Extracellular Hsp70 Stimulates Multiple Signaling Pathways in A431 Carcinoma Cells

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Abstract—Heat shock proteins (Hsp) were considered to be intracellular. However, there is evidence that stress-inducible Hsp70 is released by cells into the blood or conditioned medium of cultured cells. Previously the signaling function of exogenously added Hsp70 was proposed, referred to as chaperone function of Hsp70. The later was restricted to immune cells. Here we show that Hsp70 stimulates TLR2/4 receptors in A431 squamous carcinoma cells. Extracellular Hsp70 secreted at the initial steps of heat shock is shown to be sufficient for the EGF receptor (EGFR) transactivation. The recombinant Hsp70 stimulates tyrosine phosphorylation of EGFR and the appearance of phosphorylated forms of key components of its downstream signaling pathways: phospholipase Cγ1 (PLCγ1), transcription factor STAT3, and ERK1/2. The neutralizing antibody to EGFR has no effect on the Hsp70-induced EGFR activation, which suggests that neither extracellular Hsp70 nor other extracellular factor binds to EGFR.

Key words: extracellular Hsp70, Toll-like receptors, epidermal growth factor receptor.

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Heat shock proteins and Hsp70 (70 kD heat shock protein) in particular were considered to be intracellular, though recently they were detected in extracellular environment: in human and animal tissue fluids and in the conditioned medium of cultured cells. The appearance of extracellular Hsp70 was described for a number of human and animal diseases. Hsp70 was detected in blood of patients with hypertension [1], atherosclerosis [2], and kidney disease [3]. Some authors assume that the presence of Hsp70 in blood indicates a myocardial damage [4–6]. Hsp70 concentration is significantly increased in blood under bacterial and viral infection [7–9].

Extracellular Hsp70 mediates signal transduction performing so-called chaperone function [10]. Extracellular Hsp70 specifically binds to the outer membrane of different immune cells [10–15]. These data suggest the presence of specific receptors for Hsp70 in the outer membrane of cells. Indeed, a group of cellular receptors that bind Hsp70 was described recently. It includes Toll-like receptors (TLR) and scavenger receptors [11, 15].

TLRs from interleukin 1-related receptor group recognize structural components of various bacteria, viruses and fungi [16]. TLR binding to the ligand induces receptor activation, interaction with MyD88 adapter protein and onset of signal transduction, including the activation of serine-threonine kinases of IRAK family and activation of NF-κB, AP-1, EIK-1, CREB and STAT transcriptional factors, and even activation of MAP-kinase signal pathway [17, 18].

The studies of signal pathway induced by extracellular Hsp70 reveal that Hsp70 activates TLR2 and TLR4 receptors responsible for recognition of cell wall components of gram-positive and gram-negative bacteria respectively [10, 11, 13, 19]. The process implicates activation of NF-κB and MAP-kinases [13]. Extracellular Hsp70 is able to increase intracellular calcium, which is also dependent on TLR2/4 activation [10]. Most of these studies were performed on immune cells, whereas data concerning signal function of extracellular Hsp70 in other cells are scanty.

Hsp70 is released from cells under stress conditions such as heat shock, ionizing radiation, hyper- and hypotonia, hypoxia, and oxidative stress. Hsp70 was detected in culture medium of rat embryonic cells, human blood cells, glia cells, human carcinoma cells, pancreas, and mast cells after heat shock [20—26].

Stress conditions cause transactivation of epidermal growth factor receptor (EGFR), i.e. its ligand-independent activation. Under stress conditions the factor that interacts with the cell and triggers signal transduction is not so obvious. Previously we showed that heat shock induced Hsp70 release from A431 cells [23], on the one hand, and caused EGFR transactivation, on the other hand [27]. Therefore, we suggested that secreted Hsp70 might be implicated in EGFR transactivation.

The aim of this work was to study signal pathways activated by extracellular Hsp70 in A431 cells and to
elucidate the role of Hsp70 in the EGFR transactivation.

EXPERIMENTAL

Human epidermoid carcinoma A431 cells were from Russian Cell Culture Collection (Institute of Cytology, Russian Academy of Sciences). Cells were cultured in DMEM supplemented with 10% fetal calf serum. Subconfluent cells were treated with 100 ng/ml of Hsp70. Heat shock was induced by cultivation at 42°C for the time indicated. Cells were treated with neutralizing anti-EGFR antibodies for 3 h.

Electrophoresis, immunoprecipitation and immunoblotting. Cells were grown on 90 mm Petri dishes. After the treatment cells were lysed in a buffer containing 0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5% nonidet P40, 0.2 mM aprotinin, 0.2 mM leupeptin, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), and 20% glycerol.

For immunoprecipitation cell lysates were incubated with anti-TLR2 or anti-TLR4 antibodies for 18 h at 4°C under stirring. Then protein-A- sepharose was added according to the manufacturer’s recommendations, and probes were incubated at 4°C for 1 h. Precipitate was pelleted by centrifugation (5 min, 1000 g) and resuspended in 2-fold Laemmli buffer. Anti-Hsp70 antibodies were used for Hsp70 precipitation from the conditioned medium.

Proteins from lysates or precipitates were separated by SDS-PAGE in 7.5% gels and transferred to nitrocellulose membrane. Immunoblots were stained with polyclonal antibodies to EGFR, phospholipase Cγ (PLCγ), ERK1/2 protein kinases, STAT3 and NF-κB transcriptional factors, and phosphorylated forms of these proteins at 1 : 1000 dilution. Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antibodies were used as secondary antibodies. Immunoblots were developed with enhanced chemiluminescence method.

Materials. The following reagents were used: DMEM medium (Sigma, USA), fetal calf serum (Gibco BRL, USA), purified Hsp70 from bovine muscles kindly gifted by Dr. B. A. Margulis, and recombinant Hsp70 (Stress gene, USA), polyclonal antibodies to EGFR, ERK1, 2 protein kinases, STAT3 and NF-κB transcriptional factors and phosphorylated forms of these proteins (all antibodies were from Cell Signaling Technology, USA), monoclonal antibodies to phosphorylated tyrosine (PY20 clone), to PLCγ1 and its phosphorylated form (BD Transduction Lab., USA), to TLR2 and TLR4 (Santa Cruz, USA), antibodies to Hsp70 kindly gifted by Dr. B. A. Margulis, horse-radish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG antibodies (Sigma), nitrocellulose membrane (Immobilon P, Amersham, UK), HEPES, PIPES, Triton X-100, PMSF, EGTA (Sigma). Other reagents were produced in Russia.

RESULTS AND DISCUSSION

Extracellular Hsp70 binds a number of cell receptors, including TLR2/4 that are expressed in human keratinocytes, and in A431 cells in particular [28]. To study the Hsp70-induced activation of TLR2/4 we examined complex formation of receptors and MyD88 adapter protein with immunoprecipitation method. Proteins from lysates of untreated control cells and cells pre-treated with 100 ng/ml of recombinant Hsp70 for 15 min were immunoprecipitated with anti-TLR2 antibodies. Proteins from immunoprecipitates were separated with denaturating PAGE, transferred to nitrocellulose membrane that was subsequently stained with anti-MyD88 antibodies (Fig. 1a). Similar experiments were performed with anti-TLR4 antibodies (Fig. 1b). The results showed that Hsp70 induced interaction of MyD88 both with TLR2 and TLR4.

Additional evidences of Hsp70-induced TLR2/4 activation were obtained in the experiments monitoring activated NF-κB. Proteins from lysates of control and Hsp70-treated cells were separated with denaturating PAGE, transferred to nitrocellulose membrane, and phosphorylated (active) form of p65 protein, NF-κB subunit, was revealed after immunostaining. Figure 1c demonstrates that extracellular Hsp70 induces phosphorylation of NF-κB. The obtained data indicate the TLR2/4 activation by Hsp70. Hsp70-induced activation of TLR2/4 and NF-κB was described for the first time for immune cells [10, 13]. Here we show that Hsp70 has the same effect on epithelial cells.

A431 cells secrete Hsp70 at the early stages of heat shock [23], and EGFR is simultaneously activated in such cells [27]. To elucidate the role of extracellular Hsp70 in EGFR transactivation, we studied the effect of conditioned medium from heat shock-treated cells. Conditioned medium from A431 cells was discarded and substituted for medium from either control cells or from heat shock-treated cells (42°C, 15 min) or for Hsp70-depleted medium from heat shock-treated cells obtained after Hsp70 immunoprecipitation. After medium change cells were incubated under normal conditions (at 37°C) for 15 min, then lysed, and tyrosine-phosphorylated EGFR was revealed with immunoblotting. As we expected, no changes in phosphorylation status of EGFR were detected in cells incubated in the conditioned medium from control cell, whereas the medium from cells after heat shock induced a significant increase of tyrosine phosphorylation of EGFR (Fig. 2, bands 1, 2). Hsp70-depleted medium from heat shock-treated cells reduced tyrosine phosphorylation of EGFR (Fig. 2, band 3). The obtained results indicate that heat shock-induced transactivation of EGFR is implemented due to auto/paracrine processes and extracellular Hsp70 plays an important role there.