cells without any heat treatment.

tained from the different bands between different autoradiograms, the absorbance of each band is expressed relative to the total absorbance of the respective lane. In this paper we used these data of relative Hsp synthesis to identify a possible synergistic action when low stressor doses are applied to stressed cell cultures. No correction for total protein synthesis took place, since no significant differences in total protein synthesis values were observed between the different stressor treatment protocols used.

Luc assay in intact cells. Luc activity of an intact monolayer of pGL3-transfected H9c2 cells was determined by placing this culture in the temperature-controlled chamber of the photodetection system. The cell monolayer was approximately 7 cm below the photomultiplier tube window of the photon counter. The photon counter, in single-photon counting mode, was equipped with a Hamamatsu R550 photomultiplier tube (spectral response 280–850 nm, 1.5 kV) kept at −20 °C. Standard high-performance photon-counting electronics consisting of a low-noise preamplifier, amplifier, discriminator and ratemeter were used. Under these conditions, background or dark current of the photomultiplier amounted to 60–90 counts per second. Luciferin was added 30 min before the measurement at a final concentration of 0.1 mM.

Heat shock. Cells were exposed to heat shocks by placing the culture dishes either in a waterbath in which the temperature was regulated within 0.1 °C or in the temperature-controlled chamber of the photodetection system which was regulated within 0.2 °C. Temperature equilibration of the cell cultures took about 1 min. The photodetection system allowed quantitative measurements of luminescence either during the heat treatment itself at the chosen heat shock temperature or before and immediately after the heat treatment at 37 °C.

Results

Mild hyperthermic conditions increase heat-shock-induced synthesis of Hsps. To study the effect on H9c2 cells of mild hyperthermic temperatures after a pre-treatment with a heat shock, a protocol was selected that includes a 30-min heat shock at 43 or 44 °C followed or not by an 8-h mild hyperthermic treatment at 39.5 or 40.5 °C. It must be noted that these conditions are non-lethal: neither the mild hyperthermic conditions nor the short heat shocks affect cell survival significantly (not shown).

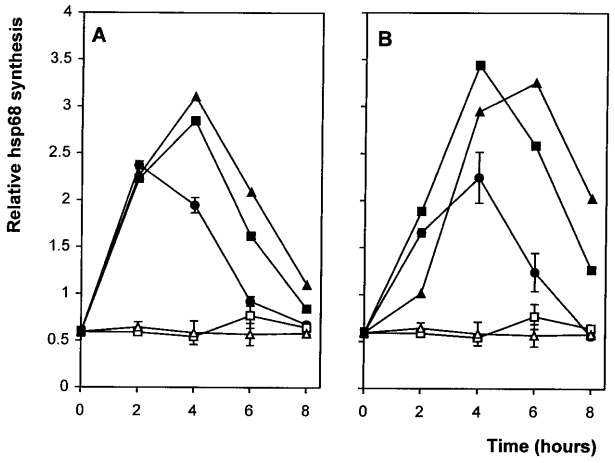

Figure 2. Hsp68 synthesis of H9c2 cells after a pretreatment (closed symbols) of 43 °C (*A*) or 44 °C (*B*) or without a pretreatment (open symbols) followed by an incubation period at 37 °C (●), 39.5 °C (□, ■) or 40.5 °C (△, ▲) at the indicated time. Time points represent Hsp synthesis during the preceding (2 h) labelling period.

Heat shock at temperatures of 43 or 44 °C induced the synthesis of Hsp. The individual Hsps were identified by their increased synthesis at higher temperatures, as described before [26]. H9c2 myoblastic cells incubated for up to 8 h under mild hyperthermic conditions (39.5 and 40.5 °C) showed no induction of Hsps. However, when the same incubation conditions were applied immediately after a short heat shock at a higher temperature (30 min 43 °C) an enhancement of Hsp synthesis was observed (fig. 1).

As an example of the stimulation of Hsp induction by a mild hyperthermic treatment temperature after a pretreatment with a heat shock, quantitative analysis of the induced Hsp68 is presented in figure 2. Whereas exposure to a mild hyperthermic treatment for 8 h did not induce any synthesis of Hsp68 in naive (non-pretreated) cell cultures, it increased the amount of Hsp68 synthesized by a factor of 1.7 in cultures previously heat shocked at higher temperatures.

The synthesis of Hsps is stimulated in the presence of thermally denatured cellular proteins. Here we hypothesize that the observed enhancement in induction under mild hyperthermic post-heat shock conditions is either the result of an increase in the amount of denatured proteins or an increase in the period during which these proteins are present. To test these hypotheses, we studied the effect of these mild hyperthermic conditions on inactivation and reactivation of firefly Luc, a protein frequently used as a model for heat-induced protein denaturation and renaturation.

Inactivation and reactivation of Luc in the presence of a protein synthesis inhibitor: the effect of mild hyperthermic conditions. Luc inactivation and reactivation in cultures of transfected H9c2 cells can be continuously assessed in intact cells, a very desirable feature for studying denaturation and renaturation kinetics. Furthermore, no artefacts will occur due to cell lysis. In figure 3, cellular luciferase activity is presented during exposure to mild hyperthermic temperatures up to 41.5 °C (fig. 3A). To study inactivation and reactivation of Luc without interference by new Luc synthesis, cells were incubated in the presence of 5 μM cycloheximide to prevent de novo synthesis of Luc. The concentration of

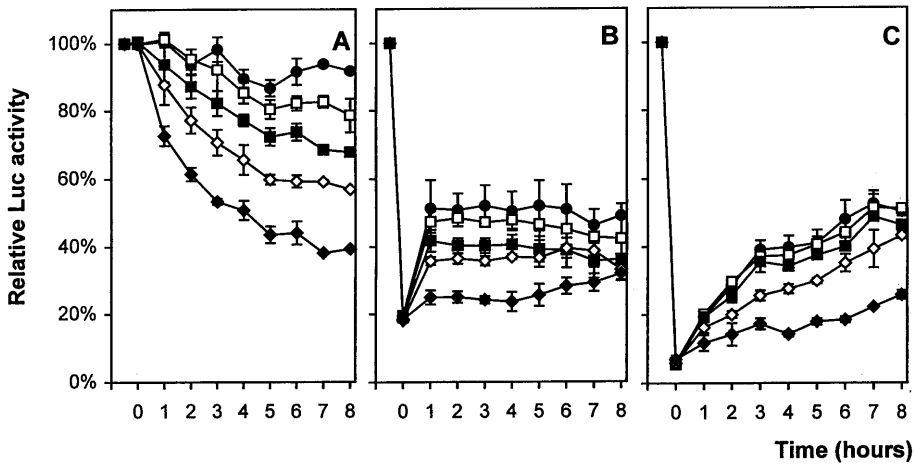

Figure 3. Relative change in Luc activity in H9c2 cells in the presence of 5 μM cycloheximide without a pre-treatment (*A*) or after pre-treatments of either 30 min at 43 °C (*B*) or 44 °C (*C*), followed by an incubation period at 37 °C (●), 38.5 °C (□), 39.5 °C (■), 40.5 °C (◇) or 41.5 °C (◆). Data points are the average values of three experiments with the standard error as error bars. Cycloheximide was added just after the pre-treatment and was present throughout the experiments.

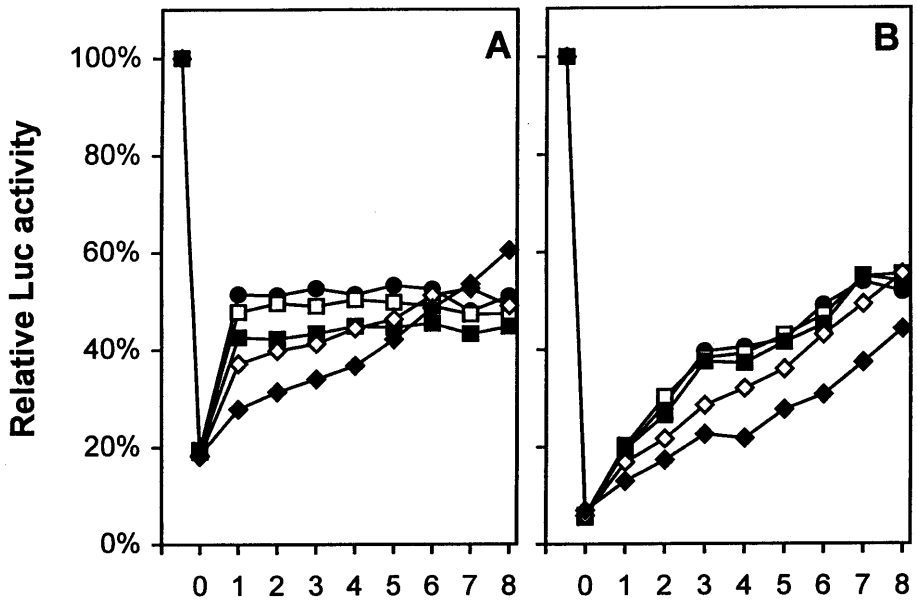

Figure 4. Renaturation of Luc activity in H9c2 cells in the presence of 5 μM cycloheximide after pre-treatments of either 30 min at 43 °C (*A*) or 44 °C (*B*), followed by an incubation period at 37 °C (●), 38.5 °C (□), 39.5 °C (■), 40.5 °C (◇) or 41.5 °C (◆). The data are the same as in figure 3 but corrections were made for the denaturation of Luc during the mild hyperthermic treatments without any prior pre-treatment. The assumption was made that the denaturation process after a pre-treatment does not change. Data points are the average values of three experiments; for better visualization error bars are omitted. Cycloheximide was added just after the pre-treatment and was present throughout the experiments.

cycloheximide used inhibits protein synthesis by more than 90% (as can be seen in fig. 3A, at 37 °C and in the absence of protein synthesis, only about a 10% decrease in Luc activity was found during a period of 8 h). At the higher temperatures, the rate of Luc inactivation increased. After a heat shock of 30 min at 43 or 44 °C, the Luc activity decreased to 15% and 5%, respectively. Reactivation of Luc in cells that were first exposed to these heat shocks is demonstrated in figure 3B, C. This figure shows that (renaturation) reactivation of Luc at the various temperatures is initially fast (first hour after heat shock) and then proceeds at a lower speed. A more detailed analysis of the kinetics of this biphasic renaturation process has been described elsewhere [24].

Furthermore, it can be seen that the activity of Luc at the higher renaturation temperatures (38.5–41.5 °C) increases at a lower rate and thus reaches a lower level of activity within the time period studied. Since some inactivation of Luc occurs in this temperature range, these primary data do not allow accurate estimation of the reactivation rates of Luc at the various temperatures. However, if it is assumed that the relative Luc inactivation in pre-heated samples is the same as that in the non-pre-heated samples, the experimentally obtained data on Luc activity can be corrected and the reactivation rates calculated. The amount of renatured Luc is approximately equal to the net increase in Luc activity at the given temperature supplemented with the amount of inactivated Luc in that period. For this correction, the Luc inactivation rate at each temperature was determined and these values were multiplied by the average amount of Luc in that period. This approximation can only be used if the amount of inactivated Luc in that period is relatively small compared to the amount present, which is indeed the case. Figure 4 shows the renaturation of Luc activity through time, as calculated by the summation of the amounts of renaturated Luc of the successive periods. From this analysis, it is concluded that the reactivation process of Luc is already inhibited at mild hyperthermic temperatures.

Effect of cycloheximide on Luc renaturation. The protein synthesis inhibitor cycloheximide was used during the period of renaturation to prevent an increase in Luc activity that might be ascribed to the synthesis of new Luc proteins. Thus the question was asked whether a change in the renaturation kinetics is related to new Luc synthesis comparing the reactivation of Luc in the absence and presence of cycloheximide.

Figure 5 depicts the effect of the mild hyperthermic treatments (fig. 5A) on Luc activity in the absence of cycloheximide. In comparison with the rate of Luc inactivation at the mild hyperthermic temperatures and in the presence of cycloheximide (fig. 3A), it can be concluded that only minor differences in the loss of Luc activity are found.

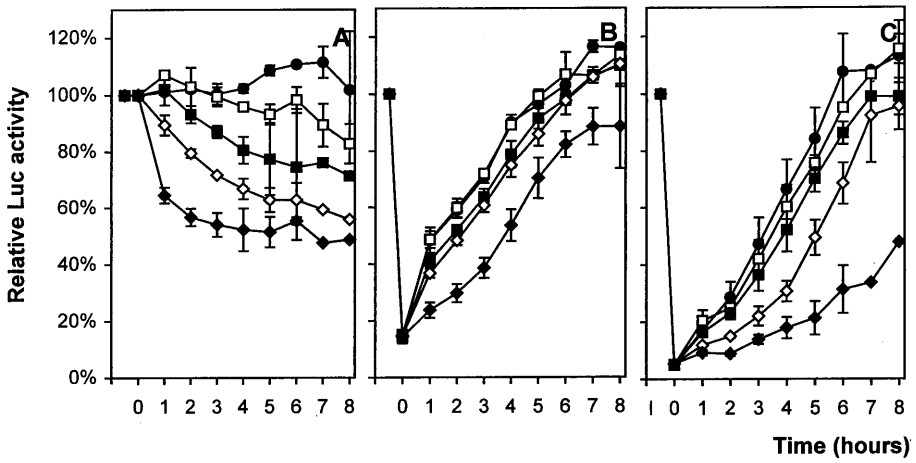

Figure 5. Relative change in Luc activity in H9c2 cells in the absence of cycloheximide, without a pre-treatment (*A*) or after pre-treatments of either 30 min at 43 °C (*B*) or 44 °C (*C*), followed by an incubation period at 37 °C (●), 38.5 °C (□), 39.5 °C (■), 40.5 °C (◇) or 41.5 °C (◆). Data points are the average values of three experiments with the standard error as error bars.

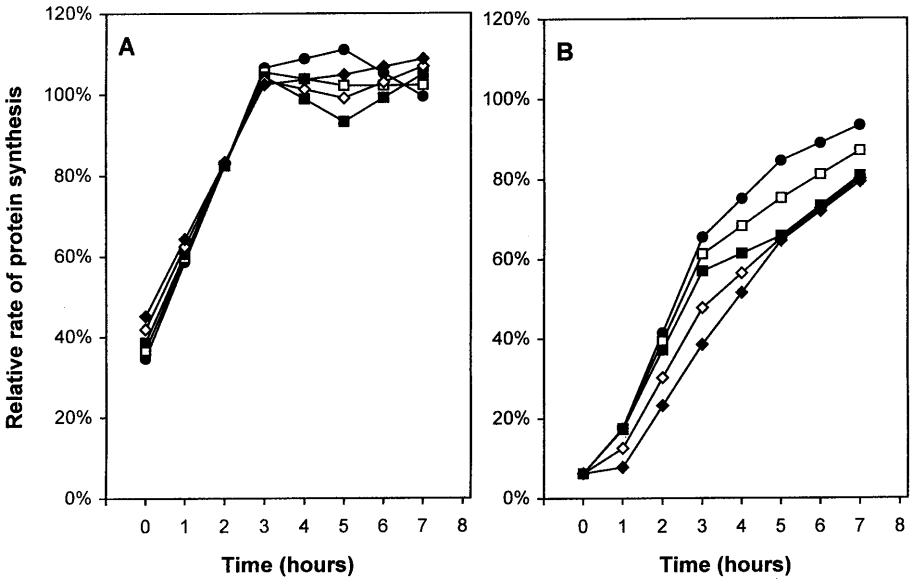

Figure 6. Recovery of the rate of total protein synthesis of H9c2 cells after a pre-treatment with a heat shock of 43 °C (*A*) or 44 °C (*B*) followed by incubation at 37 °C (●), 38.5 °C (□), 39.5 °C (■), 40.5 °C (◇) or 41.5 °C (◆).

However, the effect of cycloheximide becomes evident when the reactivation of Luc is measured from 2–3 h and onwards after heat shock pre-treatments (fig. 3B, C). During the first few hours, only small differences are observed. From the first part of the curves we conclude that cycloheximide, at least initially, does not interfere substantially with the reactivation process and that in the first few hours after heat shock, new synthesis of Luc does not substantially contribute to the increased Luc activity. However, after this period, the activity of Luc reaches substantially higher levels in the absence of cycloheximide, possibly as a result of new Luc synthesis.

To estimate the contribution of new Luc synthesis to the increased Luc activity, we assumed that in control cells an equilibrium exists between Luc synthesis and its degradation since in these cells Luc activity does not change. The synthesis rate can then be derived from the half-life of Luc. Using the decay of Luc activity after inhibition of protein synthesis by cycloheximide, the calculated half-life appeared to be 27 h ($\pm$3 h SD) in control cells and the synthesis rate was estimated to be 2.6% per hour of the amount of Luc already present.

To calculate the contribution of newly synthesised Luc to the increase in Luc activity observed during the

reactivation period, we determined the net protein synthesis rate in this period. As shown in figure 6, the rate of protein synthesis recovers within 3 h after a heat shock at 43 °C, whereas in the 44 °C-treated cells, recovery did not reach its pre-heat shock values even after a recovery period of 7 h. Luc renaturation was then calculated based on (i) the degree of Luc inactivation at the renaturation temperatures used, (ii) the data of the rate of total protein synthesis and (iii) the assumption that the new synthesis of Luc behaves as the average synthesis of proteins in the cells (fig. 7 A, B). Based on these calculations, it could be concluded that de novo synthesis of Luc makes only a small contribution to the increase in Luc activity observed after heat treatment. To illustrate the observed differences between the reactivation kinetics of Luc in the presence and absence of cycloheximide in more detail, this difference is shown in figure 7C, D: after a lag period of 2–3 h following heat shock, a large increase in Luc reactivation occurred in the cells that were not treated with the protein synthesis inhibitor, an increase which is not due to the synthesis of new Luc molecules.

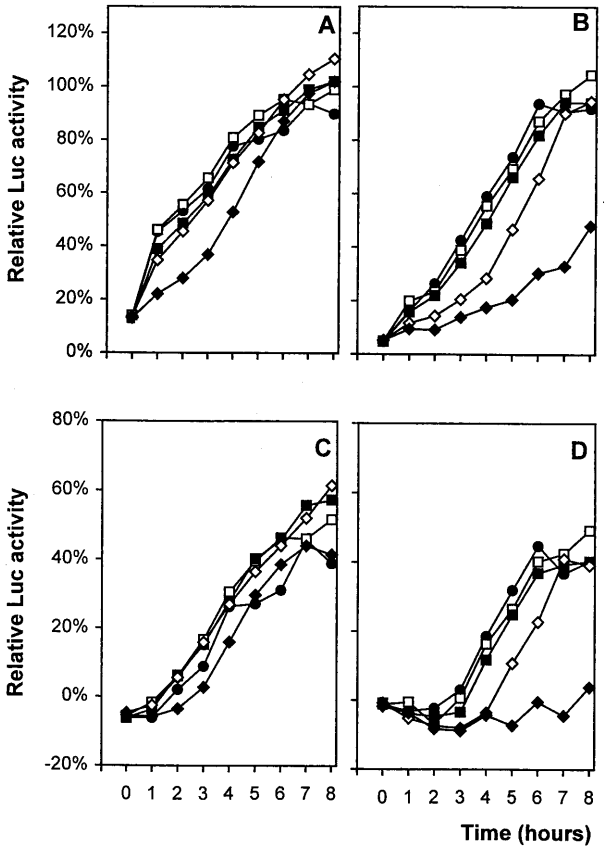

Figure 7. (*A*, *B*) Renaturation of Luc activity in H9c2 cells in the absence of cycloheximide after pre-treatments of either 30 min at 43 °C (*A*) or 44 °C (*B*), followed by an incubation period at 37 °C (●), 38.5 °C (□), 39.5 °C (■), 40.5 °C (◇) or 41.5 °C (◆). Same data as in figure 5 but corrections were made for the denaturation of Luc during the mild hyperthermic treatments without any prior pre-treatment and for Luc synthesis. The assumption was made that the denaturation process after a pre-treatment does not change and that Luc synthesis is proportional to the total protein synthesis rate. Data points are the average values of three experiments; for better visualization error bars are omitted. (*C*, *D*) Extra renaturation in the presence of protein synthesis after pre-treatments of either 30 min at 43 °C (*C*) or 44 °C (*D*), followed by an incubation period at 37 °C (●), 38.5 °C (□), 39.5 °C (■), 40.5 °C (◇) or 41.5 °C (◆). Data points represent the differences between the data shown in figure 4 and *A*, *B* in this figure. For better visualization, error bars are omitted.

Discussion

In this paper, we have shown that mild hyperthermic temperatures that do not exert any effect on synthesis of Hsps in control cells are able to stimulate this synthesis when applied to heat-shocked H9c2 rat myoblastic cells. This observation is in agreement with our previous work in Reuber H35 rat hepatoma cells [12–14]. Both the development of tolerance as well as synthesis of Hsps, which were induced by exposure to high doses, were shown to be further enhanced upon subsequent application of a low dose of the same stressor. Low doses of other agents, such as heavy metals, oxidative stressors and sulphydryl reagents, have also been shown to stimulate tolerance development and Hsp synthesis when applied to these heat-shocked hepatoma cells [15, 16].

As an extension of this work, the present paper focuses on the mechanism underlying the stimulatory action on Hsp synthesis by low doses of stress conditions that alone could not induce Hsp synthesis. According to the autoregulation model of Hsp synthesis [19, 27], the kinetics of reactivation of heat-denatured proteins might play a crucial role in the time period that Hsp70 remains induced and synthesized. Thus, we hypothesized that inhibition of protein renaturation may prolong time period that Hsps are required for binding to stress-damaged proteins, which in turn may prolong the activated state of the HSF and thus extend the induction and synthesis of Hsps. In agreement with this, Ovelgönne and Van Wijk [20] showed a prolonged presence of HSF in its activated form as well as increased levels of Hsp mRNA when heat-shocked hepatoma cells were exposed to mild hyperthermic conditions.

In this paper we focused on a possible role of the kinetics of protein renaturation in Hsp induction, by studying the reactivation kinetics of heat-inactivated Luc. Since Luc has frequently been used as a model protein to study reactivation of heat-denatured proteins [23, 28–32], we investigated whether a slight increase in

incubation temperature following a more vigorous heat shock could modulate the kinetics of heat-inactivated Luc. Renaturation of Luc was studied in the presence of the protein synthesis inhibitor cycloheximide to prevent new synthesis of Luc. From our results, it could be concluded that Luc reactivation is indeed inhibited when heat-treated cells were post-treated under mild hyperthermic temperatures.

In addition, we studied the role of protein synthesis during the process of Luc reactivation by comparing differences in reactivation in the presence or absence of a protein synthesis inhibitor (cycloheximide). During the recovery of the rate of protein synthesis and especially from 2–3 h after heat shock, differences in Luc reactivation became apparent, only a small proportion of which could be attributed to new synthesis of Luc. Thus a protein-synthesis-dependent factor, probably the synthesis of a protein, which manifests after a delay of a few hours, plays a role in the optimal renaturation of Luc.

Hsps play an important role in the protection and/or repair of damaged proteins [2]. Recently, we compared the parental cells (Rat-1) with Hsp68-overexpressing cells (HR24) and showed that Hsp68 is important for the inactivation and reactivation kinetics of Luc [24]. In the cells that stably expressed enhanced Hsp68 levels, thermal Luc inactivation decreased, and subsequent reactivation yielded higher activity levels, in comparison with the parental cell line. We therefore suggest that the increase in Hsp synthesis, especially of the inducible forms that are not present before heat shock, may lead to an increase in reactivation of Luc.

In summary, our data demonstrate that, both in the absence or presence of protein synthesis inhibitors, reactivation of Luc was inhibited when heat-treated cells were subsequently exposed to a mild increase in temperature. These results suggest that an inhibition of the protein renaturation process, and thus a prolongation of the period in which substantial amounts of denatured proteins are present, extends the time period in which Hsps are synthesized. Modulation of renaturation following stress conditions may be of interest for modulating the synthesis of Hsps and consequently the level of tolerance that is achieved.

Acknowledgements. The financial support of the HomInt organization is gratefully acknowledged.

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