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HSP-70 mitigates LPS/SKI-induced cell damage by increasing sphingosine kinase 1 (SK1)

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ABSTRACT

Heat shock proteins (HSPs) are potent protectors of cellular integrity against environmental stresses, including toxic microbial products. To investigate the mechanism of HSP-70 cell protection against bacterial lipopolysaccharide (LPS), we established a stable HSP-70 gene-transfected RAW 264.7 murine macrophage model of LPS-induced cell death. Bacterial LPS increases the activity of sphingosine kinase 1 (SK1), which catalyzes formation of sphingosine-1-phosphate (S1P). S1P functions as a critical signal for initiation and maintenance of diverse aspects of immune cell activation and function. When mouse macrophages were incubated with *Escherichia coli* LPS (1 μg/ml) and sphingosine kinase inhibitor (SKI, 5 μM), 90% of cells died. Neither LPS nor SKI alone at these doses damaged the cells. The LPS/SKI-induced cell death was partially reversed by overexpression of HSP-70 in gene-transfected macrophages. The specificity of HSP-70 in this reversal was demonstrated by transfection of HSP-70-specific siRNA. Down-regulation of HSP-70 expression after transfection of siRNA specific for HSP-70 was associated with increased LPS/SKI-induced cell damage. Overexpression of human or murine HSP-70 (HSPA1A and Hspa1a, respectively) increased both cellular SK1 mRNA and protein levels. Cellular heat shock also increased SK1 protein. These studies confirm the importance of SK1 as a protective moiety in LPS-induced cell injury and demonstrate that HSP-70-mediated protection from cells treated with LPS/SKI is accompanied by upregulating expression of SK1. HSP-70-mediated increases in SK1 and consequent increased levels of S1P may also play a role in protection of cells from other processes that lead to programmed cell death.

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1. Introduction

Heat shock proteins (HSPs), a group of stress-induced proteins, are present in all eukaryotic cells. Their synthesis is transiently increased when cells are exposed to heat shock [1]. In addition, HSPs, mainly the A isoform of HSP-70, are actively synthesized when macrophages are exposed to bacterial toxins, lipid mediators (e.g., prostaglandin A1 [2], oxygen free radicals and nitric oxide) [3]. HSP-70 exists in the cytosol of cells and functions as a molecular chaperone, guiding the folding of proteins after they are synthesized and during transfer between cellular organelles. The regulatory effect of HSP-70 on newly synthesized proteins provides protection to cells from many different noxious molecules produced by pathogens and other environmental stressors. As HSPs are involved in diverse aspects of cellular protection, the mechanisms that mediate this protection may involve multiple intracellular regulatory factors.

Gram-negative bacteremia with enteric organisms may result in a severe sepsis or systemic inflammatory response syndrome (SIRS) characterized by fever, cardiovascular collapse, coagulation abnormalities, respiratory failure and death. This syndrome, which may be associated with the presence of lipopolysaccharide (LPS) in the circulation at levels of nanograms per milliliter, is mediated by numerous cytokines and reactive molecules, including TNFα, IL-1ß, interferon-gamma (IFN-γ), NO and coagulation factors [4]. Early inflammatory responses in sepsis are typically mediated by moncyte-macrophages, while later responses are driven by lymphocyte products. These inflammatory responses can be eliminated by inhibition of macrophage production of the responsible cytokines [4]. At the cellular level, programmed cell death, manifested as cell apoptosis, can occur directly via engagement of LPS with toll-like receptors (TLRs) [5] or via LPS-stimulated production of multiple inflammatory factors that activate death pathways [6].

Treatment of the murine RAW 264.7 macrophage-like cells with LPS results in a time- and dose-dependent activation of sphingosine kinase 1 (SK1) and membrane translocation of the activated enzyme [7]. In addition, RAW 264.7 cells treated with LPS and inhibitors of SK1 undergo apoptosis [7]. SK1 catalyzes the formation

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of an active product, sphingosine-1-phosphate (S1P), from sphingosine. Sphingosine and ceramide are important intermediate products in sphingolipid metabolism and are involved in diverse cellular processes as lipid second messengers. The balance between intracellular levels of S1P and these two important sphingolipids, sphingosine and ceramide, is a key indicator of whether a cell will survive or die [8]. Ceramide is an immediate precursor of sphingomyelin and functions as a signal transducer to cause programmed cell death in many tissues and cell lines [9]. S1P promotes a variety of proliferative cellular processes including cell growth and differentiation [10]. In addition, S1P opposes the apoptosis resulting from elevated levels of ceramide or sphingosine [8,11], although it has also been shown to trigger apoptosis in various cells [10].

Among cells of the immune system, macrophages play a key role in host defense against bacterial infection, especially by intracellular bacteria. In addition to antigen processing and presentation to promote development of specific immunity, they are important effector cells for phagocytosis, bacterial killing and secretion of cytokines. They are a major source of proinflammatory cytokines, especially TNFα and IL-1ß, which they secrete in response to stimulation by LPS [12]. We have previously observed that production of these mediators in human monocyte-derived macrophages is regulated, in part, by HSP-70, which is actively synthesized when the cells are exposed to LPS; overexpression of HSP-70 down-regulates proinflammatory cytokine production [13]. In this study, we find that overexpression of HSP-70 protects RAW 264.7 murine macrophage-like cells from LPS-induced cell damage and demonstrate that this protection is associated with HSP-70-induced up-regulation of SK1. Heated cells that express elevated levels of HSP-70 also have elevated levels of SK1. These findings suggest a mechanism for a protective effect of HSP-70 against cell damage that may occur as a consequence of sepsis or other cellular insults.

2. Materials and methods

2.1. Cell culture

Murine macrophages RAW 264.7 (American Type Culture Collection, Manassas, VA) were grown in 150 mm × 15 mm flasks incubated at 37 °C in an atmosphere of 5% CO_2 in air. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 0.03% glutamine, 4.5 g/l glucose, 25 mM HEPES, 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco BRL, Gaithersburg, MD). The medium of cell culture was changed twice per week.

2.2. Construction of human and mouse HSP-70

Human and mouse genomic DNA samples were isolated from U937 cells (ATCC, Manassas, VA) and RAW 264.7 cells (ATCC, Manassas, VA), respectively. Full-length human [14] and mouse [15] inducible HSP-70 genes (HSPA1A and Hspa1a, respectively) were synthesized by PCR. The primers used for coding sequence of human (Gene bank accession number NM_005345) and mouse (Gene bank accession number M76613) heat shock protein 70 were hHSP Forward: 5′-CACCGAATTCCCACCAATGGCCAAAGCCGCGGCGATC-3′, hHSP Reverse: 5′-ACCGGTATCTACCTCCTCAATGGTG-3′, mHSP Forward: 5′-CACCGAATTCCCACCAATGGCCAAGAACACGGC-3′, mHSP Reverse: 5′-ACCGGTATCCACCTCCTCGATTGTTGGTCCTGAGCC-3′. The PCR products were initially cloned into pcDNA3.1D/V5-His-TOPO vector (Invitrogen, Carlsbad, CA). This 5.5 kb vector contains an enhancer–promoter sequence from the human cytomegalovirus gene for high-level transcription, and has a polyadenylation signal and transcription terminal sequence derived from the bovine growth hormone gene to enhance RNA stability. In addition, it encodes a C-terminal V5-hexahistidine sequence that increases the molecular size of the recombinant product by 2.2 kDa. The neomycin resistance gene on the vector was used for later selection of geneticin (G418)-resistant stable cell lines.

2.3. Gene transfection

Transfection of empty vector or vector bearing human HSP-70 (hHSP), murine HSP-70 (mHSP), or LacZ genes into RAW 264.7 macrophages cells was conducted in 4 cm diameter wells in a 6-well plate. Cells at 50% confluence in culture medium without antibiotics were transfected with recombinant constructs using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). Briefly, 1 μg recombinant DNA was added in 50 μl Opti-MEM® I reduced serum medium. The solution was then combined with diluted Lipofectamine (1 μl in 50 μl medium) and incubated for 20 min at room temperature for complex formation. The mixture of recombinant DNA and Lipofectamine was added to each well. The transfected cells were cultured in a CO_2 incubator at 37 °C for 2 days. The cells were subsequently incubated with 250 μg/ml of geneticin (G418) for further selection of gene-transfected cells based on our previous protocol [16].

2.4. Examination of overexpression of HSP-70

Overexpression of HSP-70 in cells selected by culture with G418 was verified by Western blot as previously described [17]. Cell pellets were collected and treated in 200 μl of M-per mammalian protein extraction solution (Pierce, Rockford, IL). After cell lysate, 1.5 μg of protein samples in 15 μl buffer were separated by electrophoresis using 10% polyacrylamide gels and then electroblotted onto 0.2 μm nitrocellulose membranes (Bio-Rad, CA). The blots were incubated with 1:1000 mouse monoclonal anti-HSP-70 antibody (3A3, catalogue number SC32239) (Santa Cruz Biotech Inc.) overnight at 4 °C. Then the blots were incubated with 1:1000 goat anti-mouse second antibody conjugated with alkaline phosphatase (Amersham) overnight at 4 °C. The blots were developed with 1 mg/ml of Naphthol AS-MX phosphate (Sigma) and 2 mg/ml Fast Red TR salt (Sigma) in 50 mM Tris buffer and the molecular weight of stained proteins compared to molecular weight standards (Invitrogen Inc.).

2.5. Assay of cell viability after treatment with LPS and SK inhibitor (SKI)

The viability of macrophages was monitored by Trypan Blue staining based on dye exclusion [18]. Briefly, RAW 264.7 macrophages were seeded in 4 cm diameter wells in 6-well plates (2.5×10^5 cells/well in 2 ml of culture medium). After culture for 2 days, when cells were approximately 50% confluent, they were pretreated with or without 5 μM SKI (2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole, HCl, catalogue number 567731, Calbiochem/Merck) for 60 min at 37 °C, then incubated with or without 1 μg/ml of LPS derived from *Escherichia coli* (catalogue number L4391, Sigma) for 5 days. The media was changed on day 3 after LPS treatment. After incubation, both detached and attached RAW 264.7 were suspended in DMEM and mixed with Trypan Blue solution to a final concentration 0.8 mM. The reaction was conducted at room temperature for 5 min to allow cell staining to occur. Ten microliters of stained cells were loaded onto a hemocytometer and the numbers of non-stained cells (surviving cells) and stained cells (dead cells) were counted under a microscope. Cell viability was calculated as non-stained cell number/total cell number.