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Role of SelS in lipopolysaccharide-induced inflammatory response in hepatoma HepG2 cells

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ABSTRACT

To investigate the role of SelS in bacterial lipopolysaccharide (LPS) induced inflammatory response, some parameters in LPS-stimulated HepG2 cells were comparatively studied fore-and-aft SelS silence. LPS induced the decreases of cytoplasmic glutathione peroxidase (GPx-1) mRNA expression and activity, and the increases of reactive oxygen species (ROS) level, intracellular and extracellular nitric oxide (NO) levels, inducible nitric oxide synthase (iNOS) mRNA expression and activity, and serum amyloid A1 (SAA1) mRNA expression and secreted protein level in hepatoma HepG2 cells. When SelS was suppressed by small interfering RNA (siRNA), those decreases and increases were further aggravated under LPS stimulation, respectively. In conclusion, the negative association between SelS and the LPS-induced production of ROS, NO and SAA1 demonstrated that SelS had an important role in influencing inflammatory response, and that role may be related with SelS as a central component of retro-translocation channel in endoplasmic reticulum-associated protein degradation (ERAD) and its anti-oxidative property.

Selenoprotein S (SelS; gene aliases: Tanis, SEPS1, VIMP) is a novel endoplasmic reticulum (ER) and plasma membrane resident selenoprotein that participates in the removal of misfolded proteins from the ER lumen for degradation [1]. It protects cells from oxidative damage and ER stress-induced apoptosis [2,3]. Suppression of SelS by small interfering RNA (siRNA) has been shown to result in an enhanced production and release of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in macrophages in culture in vitro [4]. SelS is described as a putative receptor for serum amyloid A (SAA) [5] and its mRNA expression in human skeletal muscle and adipose tissue is positively correlated with circulating SAA [6].

SAA, one of the acute-phase proteins and mainly produced in the liver, is an apolipoprotein of high-density lipoprotein [7]. The human SAA family consists of SAA1, SAA2, and SAA4. The first two, also known as acute-phase SAAs, dramatically increase by as much as 1000 times during inflammation. SAA3 is a pseudogene, SAA4 is known as a constitutive SAA (C-SAA) [7]. It has been suggested that both local and systemic induction of acute-phase SAA occur during inflammation and may contribute to the pathogenesis of chronic inflammatory diseases associated with amyloid deposition [8]. A major factor responsible for the development of amyloid A amyloidosis is the increased synthesis and subsequent degradation of the precursor protein SAA1 under chronic inflammatory conditions [9].

Bacterial lipopolysaccharide (LPS), an essential component of the outer membrane of gram-negative bacteria, provokes a generalized proinflammatory response in the infected host that leads to septic shock and multiple organ failure [10]. The liver is not only a major target organ of LPS toxicity, but also a primary site for the uptake and clearance of microbial products [11]. It has been suggested that hepatocytes play an important role in inflammation during sepsis [12,13]. Hepatocytes stimulated with LPS produce SAA1, reactive oxygen species (ROS), nitric oxide (NO), cytokines and so on in cultures in vitro [10,13,14]. ROS are mediators of cellular injury and involved in the onset of cellular damage during endotoxemia [15,16]. Nitric oxide acts as an intracellular messenger and regulates cellular functions such as vasorelaxation and inflammation; however, overproduced NO is oxidized to reactive nitrogen species and results in the disruption of cell signaling and uncontrolled systemic inflammation and septic shock [17].

However, few data is available about the role of SelS in LPS-induced inflammatory response. In this study, some parameters in LPS-stimulated human hepatoma HepG2 cells were comparatively studied fore-and-aft SelS silence, such as level of reactive oxygen species (ROS), mRNA expressions and activities of cytoplasmic glutathione peroxidase (GPx-1) and inducible nitric oxide synthase (iNOS), levels of intracellular and extracellular nitric oxide (NO), and mRNA and secreted protein levels of SAA1. The results showed that there was a negative association between SelS and the LPS-induced production of ROS, NO and SAA1 in HepG2 cells, which meant that SelS played an important role in influencing inflammatory response in LPS-stimulated HepG2 cells.

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Materials and methods

Reagents

LPS from Escherichia coli and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma Co. Dulbecco's Modified Eagle's Medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT), and newborn calf serum (NCS) were obtained from Gibco BRL (Gaithersburg, MD, USA); Penicillin G and streptomycinsulfate were purchased from Amersco. Trizol reagent and Lipofectamine 2000 were obtained from Invitrogen. Molony murine leukemia virus (M-MLV) reverse transcriptase and oligo (dT) were purchased from Promega. SYBR Green PCR Master Mix was obtained from Toyobo (Japan) and dNTP was from Roche. Rabbit polyclonal antibodies against SAA1 and SelS were presented by Aviva Systems Biology (China). All other reagents were of analytical or biochemical reagents.

Cell culture and siRNA transfection

Human hepatoma HepG2 cells were cultured in DMEM media containing 10% (v/v) heat-inactivated NCS, 100 U/mL penicillin G and 100 μg/mL streptomycinsulfate in a humidified atmosphere with 5% CO_2 at 37 °C. One day before transfection, HepG2 cells were split in growth medium without antibiotics, and then were transfected with chemically synthesized siRNAs after complex formation with Lipofectamine 2000 according to the manufacturer's instructions. Double-stranded oligonucleotides targeting against SelS were synthesized by Shanghai GenePharma (Shanghai, China), siRNA sequences were: 5′-GAACUAAAUGCGCAAGUUGTT-3′ (sense) and 5′-CAACUUGCGCAUUUAGUUCTT-3′ (antisense) for SelS, and 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACAC GUUCGGAGAATT-3′ (antisense) for negative control. Following transfection for approximately 24 h, fresh serum-free growth medium was replaced. Twelve hours later, the cells were treated with the fresh normal media and then incubated with 200 ng/mL LPS (dissolved in phosphate-buffered saline) for 12 or 24 h [18] and harvested for analysis.

Measurement of cell viability, reactive oxygen species and GPx-1 activity

Cell viability was assessed by MTT assay.

Level of reactive oxygen species and GPx-1 activity were determined as described in the previous paper [19].

Real-time RT PCR analysis

Total RNA was isolated from the cells using Trizol reagent according to the manufacturer's directions. Complementary DNA (cDNA) was prepared from the RNA in the presence of M-MLV reverse transcriptase, dNTP, and oligo (dT) in Tris (hydroxymethyl) aminomethane hydrochloride (Tris–HCl) buffer (50 mM, pH 8.3). The reaction mixture was incubated at 37 °C for 60 min and stopped by heating at 95 °C for 10 min.

Real-time PCR was performed with the DNA Engine Opticon 2 (MJ Research, Watertown, Massachusetts, USA), using the SYBR Green PCR Master Mix kit, according to the vendor's protocol. Primer sets for SelS were 5′-gttgcgttgaatgatgtcttcct-3′ (forward) and 5′-agaaacaaaccccatcaactgt-3′ (reverse) [2]; for GPx-1 were 5′-cgcttccagaccattgacatc-3′ (forward) and 5′-cgaggtggtattttctgtaagatca-3′ (reverse) [20]; for iNOS were 5′-cagcgggatgactttccaag-3′ (forward) and 5′-aggcaagatttggacctgca-3′ (reverse) [21]; for SAA1 were 5′-ctgcagaagtgatcagcg-3′ (forward) and 5′-attgtgtaccctctcccc-3′ (reverse) [22] and for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as an internal control were 5′-ccatgttcgtcatgggtgtgaacca-3′ (forward) and 5′-gccagtagaggcagggatgatgttc-3′ (reverse). The thermal cycle conditions were as follows: 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min for SelS and iNOS; 10 min at 95 °C followed by 40 cycles of 94 °C for 15 s, 62 °C for 30 s and 72 °C for 30 s for GPx-1; 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 95 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min for SAA1; 5 min at 94 °C followed by 25 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min for GAPDH. Expression levels of target genes were related to the expression level of the housekeeping gene GAPDH, and relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ rule [23].

Western blot assay

After treatment cells in 10 cm dishes were collected, and lysed in cell lysate (100 mM Tris–HCl, pH 8.0; 0.15 mM NaCl; 1 mM EDTA; 1 mM phenylmethane sulfonate fluoride (PMSF); 10 μg/mL aprotinin; 10 μg/mL leupeptin; 1% Tween-20). The protein content was determined using the method of Bradford with bovine serum albumin (BSA) as standard. Then 75 μl aliquots were mixed with 25 μl sample loading buffer, heated at 100 °C for 3 min, and loaded on 12.5% sodium dodecyl sulfate-polyacrylamide gels (SDS–PAGE) for electrophoresis. The fractionated proteins were transferred to nitrocellulose membranes and immunoblotted with a rabbit polyclonal antibody against SelS. The culture media were carefully collected and spun at 2000*g* for 5 min. Then the supernatant was concentrated using Millipore concentrators. The concentrated culture media were loaded on 14% polyacrylamide gels for electrophoresis and the fractionated proteins were transferred to a nitrocellulose membrane and probed with a rabbit polyclonal antibody against SAA1. The antibodies were detected using an anti-rabbit secondary antibody conjugated with horseradish peroxidase. Chemiluminescence was used to identify specific proteins according to the ECL system (Pierce).

Measurement of iNOS activity and NO level

The iNOS activity assay is based on the catalysis of L-arginine and dioxygen reaction to generate NO by iNOS which is essentially Ca^{2+}-independent [24]. One unit of iNOS was defined as a production in the NO of 1 nmol/min and was expressed in unit per milligram protein.

Intracellular and extracellular (in the medium) NO levels were determined indirectly by quantification of their oxidized products of degradation ($NO_2^-$and NO_3^-), using nitrate reductase [25] and Griess reagent according to MOSHAGE et al. [26].

Statistical analysis

All results are expressed as mean ± standard deviation (SD). The statistical significance of difference between groups was evaluated by analysis of variance, followed by the Student's *t*-test, and a *P* value less than 0.05 was considered to be statistically significant.

Results

SelS mRNA and protein expressions in HepG2 cells fore-and-aft SelS gene silence

After double-stranded siRNA transfected into human hepatoma HepG2 cells with Lipofectamine 2000, cell viability was measured by MTT assay. Results (data not shown) showed that cell viability was almost not affected by SelS siRNA or negative control (siRNA)