Research Article

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


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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 standard calcium phosphate precipitation method. Stably transfected cells were selected by incubation of the cells in culture medium containing genetin (Gibco/Life Technologies). For experiments, cells were grown as monolayers in 8-cm² dishes.

Protein synthesis, gel electrophoresis and analysis of labelled proteins. Protein synthesis was measured by incorporation of [14C]-methionine and [35S]-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 (acylamide 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-