THE INFLUENCE OF DEXAMETHASONE ON HSP70 LEVEL AND ASSOCIATION WITH GLUCOCORTICOID RECEPTOR IN THE LIVER OF UNSTRESSED AND HEAT-STRESSED RATS

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Summary: The aim of the present study was to examine the influence of dexamethasone on the levels of heat shock protein Hsp70 and glucocorticoid hormones receptor, as well as on the interaction of these two proteins in the liver cytosol and nuclei of unstressed and rats exposed to whole body hyperthermic stress. The results, obtained by quantitative immunoblotting, have shown that dexamethasone provoked a reduction of Hsp70 basal level and an increase in its stress-induced level in the nuclei, supporting the idea that this hormone may be a factor included in the regulation of Hsp70 level both under normal and stress conditions. The cytosolic reduction and nuclear elevation of the glucocorticoid hormones receptor level by dexamethasone were also observed. Co-immunopurification of Hsp70 and glucocorticoid hormones receptor has revealed that the changes of cytosolic and nuclear levels of the two examined proteins resulted in the changes of their interaction within the respective cellular compartments. Thus, 41 °C heat stress, was shown to cause at least two-fold elevation of Hsp70/GR ratio within the glucocorticoid hormones receptor heterocomplexes both in the presence and in the absence of dexamethasone. The results support the view that glucocorticoid hormones signaling pathway and heat shock system are interrelated.

Key words: Hsp70, whole body hyperthermic stress, dexamethasone, glucocorticoid receptor

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Introduction

At cellular level, all living organisms respond to unfavorable conditions, such as heat shock, by a rapid and transient synthesis of heat shock proteins (Hsps) constituting an important family of intracellular protective proteins. The most abundant, evolutionary conserved and phylogenetically ubiquitous among them is Hsp70 subfamily. In mammalian cells it comprises two main nucleocytoplasmic isoforms: stress inducible, Hsp72, and constitutive, Hsp73 (Hsc70). Hsp70 family members are essential for survival under both normal and stress conditions. They are important components of protein folding pathways and are found in complexes with many proteins, including steroid hormones receptors (1).

The role of glucocorticoid hormones in systemic defense reactions to stress has been well documented long ago (2). However, the question of their implication in molecular events underlying cellular response to stress has remained opened. The most of the numerous physiological effects of these hormones is mediated through the specific receptor protein present in almost all mammalian cells. It is well known that glucocorticoid receptor (GR) acts as hormone-inducible transcription factor, and that, when unliganded, it occurs in target cells in the form of multiprotein complexes with several Hsps, immunophilins and some other proteins (1). Assembly process of the three Hsps (Hsp90, Hsp70 and Hsp60), which comprise the core of ubiquitous and conserved assembly system, proceeds through different transition heterocomplex states (3, 4). The role of Hsp70 in this process and even its presence within the GR heterocomplexes is still a matter of debate. Hsp70 is generally known to play a chaperoning role in protein folding (5) and transport from cytosol to nucleus (6). When present in GR heterocomplexes, this protein is bound directly to the hormone-binding domain of the receptor in sub-
When appropriate, dexamethasone (5 mg/kg b.w.) was i.p. administered to adrenalectomized rats 4 h before the sacrifice. Hyperthermic stress was achieved by placing the animals anesthetized with Nesdonal (4.6 mg/100 g b.w., i.p.) into ventilated and humidified chamber preset to 44 °C until rectal body temperature, continuously monitored by a digital thermometer with suitable sonde, reached 41 °C, which usually took about 45 min. After 15 min of maintaining the rectal temperature at 41 °C, the rats were transferred to room temperature and sacrificed 2 h later. For the hyperthermic stress at 42 °C, animals were placed into the same chamber and after approximately 55 min, when their rectal temperature reached 42 °C, they were transferred to room temperature. The control (unstressed) animals were treated in the same way, except that they were kept in a hood at room temperature.

**Materials and methods**

**Chemicals**

Monoclonal antibodies N27F3-4 (anti Hsp72/73) and BuGR2 (anti GR) were the products of StressGen (Victoria, British Columbia, Canada) and Affinity BioReagents (Neshanic Station, NJ), respectively. 125I-cojugated sheep anti-mouse IgG was obtained from ICN Pharmaceuticals (Costa Mesa, CA), Protein A-Sepharose, nonimmune mouse IgG and horseradish peroxidase-conjugated goat anti-mouse IgG from Sigma (St. Louis, MO), Nesdonal from Specia (Paris, France) and dexamethasone from ICN-Galenika (Belgrade, Yugoslavia).

**Animals and treatment**

Male rats of Wistar strain (200–250 g b.w., 2.5 months old), reared under standard laboratory conditions (22 °C, 12/12 h light-dark cycle), were deprived of glucocorticoid hormones by bilateral adrenalectomy 4 days before exposure to hyperthermic stress. When appropriate, dexamethasone (5 mg/kg b.w.) was i.p. administered to adrenalectomized rats 4 h before the sacrifice. Hyperthermic stress was achieved by placing the animals anesthetized with Nesdonal (4.6 mg/100 g b.w., i.p.) into ventilated and humidified chamber preset to 44 °C until rectal body temperature, continuously monitored by a digital thermometer with suitable sonde, reached 41 °C, which usually took about 45 min. After 15 min of maintaining the rectal temperature at 41 °C, the rats were transferred to room temperature and sacrificed 2 h later. For the hyperthermic stress at 42 °C, animals were placed into the same chamber and after approximately 55 min, when their rectal temperature reached 42 °C, they were transferred to room temperature. The control (unstressed) animals were treated in the same way, except that they were kept in a hood at room temperature.

**Preparation of liver cytosol and nuclei**

The livers were perfused in situ with cold saline and homogenates were prepared in 2 vol. (w/v) of 50 mmol/L Tris buffer, pH 7.55 containing 0.25 mol/L sucrose, 25 mmol/L KCl, 10 mmol/L MgCl2 and 20 mmol/L Na2MoO4. After the first centrifugation (10 min, 6000 g, 4 °C), the supernatants were separated and re-centrifuged (1 h, 105 000 g, 4 °C) to obtain cytosols. The lipid layers floating on top of the cytosols were aspirated and cytosols stored in liquid nitrogen until use.

The pellets obtained after the first centrifugation were used to prepare purified nuclei (20) which were washed and stored in liquid nitrogen in 10 mmol/L Tris-HCl buffer pH 7.35 containing 0.25 mol/L sucrose, 3 mmol/L MgCl2 and 1 mmol/L β-mercaptoethanol.

**Immunoadsorption of GR heterocomplexes from cytosol**

The immunoadsorbent was prepared by rotating (30 min, 4 °C) 8 mL Protein A-Sepharose pellets with 2 μg BuGR2 in 400 mL TEG buffer (10 mmol/L TES, pH 7.6 at 4 °C, containing 50 mmol/L NaCl, 4 mmol/L EDTA, 10% (w/v) glycerol and 20 mmol/L Na-molybdate). After addition of 200 μL cytosol (6 mg protein), the rotation was continued for another 3 h at 4 °C (21). The immune pellets were washed three times by suspension in 1.0 mL TEG buffer and centrifugation. For nonimmune controls, the preimmune mouse IgG was used instead of BuGR2. The immunoadsorbed proteins were extracted by boiling in 5% SDS-sample buffer and subjected to SDS-PAGE.

**Immunoadsorption of GR heterocomplexes from nuclei**

Pellets of purified nuclei, containing 7 mg DNA, were resuspended in 100 mL 10 mmol/L Hepes buffer pH 7.4 containing 1 mmol/L EGTA and 5 mmol/L...
MgCl₂, and incubated at room temperature for 5 min with 10 μg DNase I. After addition of 55 μL 5 mol/L NaCl and 250 μL 10 mmol/L Hepes buffer pH 7.4 containing 1 mmol/L EGTA, 10% (v/v) glycerol and 20 mmol/L Na-molybdate, the samples were subjected to sonication (2 × 15 s at 10 MHz). The nuclear lysates were then rotated with the immunoadsorbent previously prepared as described above. Pelleted agarose beads carrying immunoadsorbed nuclear GR heterocomplexes were washed 3 times with TEG buffer, boiled for 5 min in 2×SDS-sample buffer and loaded onto SDS-polyacrylamide gels.

**Gel electrophoresis**

Proteins were resolved according to Laemmlli (22) in 7.5% SDS-polyacrylamide gels using Mini-Protein II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA). Myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa) were simultaneously run as molecular mass references.

**Immunoblotting**

Western transfer of proteins from polyacrylamide gels to nitrocellulose membranes was performed in 25 mmol/L Tris buffer, pH 8.3 containing 192 mmol/L glycerine and 20% (v/v) methanol, at 135 mA overnight in Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). Unbound sites on the membranes were blocked by 20 mmol/L Tris buffer, pH 8.3 containing 192 mmol/L glycerine and 20% (v/v) methanol, at 135 mA overnight. The membranes were incubated with 125I-labeled counter antibody. Membranes were exposed to phosphor-storage screens, scanned by PhosphorImager (Molecular Dynamics), and numerical data summarized in Figure 1A. The PhosphorImaging analysis provided numerical data summarized in Figure 1B. The results have shown that dexamethasone administration provoked only small and insignificant elevation of the basal Hsp70 level in the liver cytosol (about 10% over the unstressed level) and its somewhat more pronounced (about 25%) reduction in the nuclei.

**Miscellaneous**

Protein concentration in the cytosols and DNA content of isolated nuclei were determined by the methods of Lowry et al. (23) and Burton (24), respectively.

**Results**

To examine the influence of glucocorticoid hormones on Hsp70 level in the liver cytosol and nuclei of unstressed and heat stressed rats, the adrenalecotomized rats were administered with dexamethasone (5 mg/kg b.w.) and four hours later exposed to hyperthermic stress of different intensities (41 °C and 42 °C). Hsp70 level was determined by quantitative immunoblotting with anti-Hsp70 monoclonal antibody N27F3-4 recognizing both constitutive and inducible forms of the protein (Figure 1A). The PhosphorImaging analysis provided numerical data summarized in Figure 1B. The results have shown that dexamethasone administration provoked only small and insignificant elevation of the basal Hsp70 level in the liver cytosol (about 10% over the unstressed level) and its somewhat more pronounced (about 25%) reduction in the nuclei. When applied in combination with 41 °C hyperthermic stress, dexamethasone rendered the cytosolic Hsp70 concentration at the level achieved by the stress alone (70% over the basal level), and increased the nuclear level of the protein by about 20% over the stress-induced level (i.e. about 50% over the basal level). In combination with 42 °C heat stress, however, the glucocorticoid led to 50% and 10% increases of the cytosolic and nuclear Hsp70 concentrations, respectively, as compared to the stress-induced concentrations. Interestingly, 42 °C hyperthermia was found to affect only the nuclear level of Hsp70, increasing it for about 60% above the unstressed control.
Since the increased cellular level of Hsp70 observed after heat stress and/or dexamethasone administration might affect the composition of GR multienzyme heterocomplexes, it was of interest to examine the share of Hsp70 within the complexes after these treatments. To that end, molybdate-stabilized GR heterocomplexes were immunoadsorbed to Protein A-Sepharose with BuGR2 anti-GR antibody from the liver cytosols or nuclear lysates of untreated (control) animals and of animals exposed to 41°C hyperthermic stress and/or dexamethasone treatment. Subsequently, GR and co-immunoadsorbed Hsp70 were detec-
The results have shown that dexamethasone led to considerable reduction of GR level in the liver cytosol of both unstressed and animals heat-stressed at 41 °C (Figure 2). On the other hand, it stimulated nuclear localization of the GR exerting much more pronounced effect in unstressed than in heat-stressed animals. The cytosolic reduction and nuclear elevation of the GR level by dexamethasone were the expected findings, as it is well known that glucocorticoids provoke transformation of their receptor to the DNA-binding state and its translocation from the cytoplasm to the nucleus of target cells. However, it was found that at 41 °C hyperthermic stress affects GR concentration and its intra-cellular localization, as well, causing a 30% decrease in the cytosolic GR level and a 50% increase in its level in the nuclei in comparison with unstressed controls.

Determination of Hsp70 level within immunopurified untransformed GR heterocomplexes (Figure 3) has revealed that dexamethasone elevated Hsp70 content by approximately 70% within the cytosolic and 20% within the nuclear GR heterocomplexes of control (unstressed) rats. In combination with 41 °C hyperthermic stress, however, the hormone suplementation provoked 30% and 10% decreases in Hsp70 content within cytoplasmic and nuclear GR heterocomplexes, respectively, as compared to heat stress alone, while heat treatment led to several-fold increase in GR-associated Hsp70 level in both cellular compartments.

Taking that the normal stoichiometric Hsp70/GR ratio within the GR heterocomplexes is 1/1, it was estimated from the data presented in Figures 2C and 3C that this ratio increased to 2.5/1 for the cytosolic, and to 12/1 for the nuclear heterocomplexes after exposure of the rats to 41 °C heat stress. Supplementation with dexamethasone enlarged Hsp70/GR ratio within the cytosolic heterocomplexes of unstressed rats to 2.4/1, and diminished it within the nuclear ones to 0.2/1. When applied together with 41 °C hyperthermia, dexamethasone increased the molar ratio Hsp70/GR to 3.5/1 and to 8.5/1 within the cytosolic and the nuclear heterocomplexes, respectively (Figure 4).

When heat stress- and/or dexamethasone-related changes of the total Hsp70 concentrations in the liver cytosol and nuclei were compared with the changes of the abundance of this protein within respective GR heterocomplexes (Figure 5), it was found that the elevation of total Hsp70 level was always followed by the elevation of its concentration within the complexes in the relevant cellular compartment, albeit the elevations were not of the same magnitude. The only exception were the nuclei of the rats injected with dexamethasone in which a small reduction of Hsp70 level by the hormone was followed by a small increase in Hsp70 share within the GR heterocomplexes.

**Discussion**

The GR is a glucocorticoid hormone-activated transcription factor involved in physiological processes that serve to protect organisms against stress (2, 25). Heat shock response is a well-known cellular adaptation to stress that is mediated by a family of transcription factors termed heat shock factors (HSFs), HSF1 being the best studied one (9). Recently it has become clear that both GR and HSF1 in their inactive states occur in the cytoplasm associated with Hsps, Hsp70 and Hsp90 in particular (1, 26, 27). These data raised an idea that heat shock and GR signaling pathways are interrelated. Up to now in a number of studies the influence of heat shock on steroid receptor functions has been observed (28–30). For example, it has been shown that heat stress led to considerable loss of GR binding capacity, which coincided with a decrease in the receptor protein amount in the cytosol and its increase in the nuclei (31–33) of target cells. On the other hand, the evidence for control of the heat shock response by steroid hormones is scarce (34–36), although it has been well documented that these hormones are implicated in the regulation of Hsps basal levels (13–19).

The results obtained in this study show that administration of synthetic glucocorticoid, dexamethasone, to heat-stressed rats leads to an elevation of Hsp70 level which in the liver nuclei overcomes the elevation achieved by the stress alone. This finding supports the idea that dexamethasone may be a factor included in the regulation of Hsp70 level under stress conditions. Dexamethasone-related Hsp70 induction might be explained by a possible cooperation of HSF and GR in Hsp70 gene expression. This assumption originates from the findings of Konishi et al. (37) who noticed hyperthermia-related changes in methylation protection of the sequences surrounding some of the HSE elements in the promoter region of the rat liver Hsp70 gene, and proposed a cooperative association between HSF and another transcription factor. It is, therefore, possible to postulate HSF-GR interaction as a mechanism by which dexamethasone might modulate Hsp70 gene transcription under stress conditions.

The additional evidence for steroid hormones-dependent induction of Hsps came from Sun and Knowlton (35, 36) who observed that dexamethasone and other steroid hormones could activate HSF1 in adult cardiac myocytes and induce a 60% increase in the level of Hsp72, which then served as a protector against cardiac injury. The authors explained the observed effects of steroids by altered Hsp90-HSF1 interaction suggesting that steroid hormone receptors recruit Hsp90 from these complexes and liberate HSF1, thus enabling it to trimerize and act as an upregulator of Hsp70 gene expression. Similarly, Xiao and DeFranco (38) demonstrated that overexpression of steroid receptors in transiently transfected COS-1 cells induces HSF activity and explain this observation by a seques-
toration of Hsps from Hsp-HSF complexes and their recruitment by steroid receptors. However, these two hypotheses differ in a crucial detail. The first one postulates that the addition of steroid ligands leads to the increase in the number of steroid receptor-Hsps complexes and consequently to HSF activation. On the contrary, the second one proposes that ligand binding abolishes HSF activation by facilitating the release of Hsps from the receptor complexes and stimulating their participation in negative HSF regulation. Overexpressed androgen receptor was registered as a unique exception with the ability to activate HSF both in the absence and in the presence of the hormone (38). It is obvious that elucidation of the mechanism(s) underlying the role of steroid hormones in the regulation of Hsps level under stress conditions requires further investigations.

The fact that Hsp70 is at least transient, if not stable, constituent of GR heterocomplexes, prompted us to examine whether dexamethasone- and/or heat stress-induced alterations of cytosolic and nuclear Hsp70 levels influence Hsp70-GR interaction within the respective cellular compartments. The estimation of Hsp70/GR molar ratio within GR heterocomplexes has shown that heat stress, both in the presence and in the absence of dexamethasone, leads to at least two-fold elevation of this ratio within the complexes. It could be supposed that the role of increased Hsp70 share within the receptor heterocomplexes is to confer stabilization of the complexes and protection of the GR against harmful effects of heat, as well as to aid its transport to the nucleus. This assumption seems to be logical with respect to the well documented role of Hsp70 in GR heterocomplexes assembly process and nuclear transport (1, 6, 8). Besides, this assumption is supported by the results of our previous (32) and present studies showing that 41 °C hypertermic stress provokes a reduction of the cytosolic GR level and its parallel elevation in the nuclei.

The observation that the level of GR-associated Hsp70 depends on the total Hsp70 concentration in the relevant cellular compartment, both in the presence and in the absence of the glucocorticoid ligand, seems to be important in light of the possibility that stress and other (patho)physiological states, characterized by the elevated level of Hsp70 might affect the GR functions, and hence, modulate cellular responsiveness to glucocorticoid hormones even when the circulating levels of glucocorticoids remain unchanged.

Apart from the effects of dexamethasone on Hsp70 level and association with the GR, the results presented in this paper also reveal that whole body hypertermic stress causes an increase of Hsp70 level both in the rat liver cytosol and in the nuclei. Between the two stresses of different intensities, the more severe one (42 °C) was more effective in stimulating Hsp70 nuclear localization. These findings are in concert with the data suggesting that during stress Hsp70 accumulates in the nuclei to protect important regulatory processes, including formation of pre-ribosomal complexes, processing of pre-mRNAs and protein transport from the cytoplasm to the nucleus (39).

The results of the present study demonstrate that dexamethasone is involved in the regulation of Hsp70 and GR cytoplasmic and nuclear levels in the rat liver both under normal and stressful conditions, affecting also the interaction between these two proteins. These findings provide yet another link between the signaling pathways triggered by heat shock and glucocorticoid hormones and represent the basis for further investigations aimed at elucidating their possible physiological and medical significance.
References


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