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Role of Heat Shock Protein Hsp90 in Formation of Protective Reactions in Acute Toxic Stress

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Abstract—The involvement of heat shock protein Hsp90 in pro-inflammatory response in male NMRI mice under conditions of acute toxic stress, caused by lipopolysaccharide from Gram negative bacteria, was studied using geldanamycin, a specific blocker of the activity of this protein. It is shown that the introduction of geldanamycin lowers total intoxication of the organism upon acute toxic stress caused by endotoxin. Thus, a decrease in cytokine TNF-α, IFN-γ, IL-1, and IL-10 concentrations in blood serum of the geldanamycin-treated animals with acute toxic stress was found along with normalization of functional activity of nitric oxide producing peritoneal macrophages. Studying expression of receptor protein Tlr-4 as well of proteins of two signal cascades, NF-κB and SAPK/JNK, has shown that mechanisms of the geldanamycin protective effect are realized at the level of inhibition of Tlr-4 receptor expression, which provides for endotoxin-to-cell binding, and due to lowering the endotoxin-stimulated activation of signal cascades NF-κB and SAPK/JNK. The results suggest Hsp90 might be a therapeutic target in diseases accompanied by acute toxic stress.

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Key words: heat shock protein 90, geldanamycin, toxic stress, lipopolysaccharide, NF-κB and SAPK/JNK signal pathways, cytokines, nitric oxide, Tlr-4

Mammals react to different stresses in a quite conservative way using protective mechanisms including increased expression of heat shock proteins (Hsp). This superfamily of proteins is induced by such stimulators of protein expression as growth factors, infection, and inflammation [1]. Heat shock proteins exhibit chaperon activity, taking part in protein folding in the norm and in pathologies, thus providing for maintenance of cellular homeostasis. Besides, these proteins play an important role in stimulation of innate and acquired immunity by binding antigenic peptides and participating in formation of the T-lymphocyte-specific response [2]. The best-studied protein of this very large family is heat shock protein 70 (Hsp70) that is undoubtedly one of the main participants in formation of protective response to stress factors of different nature. Recently more attention is given to other members of the family of heat shock proteins playing a significant role in physiological and pathological reactions. Thus, Hsp90 differs from other heat shock proteins because its substrates are proteins directly involved in signal transduction. They include quite a number of proteins, from protein kinases to different types of transcription factors and proteins regulating growth and survival [3]. Hsp90 controls biosynthesis, stability, and activity of its "client" proteins, most of which are key links not only in processes of normal cell functioning but also in the organism's protective reactions in pathology, including toxic stresses caused by lipopolysaccharides (LPS) of cell walls of Gram negative bacteria. At the cell level, toxic stress involves specific receptors for LPS (Tlr4, CD14), tyrosine kinases, and mitogen-activated protein kinases (MAPK). The MAPK-mediated signals activate different transcription factors including NF-κB and ATF-2 and can result in intracellular overproduction of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1, and IL-6, and also of reactive oxygen species including nitric oxide (NO) as we showed previously [4]. Accumulation of these toxic agents in high concentrations in the organism results in total intoxication,

Abbreviations: FCS, fetal calf serum; Hsp, heat shock proteins; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases.

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often accompanied by multiple-organ insufficiency, and can result in a patient's death. Despite existence of numerous works pointing to the role of heat shock proteins in LPS-induced intoxication, the main attention of authors has been aimed at investigation of involvement in this process of another protein of this superfamily, Hsp70 (rather than Hsp90). Nevertheless, there are direct proofs of the involvement of Hsp90 in protective reactions to toxic stress in cells. First, it is known that stimulation by LPS results in formation of Hsp70 and Hsp90 clusters with known LPS-binding receptors Tlr4, CD14, and MD2 [5]. Second, most key proteins involved in signal transduction in toxic stress are either direct "clients" of Hsp90 (IKK, Mekk1, Raf), or are connected via mediators with activation of this protein (JNK, Erk, p38, Mekk2, -3, NF-κB) [6]. All this along with the known ability of Hsp90 to activate macrophages—the immune cells providing for synthesis of the most important mediators of inflammation [7]—suggests the necessity of investigation of involvement of this protein in the organism's protective reactions in septic type toxic stress.

In this work, for investigation of the role of Hsp90 in reaction to toxic stress, the activity of this protein was blocked by the specific inhibitor geldanamycin, which binds with high affinity to the ATF-binding site of Hsp90. We found earlier that *in vitro* geldanamycin can reduce activation of the NF-κB and SAPK/JNK signal pathways and stimulate Hsp70 expression in mouse lymphocytes [8]. The goal of this work was to study in *in vivo* experiments expression of Tlr4 receptor and activity of the NF-κB and SAPK/JNK signal pathways during the inhibition of Hsp90 activity by geldanamycin under conditions of acute toxic stress. The level of pro-inflammatory reaction in animals was estimated by cytokine (TNF-α, IFN-γ, IL-1, IL-6, and IL-10) concentration in blood serum and by NO production by mouse macrophages.

MATERIALS AND METHODS

Animals, toxic stress model, and inhibition of Hsp90 activity. Four groups of mice were used in this work: group 1, control; group 2, animals that obtained injection of LPS; group 3, healthy animals after injection of geldanamycin; group 4, mice after injection of geldanamycin and LPS. Male NMRI mice of 22-27 g were used in the experiments.

Each experimental group included three mice. All data are the result of three independent experiments. Acute toxic stress was caused by a single intraperitoneal injection of endotoxin at the rate of 2.5 mg/kg to animals of experimental groups 2 and 4. For blocking the Hsp90 activity, the specific inhibitor of this protein geldanamycin (Sigma, USA) was used. Geldanamycin dissolved in dimethyl sulfoxide was introduced intraperitoneally to mice of groups 3 and 4 at the rate of 2.5 mg/kg; in group 4, geldanamycin and LPS were injected simultaneously. Animals with intraperitoneal injections of dimethyl sulfoxide served as control. The time of exposure to lipopolysaccharide, geldanamycin, and their combination was 1 h.

Isolation of lymphocytes. One hour after injection, the animals were sacrificed by cervical dislocation of vertebras and subsequent decapitation. All further procedures were carried out under sterile conditions. The spleen was homogenized in a glass homogenizer, and the cells were pelleted by centrifugation at 225*g* for 5 min. After selective erythrocyte hemolysis using isotonic ammonium chloride solution, lymphocytes were washed three times with large volumes of DMEM medium. The resulting cell suspension was washed, cells were counted, and the suspension was diluted to $2.5 \cdot 10^6$ cells/ml in RPMI 1640 medium containing 0.02% gentamycin, 1% L-glutamine, and 5% fetal calf serum (FCS). Then the suspension was placed in culture flasks (Costar, USA), 15 ml in each, and incubated for 6 h at 37°C in 5% CO_2. After cultivation, cells were pelleted by centrifugation at 225*g* for 10 min and used for samples for SDS-PAGE.

Serum isolation. Blood was collected in tubes during decapitation of the animals. Blood samples were kept for 3-5 h at 4°C, centrifuged for 10 min at 225*g*, and supernatants were taken.

Measurement of cytokine production. Cytokine concentration in the blood serum was determined by enzyme-linked immunosorbent assay (ELISA). To do this, 100 μl of each tested sample (serum or 100 ng/ml solution of tested protein) were placed in each well of a 96-well plate for ELISA and left overnight at 4°C. Then 200 μl blocking buffer (phosphate-buffered saline containing 0.05% Tween-20 and 5% dry milk, pH 7.4) was placed in each well and incubated at 37°C for 90 min. After blocking, 100 μl primary antibodies were applied, and incubation was continued for 2.5 h at 37°C. The following antibodies were used in this work: polyclonal rabbit antibodies to mouse TNF-α, polyclonal rabbit antibodies to mouse IL-1α, polyclonal rabbit antibodies to mouse IL-2, polyclonal rabbit antibodies to mouse IL-6, polyclonal rabbit antibodies to mouse IL-10, polyclonal rabbit antibodies to mouse IFN-γ; all antibodies and cytokines were obtained from PeproTech (USA). Then 100 μl biotin-conjugated goat antibodies to rabbit IgG (StressGen Biotechnologies, Canada) was added, the plates were incubated at 37°C for 1 h, and then solution containing streptavidin conjugated with horseradish peroxidase (IMTEK, Russia) was added. After incubation with secondary antibodies, 100 μl of green dye ABTS (Sigma) dissolved in 0.05 M citrate buffer, pH 5.0, with 0.01% H_2O_2 was applied into each well, and plates were left at room temperature. After stable color developed during 10-40 min, the reaction was stopped by addition of 1.5 mM NaN_3 dissolved in 50 mM citrate buffer, pH 5.0. Optical density was measured at 405 nm on a Titertek

Multiscan MCC/340 spectrophotometric plate reader (Flow Laboratories, Finland). Each ELISA stage was accompanied by multiple washing of wells with phosphate salt buffer containing 0.05% Tween-20.

Measurement of nitric oxide concentration. Macrophages were isolated from peritoneal exudate of the mice. Peritoneal cells were pelleted and washed three times in DMEM medium. After that 10^6 cells/ml were suspended in RPMI 1640 medium containing gentamycin, Hepes, and 10% FCS and then placed in 24-well plates, 1 ml per well, and left for 2 h at 37°C in 5% CO_2. The supernatant was carefully removed, attached cells were washed with medium RPMI 1640, and the monolayer of macrophages was left for incubation in 1 ml phenol red-free DMEM medium containing 1 mM sodium pyruvate, 25 mM Hepes, 2 mM L-glutamine, and 3% FCS in atmosphere of humidified 5% CO_2 at 37°C for 20 h in the presence of 5 μg/ml LPS. The production of NO was measured by concentration of nitrites, the final product of metabolism of the short-lived compound NO. The nitrite in supernatants was measured using Griess reagent containing a mixture of 0.1% N-naphthylethylenediamine dihydrochloride solution in a mixture of 2.5% phosphoric acid solution with 1% sulfanilamide solution in 5% phosphoric acid at the ratio 1 : 1. Samples of supernatants obtained after macrophage cultivation for 20 h were placed in a 96-well plate, 100 μl per well, 100 μl of freshly-prepared Griess reagent was added to each well, and the plate was incubated for 10 min at room temperature. Optical density was measured at 546 nm using the Titertek Multiscan MCC/340 plate reader. A calibration curve was plotted using standard $NaNO_2$ solutions.

SDS-PAGE and immunoblotting. To prepare samples, $20 \cdot 10^6$ spleen lymphocytes were lysed using an ultrasonic disintegrator on ice with continuous stirring for 2 min. Total protein concentration was determined according to the Bradford technique using commercial Bradford solution (Sigma). Protein was precipitated with acetone, and 2-fold solubilizing buffer (50 mM Tris-HCl, 2% SDS, 25% glycerol, 5% β-mercaptoethanol, and 0.1% bromphenol blue, pH 6.8) was added at the 1 : 1 ratio. Samples were boiled for 5 min and stored at −20°C. Final protein concentration in the samples was 1 mg/ml, and 10 μl of each sample was introduced into each well. The presence of marker proteins in the samples was determined by electrophoresis in 10% polyacrylamide gel at ~120 V in concentrating gel and ~160 V in resolving gel. Tris-glycine buffer (25 mM Tris-HCl, 0.1% SDS, 250 mM Gly, pH 8.3) was use for electrophoresis. The assay specificity was checked by immunoblotting. Protein was transferred from the gel onto nitrocellulose membrane in a trans-blot chamber filled with Tris-glycine buffer for transfer (47.9 mM Tris-HCl, 0.1% SDS, 38.6 mM Gly, and 20% methanol, pH 7.4) for 1 h at 350 mA. Then the filter with transferred proteins was kept overnight in blocking buffer (10 mM Tris buffered saline, pH 7.4, containing 5% dry milk and 0.05% Tween-20) at 4°C. After blocking the primary antibodies to NF-κB, marker proteins were applied. We used rabbit polyclonal antibodies to Tlr4, NF-κB, phospho-IKKαβ (Ser176/180), synthetic phosphopeptide NF-κB (Ser176/180), and synthetic phosphopeptide SAPK/JNK (Thr183/Tyr185). All antibodies were obtained from Cell Signaling Technology (USA) and exhibited cross-reactivity to the above-mentioned mouse proteins. The membrane was incubated in antibody solution for 1 h at 37°C with continuous stirring and placed for 1 h under the same conditions into solution of biotin-conjugated goat antibodies to rabbit IgG (StressGen Biotechnologies) in 10 mM Tris buffered saline, and after that with complex containing streptavidin and horseradish peroxidase (Sigma) in 10 mM Tris buffered saline. The ECL system (Amersham, Sweden) on Kodak film was used for determination. Each step was accompanied by multiple washing of the plate with 10 mM Tris buffered saline containing 0.05% Tween-20. The content of heat shock proteins in samples was estimated by comparison of staining intensities of bands corresponding to marker proteins with those of bands of purified heat shock proteins titrated in advance before electrophoresis. Results were quantitatively evaluated using the Qapa program.

Statistic analysis was carried out using the Student's *t*-criterion.

RESULTS AND DISCUSSION

Inhibition of Hsp90 prevents activation of Tlr-4 expression under toxic stress. Figure 1 shows data on the effect of geldanamycin, an inhibitor of Hsp90 activity, on expression of Tlr-4 receptor in spleen lymphocytes of healthy mice and mice with LPS-induced acute toxic stress. Toxic stress resulted in fourfold increase in intracellular Tlr-4 receptor expression. The introduction of geldanamycin into healthy mice also increased expression of this receptor, but not as much as in the case of endotoxin. It is interesting that combined introduction of gel-

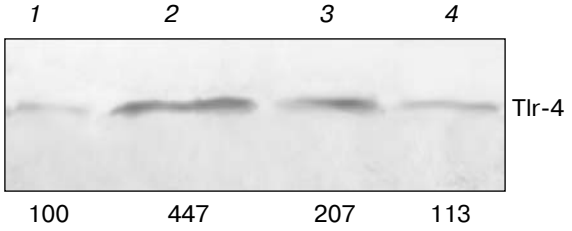

Fig. 1. Content of receptor protein Tlr-4 in mouse splenocytes. Upper panel, mouse groups: *1*) control; *2*) animals with acute toxic stress; *3*) healthy animals after geldanamycin injection; *4*) mice with acute toxic stress and geldanamycin injection. Lower panel, results of densitometry (% of group 1 (control)).

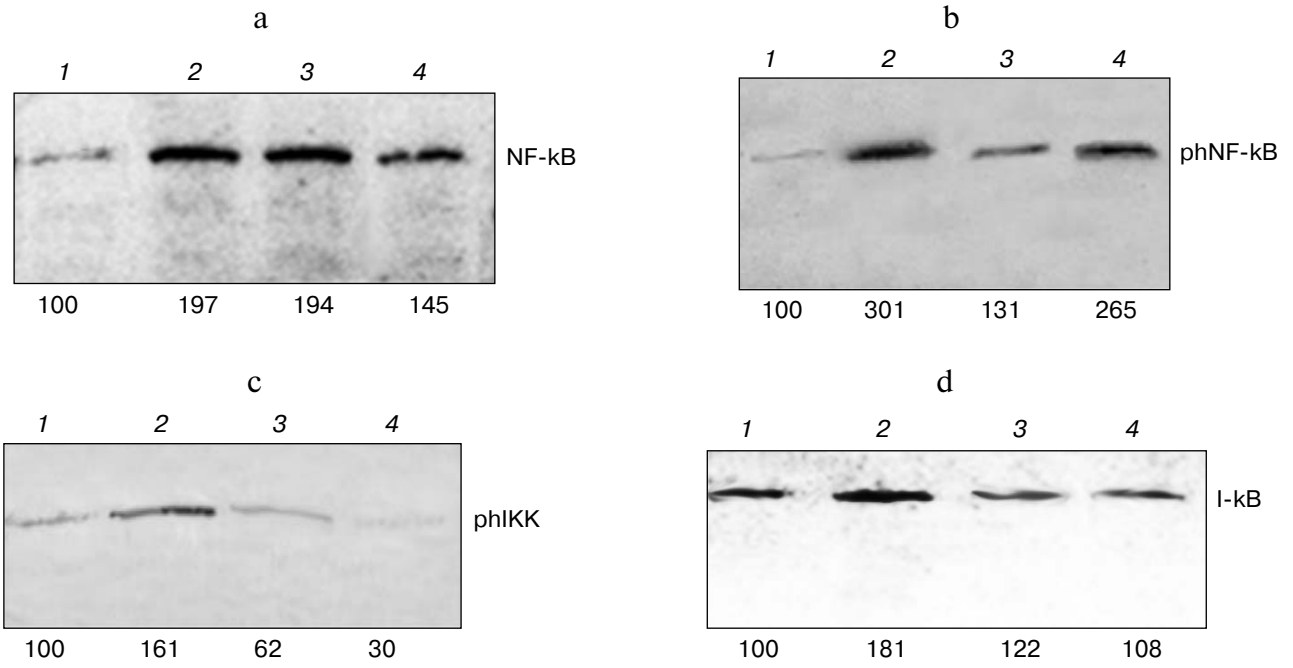

Fig. 2. Effect of geldanamycin and lipopolysaccharide injections on the NF-κB signal pathway activation, including total NF-κB (a), NF-κB phosphorylated form (b), IKKα/β phosphorylation (c), and IκB content (d) in mouse splenocytes. All designations as in Fig. 1.

danamycin and the lipopolysaccharide resulted in complete normalization of Tlr-4 receptor expression in splenocytes.

Geldanamycin decreases activation of the NF-κB signal pathway caused by endotoxin. The role of Hsp90 in activation of the NF-κB cascade in mice with acute toxic stress, caused by introduction of bacterial toxin, was estimated in this work (Fig. 2). This series of experiments shows that toxic stress is accompanied by increase in total NF-κB concentration (Fig. 2a) and, especially, of the protein phosphorylated form (Fig. 2b) in mouse splenocytes. Besides, in toxic stress an increase in the level of free IκB subunits is observed (Fig. 2d), probably due to enhanced expression of IKKα/β kinase involved in release of IκB inhibitory protein (Fig. 2c) [9]. Introduction of geldanamycin into healthy animals also increased total NF-κB concentration, although to a lower extent compared to endotoxin (Fig. 2a), but caused lowering in NF-κB phosphorylation (Fig. 2b), which points to the inhibitory effect of geldanamycin on activity of the NF-κB signal cascade. This is clearly due to the ability of this antibiotic to decrease the expression level of IKKα/β kinase (Fig. 2c), which is known to phosphorylate inhibitory subunits IκBα and IκBβ, thus releasing NF-κB for further activation. Probably due to this, no increase in IκB concentration was observed upon introduction of geldanamycin into normal mice (Fig. 2d). When together with toxic stress initiation the Hsp90 activity was inhibited by geldanamycin, expression of all studied proteins of this signal cascade significantly decreased. This concerned total NF-κB (Fig. 2a), phosphorylated NF-κB form (Fig. 2b), and kinase IKKα/β (Fig. 2c) concentrations. Thus, the activation of the NF-κB cascade following the classical pathway and, correspondingly, the possibility of translocation of the phosphorylated NF-κB form into the nucleus via IKK kinase activation under conditions of LPS-stimulated acute toxic stress is to a significant extent prevented by geldanamycin introduction.

Blocking Hsp90 activity lowers SAPK/JNK signal pathway activation in mouse splenocytes during toxic stress. It was shown previously that most proteins of the SAPK/JNK signal cascade are not Hsp90 clients, but nevertheless we found multiple increase in activation of this signal pathway not only in animals with toxic stress, but in mice after introduction of geldanamycin (Fig. 3). Surprisingly, blocking Hsp90 activity in mice in toxic stress not only did not stimulate the enhancement of

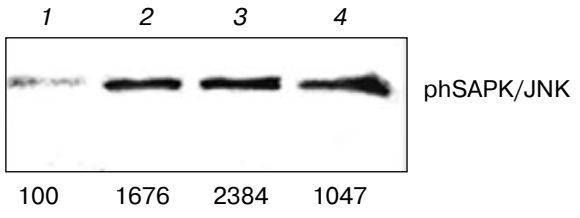

Fig. 3. SAPK/JNK phosphorylation in mouse splenocytes under toxic stress and inhibition of Hsp90 activity by geldanamycin. Designations as in Fig. 1.

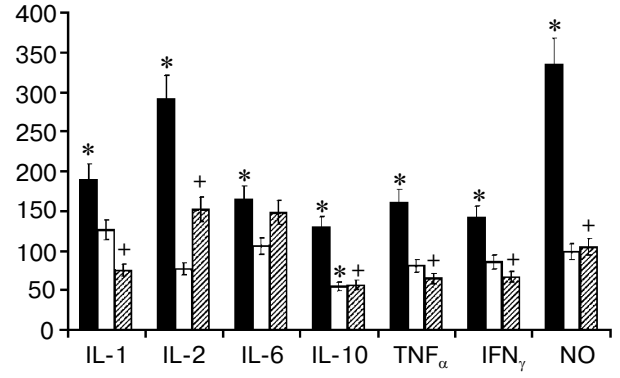

Fig. 4. Effect of geldanamycin and LPS on cytokine IL-1, IL-6, IL-10, TNF-α, and IFN-γ concentrations in blood serum and on nitric oxide (NO) production by mouse macrophages. Ordinate axis, cytokine concentrations and NO production, % of control group. Dark columns, animals under acute toxic stress; transparent columns, healthy animals after geldanamycin injection; hatched columns, mice with acute toxic stress and geldanamycin injection. * Significant difference from control, $p < 0.05$; $^{+}$ significant difference from LPS group.

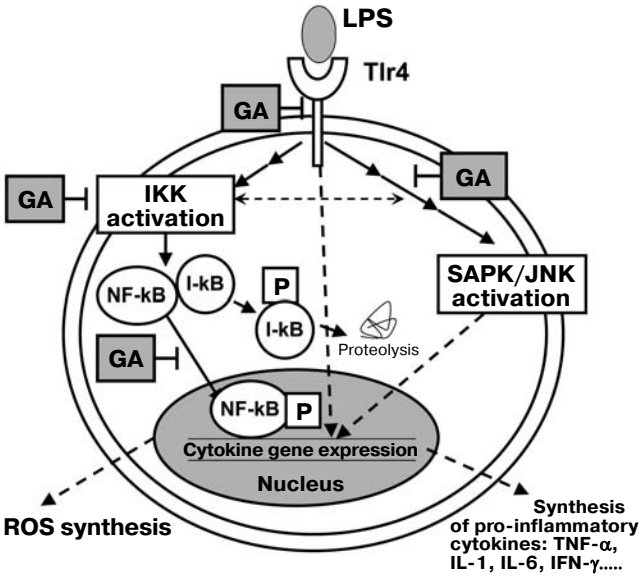

Fig. 5. Protective effect of the Hsp90 activity inhibitor geldanamycin in LPS-stimulated toxic stress. GA, geldanamycin; ROS, reactive oxygen species; P, phosphorus; I-κB-P, I-κB phosphorylated form; NF-κB-P, NF-κB phosphorylated form.

SAPK/JNK phosphorylation, but it lowered the LPS-stimulated activation of this signal pathway (Fig. 3).

Inhibition of Hsp90 activity decreases total organism intoxication during LPS-stimulated toxic stress. The found decrease in activation of key signal pathways resulted in lowering total intoxication of the organism. Figure 4 shows the results of measurement of cytokine level in blood and NO production in mouse macrophages. The introduction of geldanamycin had a slight effect on the cytokine profile in the blood of healthy animals. However, in the case of combined injections of geldanamycin and endotoxin, it prevented development of pro-inflammatory reaction to toxin, thus lowering the TNF-α, IL-1, IL-10, and IFN-γ levels to control and even lower. Geldanamycin exhibited the same effect on nitric oxide production in macrophages, and in this case its inhibitory effect on macrophage activity was also revealed after its introduction into healthy animals.

It is suggested that Hsp90 plays a critical role in pathogenesis of toxic stress caused by endotoxin, because inhibition of the activity of this stress protein lowers the level of the cell pro-inflammatory reaction via mechanisms shown in Fig. 5.

First, this happens at receptor level: the cell pro-inflammatory reaction can be lowered due to decreased expression of specific receptor Tlr-4; second, at the level of signal transduction via cascades NF-κB and SAPK/JNK by direct or mediated decrease in overexpression and phosphorylation of key participants of these pathways. And finally, blocking the Hsp90 activity results in some decrease in cell reaction to endotoxin attack at the transcription level due to decreased NF-κB phosphorylation, which reduces the probability of translocation of this transcription factor into the nucleus. As a result, all this results in reduction of total intoxication in mice under LPS-stimulated acute toxic stress. Hsp90 is now actively investigated as one of promising therapeutic targets for treatment of tumor diseases accompanied by significant stimulation of the NF-κB signal cascade [10].

Results of this work show that blockers of heat shock protein activities can also be used in therapy of acute toxic states owing to inhibition of activation of unspecific response with involvement of cascades of activated protein kinases. In fact, it was shown on different animal models that the use of Hsp90 inhibitors results in prolongation of animal life under toxic stress, inflammation, and septic states caused by bacterial endotoxins [11], reduces the endothelial barrier permeability in the case of LPS direct attack on mouse skin [12], and due to inhibition of activity of transcription factors, it attenuates LPS-stimulated eyeball inflammation [13]. All this suggests Hsp90 as a possible therapeutic target in LPS-stimulated acute toxic stress conditions and points to expediency of searching for new low-toxicity inhibitors of Hsp90 activity.

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