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Genetic Variations of Heat Shock Protein 84 in Mice Mediate Cellular Glucocorticoid Response

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Key Words

Heat shock protein 90 • Glucocorticoid receptor • Heat stress • Dexamethasone • Geldanamycin

Abstract

Heat shock protein 90 (Hsp90), encoded by *hsp84* and *hsp86* in mice, has been confirmed to modulate glucocorticoid receptor (GR) function; however, the contribution of Hsp90 in glucocorticoid (GC) sensibility/resistance has received less attention. Previously, we found that genetic variations of Hsp84 are related to differences in the *in vivo* GC-GR responses between BALB/c and C57BL/6 mice suffering from traumatic injury. To evaluate the modulation of Hsp84 polymorphisms on the GC response, we used a cellular heat-stress injury (HSI) model combined with a transgene-plasmid infection approach and assessed HSI-induced cellular damage and GR nuclear translocation, with or without dexamethasone pretreatment. We demonstrated that after HSI, fibroblasts from the C57BL/6 line exhibit higher cellular survival, higher nuclear GR levels and lower lactate dehydrogenase activity compared to those from the BALB/c line. We showed that dexamethasone-rescued HSI-induced damage is accompanied by increasing nuclear GR levels in both lines. Importantly, this protection against HSI was greater in C57BL/6 fibroblasts and was resistant to geldanamycin, a selective inhibitor of Hsp90. Importantly, transfection of the hsp84-transgene from C57BL/6 mice increased the nuclear GR levels and lessened HSI-induced damage in BALB/c fibroblasts. Our data thereby demonstrate that Hsp84 from C57BL/6 mice modulates higher cellular GC-GR responsiveness.

Introduction

Due to its multitude of therapeutic effects on various diseases, glucocorticoid (GC) is widely used to restrain the inflammatory and neuroendocrine responses against a variety of challenges, including pathogen exposure and stress [1]. Unfortunately, a subpopulation of patients either fail to respond to GC analogue-based therapy initially or fail to respond at relapse, thus presenting a challenge for researchers and clinicians in the use of GC treatment [2-4]. However, the mechanism of GC resist-

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ance is still not fully understood, and multiple mechanisms, such as pre-receptor ligand metabolism, receptor isoform expression, and receptor-, tissue-, and cell type-specific factors, contribute to generating the diversity and specificity of the GC response [3, 5-9]. Therefore, in addition to GC and the glucocorticoid receptor (GR), factors relating to GC-GR function are potential candidates for use in uncovering GC resistance and might be potential therapeutic targets.

Heat shock protein 90 (Hsp90), a ubiquitous molecular chaperone protein encoded by the hsp84 and hsp86 genes in mice, is a pivotal regulator of stress protection and influences GR function [10-13]. The variants of Hsp90 directly lead to the alteration of hormone-binding activity and the nuclear translocation ability of GR [14-17]. We previously demonstrated that genetic variations of the hsp84 gene (Genbank Accession No: AF465639) may account for the different levels of responsiveness of the GC-GR pathway and consequently *in vivo* stress resistance in mice.

To verify the variations of Hsp84 on the modulation of GR responsiveness at the molecular level, we employed a cellular heat-stress injury (HSI) model combined with a transgene-plasmid infection approach and pharmacological pretreatment with dexamethasone, and then assessed HSI-induced cell death, lactate dehydrogenase (LDH) activity and GR nuclear translocation in fibroblasts from C57BL/6 and BALB/c mice. Moreover, we evaluated the effect of Hsp84 variations on sensitivity to various doses of geldanamycin (GA), a selective Hsp90 inhibitor, to further explore the potential mechanisms of the Hsp90-mediated GC response.

Materials and Methods

Primary fibroblast culture

Primary fibroblasts were obtained from the back skin of 4-6 week-old BALB/c and C57BL/6 mice. Fibroblasts were cultured as previously described [18]. Briefly, cells were incubated in a humidified atmosphere of 5% CO_2 and grown in Dulbecco's minimum essential medium (DMEM, Hyclone) supplemented with 10% foetal bovine serum (FBS, Hyclone), 50 units/ml penicillin and 50 μg/ml streptomycin. Fibroblasts at passage 3-5 were used for all experiments.

Construction and transfection of transgene-plasmids

The original Hsp84 expression plasmid from BALB/c, named K390S, was kindly provided by Dr. Françoise Cadepond (Institut National de la Santé et de la Recherche Médicale, France). The plasmid from C57BL/6, named TCB2D, was constructed in our lab. The full-length sequences of murine hsp84 from both strains of mice were PCR amplified using the plasmids K390S and TCB2D as templates and the following primers: 5'-CTC GCT AGC ACT ACT CGG CTT TCT CGT-3' and 5'-CTT CTC GAG GCT ACC AAC CCT GCT ATC TGT G-3'. These primers (Takara) include specific cleavage sequences for the restriction endonucleases NheI and XhoI. After being digested and prepared with NheI and XhoI (Promega), the cleaved products were cloned into the pcDNA3.1 (+) plasmid (Invitrogen). The construct-expressing plasmids were named pcD3-BH84 (containing the full-length hsp84 gene from the BALB/c mouse) and pcD3-CH84 (containing the full-length hsp84 gene from the C57BL/6 mouse).

The plasmids were transfected into cultured fibroblasts from BALB/c mice using the Lipofectamine™ transfection reagent (Invitrogen). Cells were first seeded into 96-well plates at a density of 10^4 cells/100 μl per well and incubated for 24 h before transfection, and then the cells were transfected with 100 ng of Hsp84 plasmid (pcD3-BH84 or pcD3-CH84). Cells transfected with the pcDNA3.1 (+) plasmid were used as a control.

Cellular heat-stress injury (HSI) model and drug treatments

For cellular heat-stress injury experiments, fibroblasts were processed with a one-hour incubation at 42°C in the presence of 5% CO_2 and then cultured at 37°C after this heat stress until the cells were used for post-HSI assessments of survival rate and LDH activity (30 min post-HSI) or for Western blots (4 h and 24 h post-HSI). Cells were incubated with 50 nM Dexamethasone (Dex), and either 0.1, 1 or 10 μM GA (LC Laboratories, USA) for one hour prior to heat stress.

Assessment of cellular survival rates

The survival rate of the cells was measured to observe the extent of injury and to determine the distinctions between the two strains of cells under various conditions. Thirty minutes after HSI, cellular survival rates were examined using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Fibroblast cells were incubated at 37°C in 96-well plates with 20 μl of MTT solution (5 mg/mL in PBS) for 4 hours. DMSO (100 μl per well) was then added to solubilise the crystals in the viable cells and the absorbance was read at 490 nm using a Bio-TEK Synergy HT reader (BioTek Instruments, Inc.). The optical density (OD) values represent the quantity of viable cells.

Measurement of LDH activity

LDH is released into the culture supernatant when cells are damaged, and the concentration of LDH indicates the extent of cell injury. LDH activity (U/L) was assayed with an LDH assay kit (Nanjing Jiancheng Research Institute, China). The optical density was measured at 570 nm.

Western blots assay

Western blots were used to examine the cytoplasmic and nuclear expression of GR to indirectly assay translocation capacity. The translocation capacity was calculated according to the ratio of cytoplasmic to nuclear localisation of GR. Cellular

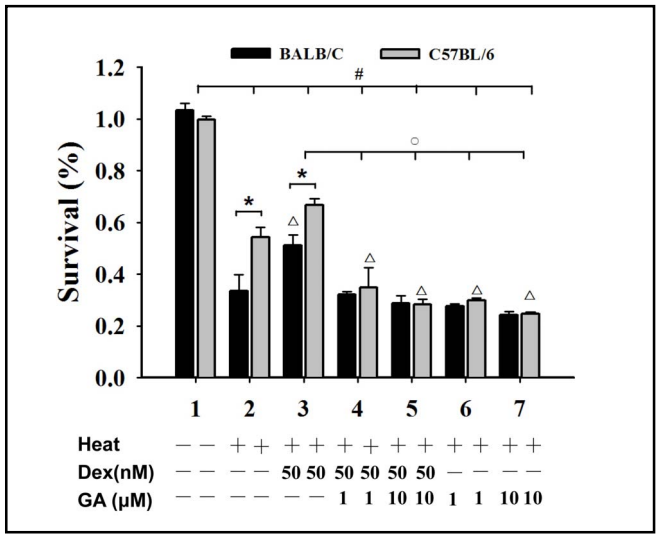

Fig. 1. Alterations of survival rates in murine fibroblasts. Cells were pretreated with Dex and GA for one hour before heat stress, and the cells were examined half an hour after heat stress. The survival rates (%) = OD value / control (group 1) OD value. # indicates P<0.05, comparing the corresponding groups to the control group (group 1); △ indicates P<0.05, comparing the corresponding groups to the heat stress group (group 2); O indicates P<0.05, comparing the corresponding groups to the Dex group (group 3); * indicates P<0.05, comparing BALB/c fibroblasts to C57BL/6 fibroblasts in the same treatment group. Data are expressed as the mean ± S.E.M. One-way ANOVA and the Student-Newman-Keuls test were used, n=6 wells per group.

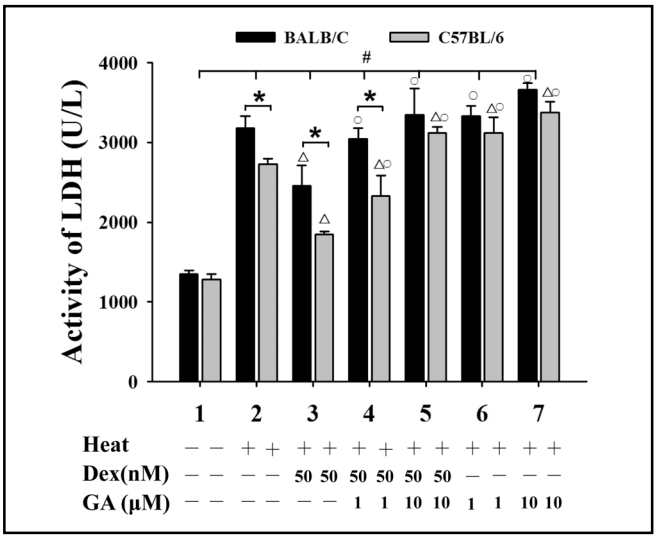

Fig. 2. Alterations of LDH in murine fibroblasts. Cells were pretreated with Dex and GA for one hour before heat stress and the cells were examined half an hour after heat stress. # indicates P<0.05, comparing the corresponding groups to the control group (group 1); △ indicates P<0.05, comparing the corresponding groups to the heat stress group (group 2), O indicates P<0.05, compared to the Dex group (group 3); * indicates P<0.05, comparing BALB/c fibroblasts to C57BL/6 fibroblasts in the same treatment group. Data are expressed as the mean ± S.E.M. One-way ANOVA and the Student-Newman-Keuls test were used, n=6 wells per group.

lysates were prepared as described in the literature [19] with some modifications, then separated by 11% SDS-PAGE and transferred onto PVDF membranes. After blocking for an hour with 5% skim milk in PBS, membranes were incubated with antibodies against GR (1:500, H300, Santa Cruz) then incubated with a peroxidase-conjugated anti-rabbit antibody (1:5,000, Santa Cruz). Bands were detected with a chemiluminescent reagent. Optical densities of the GR immunoreactive bands were measured on a UVP BioImaging acquisition and image analysis system (Ultra-Violet Products Ltd, UK). An antibody against β-actin (1:5,000, A3854, Sigma) was used to normalize GR immunoreactivity.

Statistical analysis

All data are presented as the mean ± S.E.M. Statistical analyses were performed by one-way ANOVA and the Student-Newman-Keuls Test. A $P < 0.05$ was considered to be significant.

Results

Fibroblast survival rates

The survival rates of the two strains of fibroblasts remained almost unaltered when treated with two doses of GA versus non-treated control cells. However, HSI significantly decreased the survival rates of both lines to 33.6% and 54.4% for BALB/c and C57BL/6, respectively, which is, independent of GA treatment (p<0.05). When treated with Dex, HSI-induced cell death was partially recovered in both strains, while C57BL/6 fibroblasts showed a higher survival rate than BALB/c fibroblasts (p<0.05). Importantly, the Dex-mediated cellular protection against HSI was blunted or diminished between both strains of cell when co-treated with Dex and GA along with HSI. No difference in survival rate observed between BALB/c and C57BL/6 strains (p>0.05) and all fibroblasts were severely damaged. In addition, the survival rates of both lines were slightly increased when Dex and GA was administrated simultaneously than the group GA was treated alone under heat stress (Fig. 1).

LDH activity assay

To further evaluate strain-selective susceptibility to heat stress, LDH activity was measured as a cellular biomarker reflecting the extent of heat stress-induced cellular damage. Cellular LDH release was increased substantially post-HSI, but there was no dominant difference between the strains under normal conditions, re-

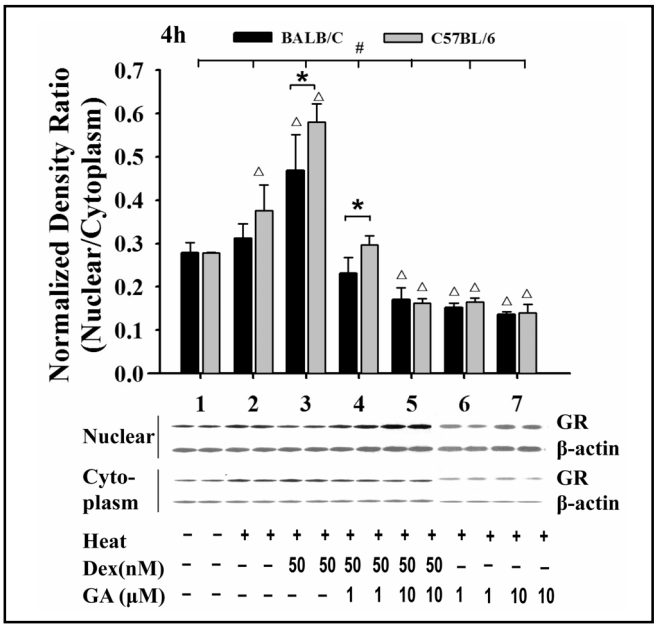

Fig. 3. Alterations of nuclear translocation of GR 4 hours after heat stress. Cells were pretreated with Dex and GA for one hour before heat stress and the cells were examined 4 hours after heat stress. The capacity of GR translocation is indicated as a normalized density ratio [(Nuclear GR/Nuclear β-actin)/(Cytoplasmic GR/Cytoplasmic β-actin)]. △ indicates P<0.05, comparing the corresponding groups to the control group (group 1); #indicates P<0.05, compared to the Dex group (group 3); * indicates P<0.05, comparing BALB/c fibroblasts to C57BL/6 fibroblasts in the same treatment group. The results are representative of 3 independent experiments and expressed as the mean ± S.E.M. One-way ANOVA and the Student-Newman-Keuls test were used.

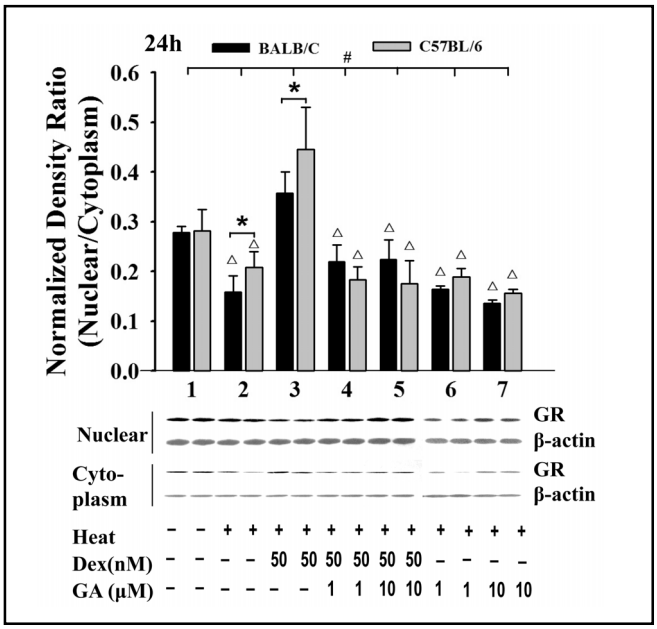

Fig. 4. Alterations of nuclear translocation of GR 24 hours after heat stress. Cells were pretreated with Dex and GA for one hour before heat stress and the cells were examined 24 hours after heat stress. The capacity of GR translocation is indicated as in Fig. 3. △ indicates P<0.05, comparing the corresponding groups to the control group (group 1); #indicates P<0.05, compared to the Dex group (group 3); * indicates P<0.05, comparing BALB/c fibroblasts to C57BL/6 fibroblasts in the same treatment group. The results are representative of 3 independent experiments and expressed as the mean ± S.E.M. One-way ANOVA and the Student-Newman-Keuls test were used.

gardless of whether GA was added. Moreover, Dex treatment significantly decreased HSI-induced LDH release indicating that Dex protects fibroblasts from HSI. However, combined treatment of GA with Dex decreased Dex-mediated protection against HSI by showing an increased LDH releasing. Noticeably, Dex-mediated protection was further blunted by 10 μM GA treatment (versus 1 μM GA) devoid strain difference, whereas 1 μM GA treatment resulted a lower LDH release in C57BL/6 strain versus BALB/c (P<0.05). These results indicate that high doses of GA may offset the difference between the two strains of cells (Fig. 2).

Nuclear translocation of GR under heat stress conditions

To explore the modulatory role of Hsp90 on GR nuclear translocation, the nuclear and cytoplasmic levels of GR were assessed by Western blot. We found that the cytoplasmic translocation of GR was increased substantially both at 4 and 24 hours post-heat stimulus. In addition, pretreatment with Dex increased the nuclear GR levels in the groups of both HSI alone and HSI combine GA treatment (versus corresponding non-Dex pretreated groups, p<0.05). The Dex-mediated protection was more obvious at 1 μM GA co-treated group versus 10 μM GA co-treatment. These results indicate the protective effect of Dex by facilitating GR nucleotranslocation. In contrast, GA inhibits the translocation of GR into the nucleus by specifically binding to the ATP domain of Hsp90 and disrupting the GR complex. Furthermore, C57BL/6 fibroblasts displayed higher levels of nuclear translocation versus BALB/c fibroblasts at the lower dose (1 μM GA) 4 h after stress (Fig. 3). No significant difference was observed in the translocation of GR at either dose of GA 24 h post-HSI (Fig. 4). The overall expression levels of GR tend to increase during the early stage after HSI then decrease afterwards in late stage.

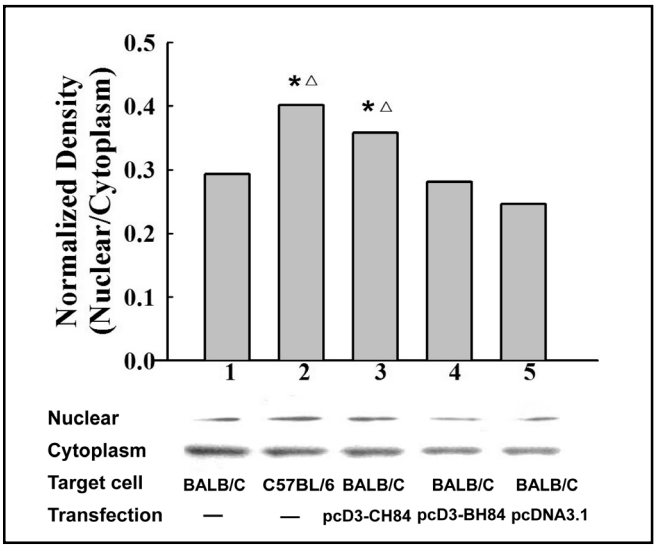

Fig. 5. The Hsp84 expression plasmids from C57BL/6 mice enhanced GR translocation. BALB/c fibroblasts were transfected with 100 ng per well of the corresponding plasmids. The cells were examined 24 hours after transfection. GR translocation is indicated by normalized density (nuclear GR level/ cytoplasmic GR level). * indicates $P<0.05$, comparing the corresponding groups to the control group (group 1); △ indicates $P<0.05$, comparing the corresponding groups to group 4.

Effect of HSP84 transfection on GR levels

In order to verify whether the alteration in the conformation of Hsp90 is the key factor responsible for the differences in glucocorticoid susceptibility, the pcD3-CH84 and pcD3-BH84 plasmids were transfected into BALB/c fibroblasts (which have a lower susceptibility to glucocorticoid than C57BL/6 fibroblasts). Western blots were used to measure the nuclear and cytoplasmic levels of GR and to assess the translocation capacity of GR. Our results show that at the time-point of 24 h post-plasmid transfection the nuclear and cytoplasmic levels of GR were no significant difference between pcD3-CH84-, pcD3-BH84- and pcDNA3.1-transfected cells, when compared with blank control cells (Fig. 5). However, the ratios of nuclear-to-cytoplasmic GR in pcD3-CH84-transfected BALB/c cell and naïve C57BL/6 cells were higher than those in pcD3-BH84-transfected or naïve BALB/c cells. This suggests that the strains-related different glucocorticoid susceptibility of fibroblasts is attributed to differences of GR translocation between BALB/c and C57BL/6, partially mediated by cellular Hsp90. The two Hsp90 expression plasmids used in present study perform a different function while C57BL/6 fibroblasts exert a high-performance form of Hsp90.

Discussion

It has been well recognized that GC resistance is the major challenge preventing the use of GC treatment against various diseases [20, 21]. The present study was designed to address two questions: (i) To determine whether factors beyond glucocorticoid and its receptor contribute to the GC response. (ii) To verify whether our previously demonstrated Hsp90 polymorphisms are responsible for the different GC responses and the consequent cellular protection against heat-stress injury (HSI) *in vivo*. Most studies to date have focused on the role of the GC levels and the isoforms of GR on the efficacy of the GC-GR pathway [22-26]. However, this concept has recently been extended and other components, such as Hsp90, with its conserved genomic features, have been implicated in GC resistance [27]. Moreover, the genetic variations of Hsp90 have been suggested to play an important role in the response to traumatic injury by regulating the GC-GR pathway [28].

Four major findings were demonstrated in the present study and are associated with the different conformations of Hsp84 found in the C57BL/6 and BALB/c mouse stains: *(i)* Compared to the BALB/c line, fibroblasts from the C57BL/6 line showed higher GR nuclear translocation, greater cellular survival and lower LDH activity after HSI. *(ii)* Dexamethasone (Dex) treatment rescued HSI-induced cellular damage with enhanced nuclear GR levels. Importantly, this Dex-mediated protection was more efficient in the C57BL/6 line. *(iii)* Geldanamycin (GA), a selective inhibitor of Hsp90, blocked the Dex-mediated protection against HSI. Interestingly, a low dose of GA (1 μM) blocked the Dex-mediated protection more efficiently in the BALB/c line during the early stage (4 hours after heat stimulus), while a high dose of GA (10 μM) blocked the protection almost equally between the two lines. *(iv)* Most importantly, plasmid transfection of the hsp84 gene from the C57BL/6 line blunted HSI-induced cell damage in BALB/c fibroblasts and was accompanied by an increase in the nuclear GR levels. Our data thereby demonstrate that Hsp84 from C57BL/6 mice causes a higher cellular GC-GR response.

Notably, the finding of a higher GC-GR response in C57BL/6 mice after *in vitro* cellular injury models was also confirmed by our previous study using an *in vivo* whole body traumatic injury model, which is tightly associated with a resistance to injuries [29]. As expected, pretreatment with Dex enhanced the nuclear translocation of GR in both cell lines during heat stress, and consequently rescued cellular death and reduced LDH release.

This Dex-mediated protection against heat-stress is thought to be due to an increase in the efficiency of GC-GR function, as indicated by higher nuclear GR assembly and glucocorticoid response element (GRE) binding [30-32]. The crucial protective role of Hsp90 was confirmed pharmacologically by the blocking effect of geldanamycin (GA), the specific Hsp90 inhibitor. GA has been confirmed to be able to disrupt the Hsp90-mediated GR heterocomplex and result in alteration of GR heteroprotein complex composition and a marked decline in the dexamethasone activity by specifically binding and blocking the ATP-binding domain of Hsp90 [33].

Under heat-stress conditions, both cell lines were substantially damaged while endogenous GC was mainly responsible for these effects. But C57BL/6 lines showed a stronger tolerance towards heat injury than other lines as shown by the different sensitivity to internal GC. We speculated that the conformation of hsp84 from C57BL/6 mice facilitates the nuclear import of GR before binding to the GRE and nuclear export after the regulation is finished. In addition, it is also possible that the hsp84 conformation of C57BL/6 mice is better suited for refolding and maintaining impaired proteins than that of BALB/c mice. Likewise, C57BL/6 strains displayed greater sensitivity to exogenous GC and Dex than BALB/c lines. Interestingly, while a high dose of GA (10 μM) diminished Dex-mediated protection in two cell lines by completely blocking Hsp90, pretreatment with a low dose of GA (1 μM) only partially reduced Dex-mediated protection against HSI in C57BL/6 fibroblasts, but largely abolished Dex-mediated protection in BALB/c lines. This observation raises the interesting possibility that the Hsp84 structure of C57BL/6 mice may account for the higher sensitivity of GC to modulate the GC binding activity and nuclear translocation of GR [34-39], and may lead to the resistance to GA, although more detailed and extensive experiments are needed to further evaluate these phenotypes. Most importantly, genetic manipulations performed in the present study using plasmid transfections further confirmed that Hsp84 from the C57BL/6 line mediates the higher GC-GR responsiveness, as depicted by the observation of increased nuclear GR levels in BALB/c fibroblasts, which indicates an improvement in GR trafficking, whereas the transfections of Hsp84 from the BALB/c line showed no effect on the GC-GR pathway.

Therefore, our study showed that a conformational or structural alteration, rather than changes in expression levels, of Hsp84 results in the differences observed between the two cell strains studied. Meanwhile, we cannot exclude the possibility that other isoforms, i.e., Hsp86, may also be involved in the regulation of GC-GR effects. In fact, increasing data support the crucial role of a structural alteration in Hsp90 on the regulation of GR function. For instance, five single nucleotide polymorphisms (SNPs), resulting in the substitution of amino acids, have been confirmed to cause a decrease in GR function [40]. Moreover, a Q488H SNP in the human Hsp90α gene was shown to lead to a defect in yeast growth and development [28]. In the above study, MacLean et al. [28] demonstrated the mechanism of Hsp90's involvement in the alteration of GR function. While we cannot exclude the possibility that Hsp86, or other isoforms, also contribute to the regulation of GC-GR effects, further studies of the combined mutations in other genes, some of the unverified isoforms, or mutations in the Hsp90-binding domain of GR may identify other factors that act simultaneously.

Our findings suggest that the GC response mediated by hsp90 polymorphisms may provide a potential mechanism linking clinical GC resistance to the population of normal GC-GR features, as observed, for example, in patients with regular GC levels, GR isoforms and response on the HPA axis. Given the critical role of Hsp90 in the modulation of GR functions, it is not surprising to find that the GC response occurs differently in the cells with genetic variations of Hsp90, as was observed for C57BL/6 fibroblasts, which mediated a higher nuclear distribution of GR and were more resistant to heat-stress, either under naïve or Dex-pretreated conditions. We further confirmed the higher Hsp90-mediated GC response of the C57BL/6 line via transgene manipulation of cells subjected to cellular stress. Our data also suggest that the modulation of Hsp90 is an interesting avenue to further investigate its role in GC resistance and to improve GC treatment efficiency.

Abbreviations

Dex (Dexamethasone); GA (geldanamycin); GC (glucocorticoid); GR (glucocorticoid receptor); Hsp90 (heat shock protein 90); LDH (lactate dehydrogenase).

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