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Original Paper

Extracellular HSP70 Activates ERK1/2, NF-κB and Pro-Inflammatory Gene Transcription Through Binding with RAGE in A549 Human Lung Cancer Cells

Nauana Somensi[a,b] Pedro Ozorio Brum[a] Vitor de Miranda Ramos[a] Juciano Gasparotto[a] Alfeu Zanotto-Filho[c] Diana Carolina Rostirolla[a] Maurilio da Silva Morrone[a] José Claudio Fonseca Moreira[a] Daniel Pens Gelain[a,b]

[a]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, [b]Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, [c]Departamento de Farmacologia, Universidade Federal de Santa Catarina, Florianópolis, Brazil

Key Words
eHSP70 • RAGE • ERK • NF-κB • Inflammation • Lung cancer

Abstract
Background/Aims: Heat shock protein 70 (HSP70) has been recently described with extracellular actions, where it is actively released in inflammatory conditions. Acting as DAMPs (damage associated molecular pattern) , extracellular HSP70 (eHSP70) interacts with membrane receptors and activates inflammatory pathways. At this context, the receptor for advanced glycation endproducts (RAGE) emerges as a possible candidate for interaction with eHSP70. RAGE is a pattern-recognition receptor and its expression is increased in several diseases related to a chronic pro-inflammatory state. One of the main consequences of RAGE ligand-binding is the ERK1/2 (extracellular signal–regulated kinases)-dependent activation of NF-kB (nuclear factor kappa B), which leads to expression of TNF-α (tumor necrosis factor alpha) and other cytokines. The purpose of this work is to elucidate if eHSP70 is able to evoke RAGE-dependent signaling using A549 human lung cancer cells, which constitutively express RAGE. ***Methods:*** Immunoprecipitation and protein proximity assay were utilized to demonstrate the linkage between RAGE and eHSP70. To investigate RAGE relevance on cell response to eHSP70, siRNA was used to knockdown the receptor expression. Signaling pathways activation were evaluated by western blotting, gene reporter luciferase and real time quantitative PCR. ***Results:*** Protein eHSP70 shown to be interacting physically with the receptor RAGE in our cell model. Treatment with eHSP70 caused ERK1/2 activation and NF-κB transactivation impaired by RAGE knockdown. Moreover, the stimulation of pro-inflammatory cytokines expression by

Nauana Somensi

Departmento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul - Rua Dr Ramiro Barcelos, 2600, anexo, Porto Alegre, Rio Grande do Sul (Brazil)
Tel. +55 (51) 33085577, E-Mail nauana.somensi@gmail.com

eHSP70 was inhibited in RAGE-silenced cells. Finally, conditioned medium of eHSP70-treated A549 cells caused differential effects in monocytes cytokine expression when A549 RAGE expression is inhibited. **Conclusions:** Our results evidence eHSP70 as a novel RAGE agonist capable of influence the cross-talk between cancer and immune system cells.

Introduction

Heat shock proteins (HSP) are highly conserved proteins found in all prokaryotes and eukaryotes. Heat shock protein 70 (HSP70) has been well characterized as an intracellular molecular chaperone [1] involved in nascent and damaged intracellular protein refolding, also being able to promote protein ubiquitination and proteasomal degradation [2]. A wide variety of stressful and biological alterations, including environmental, pathological or physiological stimuli induce a marked increase in intracellular HSP synthesis [3-5]. Many studies show that HSP70 can also be found outside the cell, especially under specific stress conditions [6-8]. It was first believed that origin of extracellular HSP was due to cell necrosis [9], but other studies described the active secretion of HSP70 by alternatives pathways [10-13]. In extracellular environment, HSP70 can form complexes with peptides followed by antigen cross-presentation, or HSP70 itself can interact with receptors present in APCs, like macrophages and dendritic cells, surface [7, 14, 15]. Acting as damage associated molecular pattern (DAMP) and chemokine, extracellular HSP70 (eHSP70) is capable to create potent innate immune response: it induces secretion of inflammatory cytokines (TNF-α, IL-1β, IL-6), inducible nitric oxide synthase (iNOS) expression and nuclear translocation of Nuclear Factor-κB (NF-κB) [16]. Growing number of surface receptors for HSPs, such as Toll-like receptor (TLR) and CD14, have been associated with phagocytosis and are involved in the induction of pro-inflammatory response [7, 17].

The receptor for advanced glycation endproducts (RAGE) is a transmembrane receptor of the immunoglobulin superfamily that can recognize some patterns associated to pathogen (PAMPs) and damage (DAMPs) capable of binding a broad repertoire of ligands [18]. RAGE has constitutive expression during embryonic development, but in adulthood its expression is regulated. Exceptionally, RAGE has been found to be constitutively expressed at high levels in skin and lung [19]. In other tissues, RAGE expression may be induced by accumulation of its ligands and/or inflammatory mediators.

In many pathologies RAGE is the major receptor for molecules that mediate disease progression and chronic inflammatory state, such as rheumatoid arthritis, Alzheimer's disease, arteriosclerosis, chronic kidney disease and some cancers [20]. Accumulation of agonists at sites of tissue injury and inflammation has been found to induce intracellular activation of diverse cellular pathways. Sustained stimulation of RAGE promotes a positive feedback mechanism that reinforces its own expression, mediated mainly by NF-κB [21]. Thereby, increase of RAGE expression is a progression factor to amplifying or sustain immune and inflammatory responses [22].

At this context, RAGE emerges as a potential mediator of eHSP70 signaling actions. The expression and secretion of this chemokine is increased in several diseases related to a chronic pro-inflammatory state, concomitantly with accumulation of other RAGE ligands. Besides, many intracellular pathways believed to be elicited by eHSP70 solely via other receptors that recognize molecular patterns (e.g. TLR, CD14) are strongly influenced by RAGE, such as ERK1/2-mediated activation of NF-kB. Furthermore, an ELISA screen assay proposed a potential interaction between RAGE and extracellular HSP70 [23], while a recent work by our group proposed an interaction model by molecular docking and evidenced a physical interaction between RAGE-eHSP70[24]. However, since the activity of HSP70 as molecular chaperone allows nonspecific interactions with diverse proteins, the aim of the current study was to investigate if the eHSP70-RAGE interaction is able to induce a functional activation of RAGE in a cellular model, in order to establish eHSP70 as a functional RAGE agonist. With this purpose, the effect of eHSP70 on the regulation of classical RAGE downstream targets

(ERK1/2 and NF-kB) and over pro-inflammatory mRNA transcription in A549 human non-small lung cancer cells were assessed, as these cells constitutively express RAGE. To establish the importance of RAGE in the effects evoked by eHSP70, A549 cells were transfected with RAGE siRNA.

Materials and Methods

Cell Culture

The human non-small cell lung cancer (NSCLC) cell line A549 (kindly donated by Prof. Claudia Simoes, Universidade Federal de Santa Catarina, Brazil) was grown in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1× antibiotic/antimycotic solution (Sigma-Aldrich). Cells were cultured in at 37°C in an atmosphere containing 5% CO_2. When cultures reached confluence, adherent A549 were trypsinized and seeded on culture plates at a density of approximately 20 x 10^3 cells/cm^2. When performing phosphorylation and immunoprecipitation experiments, 10% SFB medium was replaced for fresh serum-free RPMI medium two hours before eHSP70 treatments; for long-time exposition treatments, culture medium was replaced for fresh serum-free RPMI medium along with the treatment. The human myeloid monocyte cell line U937 (kindly donated by Prof. Fábio Klamt, Universidade Federal do Rio Grande do Sul, Brazil) was grown in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 × antibiotic/antimycotic solution (Sigma-Aldrich). When reached confluence, non-adherent U937 was washed and resuspended in A549 conditioned medium. After 24 hours medium was replaced for fresh 10% FBS RPMI medium and maintained for additional 24 hours before harvest for RT-qPCR analysis.

Chemicals and Treatments

Human recombinant HSP70 endotoxin-free was purchased from Abcam. Human recombinant HMGB1, U0126, cisplatin and propidium iodide were purchased from Sigma-Aldrich. RAGE antagonist FPS-ZM1 was purchased from Merck-Millipore and dissolved in DMSO at 1 mg/mL. All stock solutions aliquots were stored at -20°C. All chemicals were diluted in serum-free medium to perform treatments. Pharmacological inhibitors were added to the cells 30 minutes before adding eHSP70.

Immunoprecipitation

A549 cells were treated with eHSP70 or HMGB1 for thirty minutes and collected in cold PBS. Cells were centrifuged at 5, 000 x g for 5 minutes and lysed in extraction buffer (pH7.4) containing 20 mM HEPES, 0, 8% IGEPAL, 10 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 2 mM PMSF in the presence of a mixture of protease inhibitors (Roche Applied Science). Cells were incubated on ice for 30 minutes and centrifuged for 1 minute at 14, 000 x g, 4°C, and then the supernatant was transferred to a new tube. To remove non-specific binding, cells were incubated with 25 µL protein A/G (Pierce) for 15 minutes on ice and centrifuged at 2000 x g for 1 minute. A volume of 50 µL of supernatant was collected as lysate control and the remaining were mixed to NaCl 0.9% (proportion of 1:1 to extraction buffer). Cell homogenates were incubated overnight with polyclonal anti-RAGE (Santa Cruz, sc-5563), anti-HSP70 (Santa Cruz, sc-1060) or anti-HMGB1 (Santa Cruz sc-26351) at 2 µg per mg of total protein by reaction. The solutions were then incubated with 50 µL protein A/G for 4 hours by gentle rocking at 4°C followed by centrifugation for 30 seconds at 3000 x g (4°C). Pellet was washed three times with 500 µL of cell lysis buffer:NaCl 0.9% (1:1). Immunoprecipitates were resuspended in 2x Laemmli buffer (65.8 mM Tris-HCl, 26.3% glycerol, 2.1% SDS, 0.01% bromophenol blue, pH 6.8) with 10% 2-mercaptoethanol and then immunoblotted.

Protein proximity assay

The detection and visualization of the interaction between extracellular HSP70 and RAGE were carried out using a Duolink In Situ kit from Sigma-Aldrich. This assay facilitated the detection, visualization, and quantification of protein interactions in cell samples prepared for microscopy based on the proximity of fluorescent probes to the target primary antibodies. Cells were seeded in 24-well plates and then treated with 10 µg HSP70 and/or FPS-ZM1 15 minutes before, enabling receptor blockade. After 30 minutes, the cells were washed with PBS 1X and fixed with 4% paraformaldehyde for 30 min. Blocking solution was then added, and cells were incubated for 30 minutes at 37 °C. Samples were incubated overnight in a humidified

chamber with the primary antibodies anti-RAGE (Abcam ab37647) at a 1:1000 dilution; and anti-HSP70 (Milipore mAb3846) at a 1:700 dilution. PLA probes were added, and the samples were incubated in a humidified chamber for 60 minutes at 37 °C. Next, a ligation-ligase solution was added, and the samples were incubated for 30 minutes at 37 °C. Finally, an amplification-polymerase solution was added, and the samples were incubated for 100 min at 37 °C. Samples were washed and diluated in DAPI Medium solution for 15 minutes. The images were obtained with a Microscopy EVOS® FL Auto Imaging System (AMAFD1000 - Thermo Fisher Scientific; MA, USA).

Western Blotting

After treatments, cells were washed in cold PBS and collected in RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1 % Triton X-100, 1 % bile salt, 1 mM Na_3VO_4, 1mM PMSF, pH 7.5), sonicated and protein lysates were clarified by centrifugation. Protein quantification was performed by BCA Protein Assay kit (Sinapse), denatured in Laemmli 4 X 10% 2-mercaptoethanol buffer and heated at 95 °C for 5 minutes. A total of 30 µg of protein was loaded in 11% polyacrylamide gel and transferred by semi-dry system (Trans-blot® SD transfer cell Bio-Rad) to a nitrocellulose membrane (Hybond-ECL, GE Healthcare Lifesciences). Membranes were washed in Tris-buffered saline Tween-20 [TBS-T; 20 mM Tris– HCl, pH 7.5, 137 mM NaCl, 0.05 % (v/v) Tween 20] and blocked in solution of TBS-T with 5% BSA or non-fat milk for 1 hour at room temperature. After, membranes were washed three times in TBS-T and incubated overnight at 4°C with primary antibodies in a dilution of 1:1000: anti-RAGE (Abcam ab37647), anti-RAGE (Santa Cruz , sc-5563), β-actin (Sigma, #1978), anti-phospo-ERK1/2 (Thr202/Tyr204) (Cell Signaling, #4376S and #9101), anti-ERK1/2 (Cell Signaling, #9102), anti-phospho-JNK (Cell Signaling, #9251), anti-JNK (Santa Cruz, sc-571), anti-phospho-p38 (Cell Signaling, #9215S), anti-p38 (Santa Cruz, sc-7972). Afterward, membranes were washed and incubated with species-specific HRP-conjugated secondary antibodies at 1:3000 in TBS-T with 5 % BSA for 2 hours at room temperature. The blot was analyzed by adding SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific) and captured by CCD camera (GE ImageQuant LAS 4000).

siRNA knockdown

Human RAGE (#9438) and scrambled sequence (AM4635) small-interfering RNA were purchased from Invitrogen. Reverse transfection of siRNAs was performed using siPORT™ NeoFX™ Transfection Agent (Ambion®, Applied Biosystems Inc.) and Opti-MEM following manufacturer's instructions. A549 cells were transfected with 25 nM and 50 nM of siRNAs for 48 hours in 10 % SFB medium without antibiotic. Knockdown efficiency was evaluated by RT-qPCR and western blotting.

Real Time qPCR

After treatments, cells were washed in cold PBS buffer and samples and scrapped in TRIzol reagent (Thermo Fisher Scientific). The RT-qPCR analysis was performed as previously decribed [25]. RNA extraction was performed following manufacturer's instructions. RNAs were treated with DNase I (Invitrogen®) following reverse transcription (SuperScript III First-Strand Synthesis SuperMix Invitrogen®). Real time quantitative polymerase chain reaction (RT-qPCR) was performed using GoTaq qPCR MasterMix (Promega), 150 ng cDNA and 100nM of each primer: GAPDH (forward: 5' AATCCCATCACCATCTTCCAG 3', reverse: 5' TTCACACCCATGACGAACAT 3'); GNB2L (forward: 5' GAGTGTGGCCTTCTCCTCTG 3', reverse: 5' GCTTGCAGTTAGCCAGGTTC 3'); B2M (forward: 5' TGCTGTCTCCATGTTTGAT 3', reverse: 5' TCTCTGCTCCCCACCTCTA 3'); RAGE (forward: 5' GTGAAGGAACAGACCAG 3' , reverse: 5' GAAGCTACAGGAGAAGGTGG 3'); TNF-α (forward: 5' GATCCCTGACATCTGGAATCTG 3', reverse: 5' GAAACATCTGGAGAGAGGAAGG 3'); IL1-β (forward: 5' GCATCCAGCTACGAATCTC 3', reverse: 5' ATTCTCCTGGAAGGTCTGT 3'); IL-6 (forward: 5' AATGAGGAGACTTGCCTGGTG 3', reverse: 5' GCAGGAACTGGATCAGGACTTT 3'); IL-13 (forward: 5' GTCATTGCTCTCACTTGCCTTG 3', reverse: 5' GTCAGGTTGATGCTCCATACCA 3'); TGF-β (forward: 5' TTCCTGCTTCTCATGGCCAC 3', reverse: 5' GAGGTCCTTGCGGAAGTCAA 3'). Results were expressed in relation to constitutive normalization gene (ΔCt) and internal non-treated control group (ΔΔCt).

Reporter Gene (Luciferase) Assay

A549 cells were plated in 96-well plates and after 24 hours transfected with 100 ng of a vector containing a responsive element to NF-κB driving firefly luciferease (pGL4.32 Luc2P-NF-κB® Promega) and

10 ng constitutive Renilla-luciferase construct (pRL-TK® Promega) per well. The transfection was carried by ViaFect® reagent (Promega) and Opti-MEM. siRNAs particles were transfected along with the plasmids. Cells were also transfected with GFP vector to monitor the efficiency of transfection, and an empty vector was used as negative control. When more than 60% of cells display green fluorescence, NF-κB-luciferase and Renilla transfected cells were treated. At the end of treatments, the cells were lysed and luciferase activity was assessed using the Dual-Glo® Luciferase Assay System (Promega). Results are expressed in ratio Luciferase/Renilla luminescence relative to control groups.

FACS Analysis

For cell cycle analysis, A549 cells were treated with eHSP70 (1 μg/mL) and cisplatin (10 μg/mL) for 48 and 72 hours. After these times, 12-well plated cells were trypsinized, centrifuged and washed two times by adding 1 mL of PBS, centrifuging at 1, 000 x g for 5 minutes, and the pellet was resuspended in 500 μL of cycle buffer (PBS 1x, 1 mg/mL spermine, 5 mM EDTA, 0, 1% IGEPAL, 3 μg/mL propidium iodide, 10 μg/mL RNAse, pH 7.4). Cells were mixed and incubated on ice for 10 minutes and DNA content was determined by flow cytometry. Ten thousand events were counted per sample. FACS analyses were performed in the Flowjo tree star Software (Ashland, OR, USA) [26].

Cell Viability Sulforhodamine B Assay

This colorimetric assay evaluates cell numbers indirectly by staining total cellular protein with SRB [27]. A549 cells were plated onto 96-well plates and when culture reached 60% confluence, the culture medium was removed. Increasing concentrations of eHSP70 and FSP-ZM1 or 50nM of siRNAs were added to the cells. After 24 hours of treatment, culture medium was discarded and cells were fixed with 10 % trichloroacetic acid for 1 h at 4°C. Plates were then stained for 30 minutes with 0.4% (wt/vol) sulforhodamine B (SRB) dissolved in 1% acetic acid. Overdye was removed by four washes with 1% acetic acid, until only the dye adhering to the cells was left. Stained cells were dissolved in 100 μL of 10 mM Tris base solution (pH 10.5). The absorbance (OD) of each well was read using SpectraMAX i3 (Molecular Devices) spectrophotometer set at 515 nm. Cell survival was measured as the percentage absorbance compared to the absorbance of a control (non-treated cells).

Statistical Analyses

Analyses were performed with GraphPad Prism 5 and GraphPad Prism 7 softwares. Statistical tests were determined by one-way ANOVA followed by Tukey's post hoc test for multiple groups comparison or Bonferroni post hoc test to compare selected groups (two parameters) when applicable. Two-way ANOVA followed by Tukey post hoc test was applied to compare multiple groups (two parameters). A $p<0.05$ value was considered statistically significant. Results are expressed as mean values ± standard error (SEM). All data presented here are the result of three or more independent experiments. For HeatMap analysis, we first calculate standard score by using the formula $z = \frac{x-\mu}{\sigma}$, where x is the calculate value, μ is the mean of the population and σ is the standard deviation of the population. Graph was designed by GraphPad Prism 7 software.

Results

RAGE physically interacts with eHSP70

In a previous work, our group characterized the physical interaction between RAGE and eHSP70 by molecular docking and proximity assay using A549 cells . To investigate the mechanism and biological consequence of eHSP70 on cells, we sought to first confirm the engagement of this protein to RAGE in our cells by immunoprecipitation assay (IP). Human lung carcinoma (A549) cell line, which constitutively expresses RAGE, was treated with eHSP70 for 30 minutes prior to IP. As shown in Fig. 1 A, IP of treated cell extracts with anti-HSP70 antibody followed by WB with anti-RAGE on the precipitate, demonstrated the interaction of eHSP70-RAGE. The same result was found when cell extract was immunoprecipitated with anti-RAGE followed by WB with anti-HSP70. The content of RAGE co-precipitating with eHSP70 seems to be higher compared to HMGB1-RAGE co-precipitate,

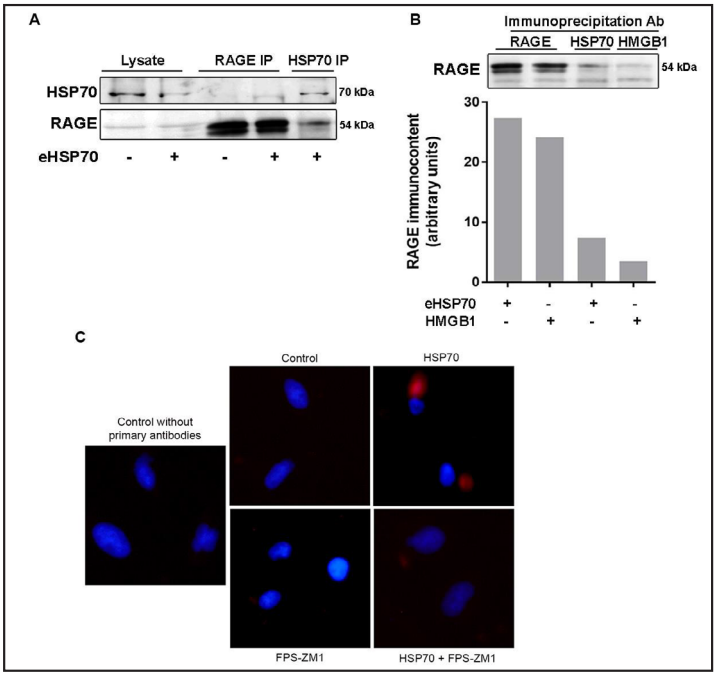

Fig. 1. eHSP70 physically interacts with RAGE. A549 cells were treated with HSP70 or HMGB1 for 30 minutes and immunoprecipitation assay was carried with antibodies against RAGE, HSP70 and HMGB1. Western Blotting analysis was performed using A) anti-RAGE and anti-HSP70 antibodies for HP70-treated cells and B) anti-RAGE to compare HMGB1 and HSP70 treated-cells. A549 were also incubated with eHSP70 and/or FPS-ZM1 and protein proximity was evaluated by duolink assay. C) Microscopy analysis indicates blue fluorescence indicates DAPI marking of nucleus and red fluorescence only exists when both marked proteins are really close, indicating interaction.

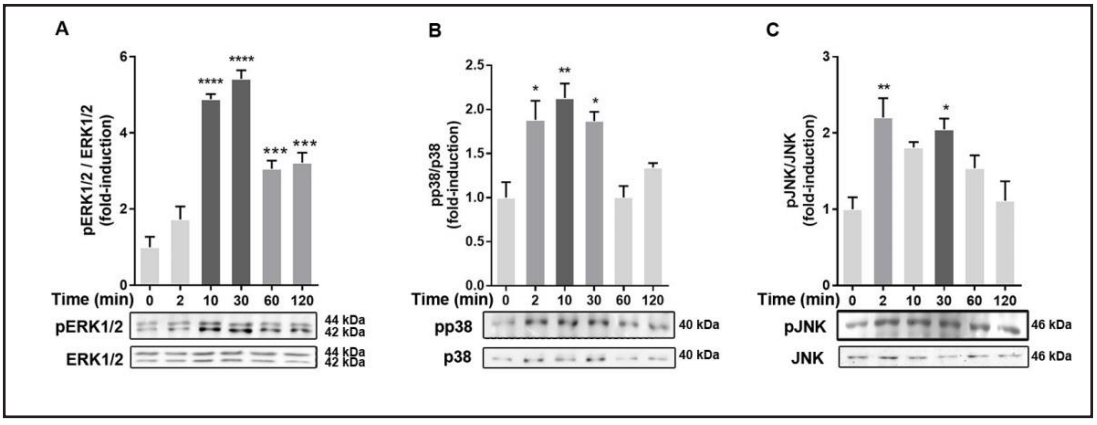

Fig. 2. eHSP70 enhances MAPK phosphorylation in a time-dependent manner.A549 were incubated with eHSP70 (1µg/µL) in a time-curve (up to 120 minutes) assay and the immunocontent of proteins was assessed by western blot: D) ERK1/2, E) p38, F) JNK (phosphorylated and total isoforms). Graphs present mean ± SEM values (two-way ANOVA statistical analysis followed by Tukey: * $p<0.05$,** $p<0.001$ compared to control group).

used as a positive control for RAGE binding (Fig. 1 B). We also performed a protein proximity assay which confirmed this observation (Fig. 1C). Red fluorescence is the result of proximity between eHSP70 and RAGE, which was observed on the outer edges of the cell in contrast to DAPI blue fluorescent nucleus. When the cells were pre-incubated with RAGE antagonist (FPS-ZM1) prior to eHSP70, the red fluorescence was decreased, indicating that eHSP70 is not interacting with RAGE.

eHSP70 enhances MAPK phosphorylation in a time-dependent manner

Mitogen-activated protein kinases (MAPKs) are a family of proteins involved in a wide range of cell responses mediated by inflammatory stimuli and previous works observed that RAGE activation leads to fast cell responses involving MAPK signaling cascade [28-32]. Thus,

we analyzed the effect of eHSP70 on the activation state of the MAPKs ERK1/2, p38 and JNK. Recombinant eHSP70 (1µg/mL) was added to medium bathing A549 cells for increasing periods up to 120 min and immunoblots were performed to detect the phosphorylated (active) forms of these protein kinases. As shown in Fig. 2, the activity of ERK 1 and 2 (Fig. 2 A), p38 (Fig. 2 B), JNK (Fig. 2 C) was significantly increased by eHSP70 in a time-dependent manner. In 10 minutes of exposition, there was a peak in MAPK phosphorylation and this activation signal continues until 30 minutes. After this time, the phosphorylation of kinases decreases.

eHSP70 activates ERK1/2 through RAGE, but not p38 and JNK.

Previous studies have shown that some RAGE agonists are also able to bind others receptors also involved in stress and inflammation, such as TLRs [30], and may initiate a cellular response via MAPK. In addition, eHSP70 has been described as TLR ligand and to play a key role in inflammation progression [17]. Considering these observations, we focused to determine the extent of RAGE involvement in MAPK phosphorylation evoked by eHSP70.

We first set out the RAGE knockdown by siRNA transfection assay (Fig. 3). Levels of mRNA and protein expression decreased when cells were transfected with highest RAGE siRNA concentration (50 nM) up to 48 hours (Fig. 3 A and B). Varying periods of transfection were tested and shorter times of incubation yielded more pronounced inhibition in RAGE mRNA expression (data not shown). Nonetheless, RAGE protein levels were more efficiently decreased after 48 hours of transfection with 50 nM of siRNA sequence. Phase contrast microscopy shows no change in cell morphology (Fig. 3 C) and SRB-incorporation assay

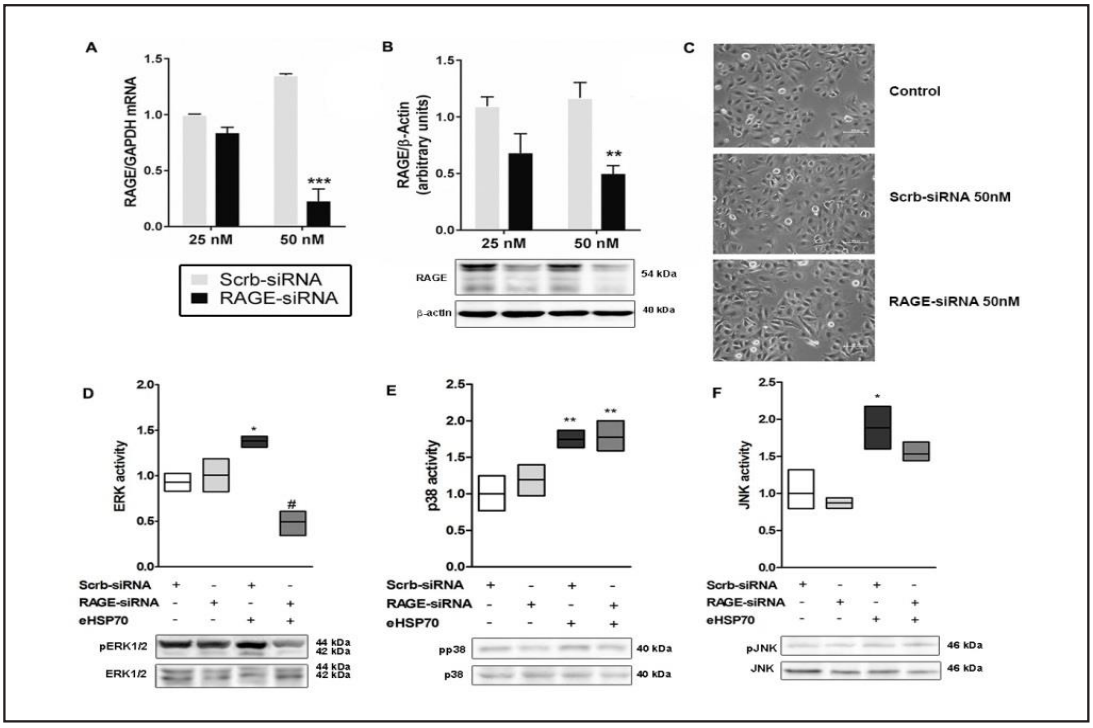

Fig. 3. eHSP70 activates ERK1/2 through RAGE. RAGE knockdown was performed with small interference RNA (siRNA) methodology; siRNA with scrambled sequence (control) and RAGE-specific sequence were transfected in concentrations of 25 nM and 50 nM. After 48 hours samples were collected and RAGE content was measured by A) RT-qPCR mRNA expression and B) Western blot protein immunocontent (ANOVA followed by Tukey: ** p<0.01, *** p<0.001). C) Morphology assessment of 48 hours after transfection. Transfected cells were treated with eHSP70 (1µg/µL) for 30 minutes and phosphorylation of D) ERK1/2, E) p38 and F) JNK was evaluated (ANOVA followed by Bonferroni: * p<0.01, ** p<0.001 compared to control, #p<0.0004 compared to scrambled-transfected cells treated with eHSP70).

shows that silencing does not affect the cell viability (Fig. 4). Together, these results allowed us to use RAGE siRNA at 50 nM to knockdown the receptor for following experiments.

Next, RAGE-silenced A549 cells were stimulated with eHSP70 for 30 minutes and MAPKs activation were evaluated by western blotting (Fig. 3 D, E, F). eHSP70 activated ERK1/2 in a RAGE-dependent manner (Fig. 3 D), once RAGE silencing impaired the phosphorylation of these kinases. On the other hand, activation of p38 and JNK were not affected by RAGE knockdown, suggesting that this effect is not dependent on RAGE activation (Fig. 3 E and F).

eHSP70 stimulates NF-κB activation via RAGE and ERK1/2

Activation of RAGE has been found to induce many cell responses, which lead the activation of transcription factors associated to cell proliferation, migration and inflammation, like NF-κB [33]. Here, we determined the transcriptional activation of eHSP70-induced NF-κB by reporter gene assay (Fig. 5 A). There was a 3-fold increase in luciferase luminescence by eHSP70 compared to control, indicating that eHSP70 activates NF-kB transcriptional activity. RAGE knockdown by siRNA transfection blocked NF-kB activation by eHSP70. This effect was also observed with RAGE pharmacological inhibition with FPS-ZM1 (1 μg/mL). Pharmacological inhibition of MEK1/2 by UO126 (10 μM) also led to blockage of NF-κB activation by eHSP70. These results evidenced the role of eHPS70 on RAGE/ERK/NF-κB pathway.

eHSP70 positively regulates the feedback of RAGE expression

It is known that RAGE itself is an NF-κB regulated target gene, exhibiting a functional binding site for NF-κB in its proximal promoter [34]. To investigate if eHSP70 stimulus is able to regulate RAGE expression, A549 cells were treated with eHSP70 for 24h and RAGE mRNA expression and protein immunocontent was evaluated by RT-qPCR and western blot, respectively (Fig. 5, B and C). We found a positive regulation of RAGE expression at mRNA and protein levels by eHSP70, once those contents increased significantly when compared to control group. We also confirmed that RAGE is essential for this cellular response evoked by eHSP70, once commercial antagonist FPS-ZM11 blocked the positive feedback. Glycated albumin (AGE), which is a well described RAGE agonist, was used as experimental positive control, but, interestingly, it did not significantly alter RAGE levels compared to control group or eHSP70 stimulated group.

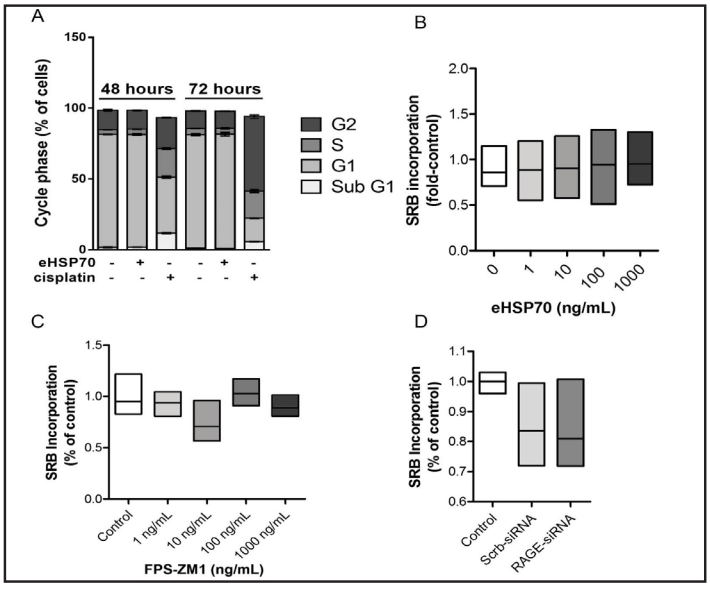

Fig. 4. eHSP70 did not change A549 cell cycle and viability. RAGE antagonist and siRNA treatments also did not affect cell viability. (A) A549 cells were incubated with eHSP70 or cisplatin (control for induction of cell cycle changes) for 48 and 72 hours. Cells were labeled with propidium iodide and staining was analyzed by fluorescence-activated cell sorting (FACS). A549 cells were treated with (B) eHSP70, (C) FSP-ZM1 in a concentration-curve or with (D) siRNA 50 nM and SRB incorporation was evaluated to measure number of cells. Treatments did not change cell viability (ANOVA followed by Tukey with p=0.05).

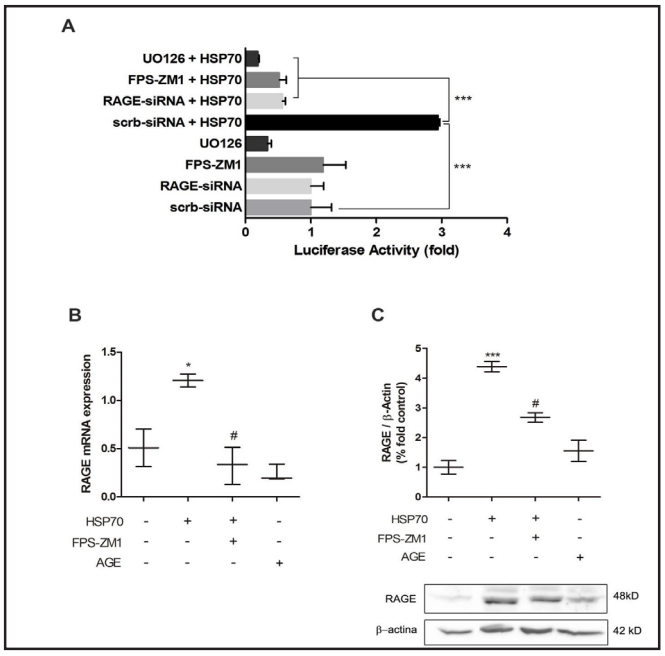

Fig. 5. Through RAGE, eHSP70 enhances NF-κB activation and RAGE itself down-regulation. First, ene reporter assay was performed in cells knockdowned with siRNA or pretreated with pharmacological inhibitors FPS-ZM1 and UO126 cells before eHSP70 treatment (12 hours). Luciferase activity was measured and results are expressed as fold-induction of Luciferase/Renilla luminescence control (ANOVA followed by Tukey: *** p<0.0001). After that, A549 cells were treated with eHSP70 and FPS-ZM1 for 24 hours and the B) mRNA expression and C) immunocontent of RAGE were evaluated (ANOVA followed by Bonferroni: * p<0.03, *** p<0.0003 compared to control, #p<0.003 compared to cells treated with eHSP70).

Table 1. eHSP70 induces RAGE-dependent pro-inflammatory cytokine transcription in A549 cells. RAGE silenced A549 cells were incubated with eHSP70 for 12 hours and RNA was extracted to determine cytokine translation by RT-qPCR. TNF-α, IL-1β ,IL-6 , IL-13 and TGF-β mRNA expression are expressed in relation to GNB2L mRNA levels. Statistical analysis were performed using ANOVA followed by Bonferroni (* p<0.05 and ** p<0.001 compared to control; # p<0.01 compared to RAGE knockdowned A549 cells treated with HSP70)

	Scrb-siRNA	RAGE-siRNA	Scrb-siRNA + HSP70	RAGE-siRNA +HSP70
TNF-α	0.7494 ± 0.3462	0.7994 ± 0.2375	1.4 ± 0.4546*	0.6804 ± 0.2101#
IL-1β	0.7781 ± 0.4669	0.3992 ± 0.2943	2.867 ± 0.1872 **	0.582 ± 0.2736 #
IL-6	1.068 ± 0.2776	0.7442 ± 0.1806	2.344 ± 0.5795 *	0.6369 ± 0.4117 #
IL-13	2.362 ± 0.5030	2.173 ± 0.1743	0.6378 ± 0.09187 **	2.264 ± 0.5148 #
TGF-β	0.6446 ± 0.5194	0.6788 ± 0.2826	1.513 ± 0.1365	1.025 ± 0.8332

eHSP70 induces RAGE-dependent pro-inflammatory cytokine transcription in A549 cells

Many of the extracellular ligands that elicit RAGE activation are worth noticing to be involved in acute and chronic immune responses [18]. In turn, HSP70 is released to extracellular medium in conditions of stress and inflammation, and once there, eHSP70 may induce cytokine production [7]. To determinate if eHSP70 is able to activate inflammatory responses through RAGE, A549 cells were treated with eHSP70 for 12 hours and the expression of different cytokines that regulate inflammatory processes was evaluated (Fig. 6 A). RT-qPCR analysis shows that TNF-α, IL-1β and IL-6 expression markedly increased with eHSP70 treatment, and this effect was blocked when RAGE was knockdowned (Table 1, Fig. 6 A). Also, eHSP70 reduced IL-13 mRNA expression, and RAGE knockdown upturned this effect. Expression of TGF-β appears to increase with eHSP70 stimulation, but it did not significantly change compared to control.

Conditioned medium from A549 stimulated with eHSP70 modulates immune response

To address if modulation of cytokines expression by eHSP70 in lung cancer cells is a relevant step in cancer immune response regulation, collected culture supernatants from A549 cells treated with eHSP70 were added to U937 monocyte cell line and samples were collected for RT-qPCR analysis (Table 2, Fig. 6 B). A549 conditioned medium of eHSP70-

Table 2. Conditioned medium from A549 stimulated with eHSP70 modulates immune response. U937 monocyte cells were treated with conditioned medium from A549 cells previous stimulated with eHSP70. RNA was extracted to determine TNF-α , IL-1β ,IL-6 , IL-13 and TGF-β mRNA expression by RT-qPCR. Results are expressed in relation to B2M mRNA levels. Statistical analysis were performed using ANOVA followed by Bonferroni (** p<0.001 compared to control; # p<0.01 compared to RAGE knockdowned A549 cells treated with HSP70)

		Conditioned medium fromA549-treated cells			
	U937 control	Scrb-siRNA	RAGE-siRNA	Scrb-siRNA + HSP70	RAGE-siRNA + HSP70
TNF-α	1.003 ± 0.09674	1.054 ± 0.2402	0.8594 ± 0.4197	0.5456 ± 0.1324	1.169 ± 0.3594
IL-1β	1.079 ± 0.5363	1.011 ± 0.1976	1.387 ± 0.4723	0.5409 ± 0.06922	1.878 ± 0.6171 #
IL-6	0.6860 ± 0.3057	0.9081 ± 0.2354	1.612 ± 0.1658	0.3163 ± 0.03409**	1.937 ±0.1578 #
IL-13	1.061± 0.5030	1.638 ± 0.3881	1.452 ± 1.018	11.36 ± 3.251**	2.039 ± 0.6882 #
TGF-β	0.8575 ± 0.1835	0.9339 ± 0.2509	0.8587 ± 0.1259	0.9614± 0.5716	1.307 ± 0.5947

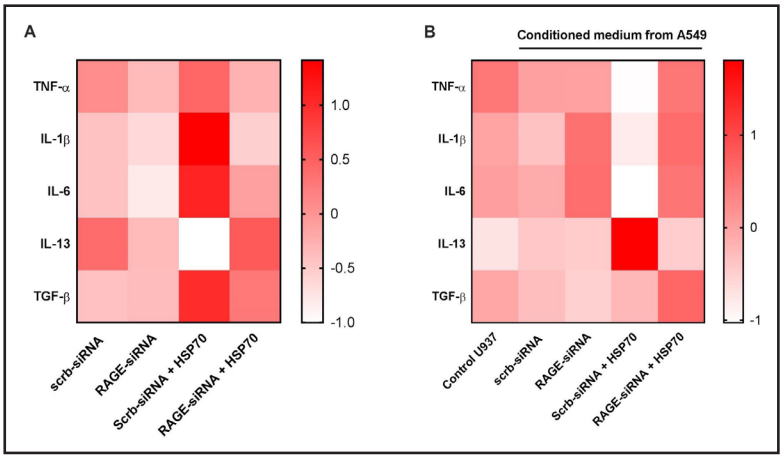

Fig. 6. eHSP70 induces RAGE-dependent pro-inflammatory cytokines mRNA transcription in A549 cells and conditioned medium from these cells modulate immune response. A) RAGE silenced A549 cells were incubated with eHSP70 for 12 hours and RNA was analyzed by RT-qPCR. B) U937 monocyte cells were treated with conditioned medium from A549 cells previous stimulated with eHSP70. The cytokines mRNA levels of U937 were measured by RT-qPCR. All cytokines expression were converted to standard score and plotted in a HeatMap. White color represents the lowest expression level and red indicates high mRNA levels.

treated cells attenuates expression of pro-inflammatory cytokines and led to a rise in expression of anti-inflammatory IL-13 mRNA. The conditioned medium of RAGE-silenced A549 cells treated with eHSP70 did not cause any effect on U937 pro-inflammatory activation. Expression of TGF-β in U937 was not affected by any treatment.

Discussion

HSP70, an intracellular chaperone, has been identified as a modulator of pro-inflammatory cytokines when released from the cytoplasm into the extracellular milieu [11, 12, 35]. Its role in the extracellular environment has been shown to be of great relevance, since it has been used as one of the serum biomarkers described for some diseases, such as gastric cancer [8, 36] . Previous studies reported that eHSP70 can bind many receptors present in APCs and the differences in binding affinities between each cell type is probably due to differences in eHSP70 receptors expression [17, 37]. RAGE is a promiscuous receptor that interacts with several inflammation and stress mediators including AGEs, HMGB1, S100

family proteins, DNA and RNA to activate the inflammatory pathway [38]. In most adult tissues, RAGE expression is suppressed in normal conditions, except in lungs and some endothelial cell types. In this study, we used a human lung cancer-derived A549 cell line, which constitutively expresses RAGE, and validated the interaction between this receptor and eHSP70 by a cellular study-based approach. Since the interaction between eHSP70 and RAGE has already been reported [23, 24], our immunoprecipitation and protein proximity image assay results confirmed this specific binding around the cell, leading us to use this cell model to investigate functional signaling responses evoked by eHSP70-RAGE engagement. It is important to note that the evaluation of functional responses mediated by RAGE is a major step to establish eHSP70 as a RAGE agonist, since HSP70 is able to interact with a myriad of proteins due to its chaperone activity. In this context, the present data demonstrating that eHSP70 activates ERK1/2 phosphorylation, NF-kB transcriptional activity and pro-inflammatory gene transcription, while RAGE knockdown hinders all these effects (some of them to control levels); together with previous data characterizing the RAGE-eHSP70 interaction, our results strongly indicate eHSP70 as a functional RAGE agonist.

The interaction between RAGE and ligand can activate many several cellular pathways. The type of response is signal-tissue specific and depends on the type of ligand, the precise environment and the cell type. In general, RAGE engagement by its agonists leads to the activation of key elements of the MAPK signaling pathways, which include ERK1/2, p38 and JNK [33]. On the other hand, eHSP70 complexed to peptides promote activation of JNK through TLR2 and TLR4 [39]. Our study showed that eHSP70 itself can induce activation of ERK1/2, p38 and JNK (Fig. 2 A, B and C). Interestingly, RAGE mediates only ERK1/2 responses evoked by eHSP70: phosphorylation of these kinases decrease when RAGE expression was inhibited by siRNA transfection, while eHSP70-mediated p38 and JNK activation were not affected when RAGE was knockdowned (Fig. 3 D, E and F).

JNK and p38 pathway are correlated with proliferation, differentiation and changes in cell cycle [39, 40], responses that are not evoked by eHSP70 in A549 cells, as shown by FACS analysis of cell cycle progression and SRB-incorporation viability assays (Fig. 4 A and B). Since cell response is not an isolated event, but dynamically activates many pathways to set a specific effect, many studies also describe involvement of ERK in those events [41]. The rapid phosphorylation of ERK1/2 is described as a typical intracellular response to RAGE activation [42]. Importantly, ERK activation is also correlated with inflammation studies that involve RAGE intracellular domain docking of ERK, leading to activation of kinase activity [30, 43]. These results, together with previous evidence showing the effect of eHSP70 through TLRs, strongly indicate that the modulatory actions of eHSP70 are dependent on multiple receptors activation. In this context, the multiple effects evoked by eHSP70 in different cells/tissues may be ascribed to the differential expression of TLRs subtypes and RAGE by each cell type.

By gene reporter luciferase assay, we showed that eHSP70 itself actives NF-κB (Fig. 5 A). RAGE siRNA transfection impaired luciferase luminescence, and this result was confirmed by RAGE antagonist treatment. Downstream activation of ERK1/2 was also involved in gene response, once pharmacological inhibitor UO126 blocked the effect of eHSP70 on NF-κB activation. These results showed eHSP70 is able to evoke cellular downstream NF-κB gene activation through RAGE ligation and ERK signaling pathway. This is a significant data as the ERK1/2-NF-κB signaling axis is considered a classical hallmark of RAGE-mediated signal transduction.

It is known that RAGE engagement by its agonists triggers feedback looping on receptor expression; once NF-κB is activated, it can bind to responsive element on RAGE promoter [34], and the expression of this gene will be regulated by the transcript factor. Our results showed that RAGE expression and immunocontent after stimulation of A549 cells with eHPS70 treatment increased (Fig. 5 B and C). Also, the receptor was shown to be essential for this cellular response, since blocking of RAGE by antagonist FPS-ZM1 inhibited the feedback. Together with previous experiments (Fig. 5 A), these data reveal that eHSP70 activation of NF-κB leads to RAGE alterations (expression and immunocontent) in our cell model.

Recently, there has been considerable interest in the biological role of HSP70 molecular chaperone in the extracellular space, particularly in the modulation of immune response. It has been described that eHSP70 can bind with high affinity to plasma membrane receptors, eliciting activation of NF-κB and regulating the expression of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 in human monocytes [7]. In the present study, eHSP70 was able to drive the expression of these cytokines in A549 cells, a human non-small lung cancer line. TNF-α is an inflammatory mediator that has been implicated in carcinogenesis and can be produced at early stages of cell pro-inflammatory activation. TNF-α, IL-1β and IL-6 promote cancer invasiveness [44] and malignancy [45], respectively; mRNA expression levels of these pro-inflammatory mediators were observed to be modulated by eHSP70 in a RAGE-dependent fashion. Importantly, RAGE knockdown was able to completely block the effect of eHSP70 on modulatory cytokines.

It is well described that eHSP70 promotes cytokine production in APCs via TLR and other c-type lectin members, but it was also reported that eHSP70 can keenly bind to non-APC cell lines, especially those from epithelial or endothelial background [46]. Our results indicate that RAGE is an important recognizer of eHSP70, and particularly in our cell model this receptor is essential to promote cytokines expression by eHSP70. This is probably because RAGE is the major pattern recognizer in A549 lung epithelial cell line, and its constitutive expression is higher than TLR and other c-type lectine receptors. Similar effects by HMGB1 were described in normal human bronchial epithelial (NHBE) cells, once RAGE blockage impairs the production of inflammatory cytokines stimulated by this agonist [30].

Expression of IL-13 was also decreased by eHSP70 via RAGE. IL-13 is a M2 cytokine that act as central mediator of the physiologic changes induced by allergic inflammation, but also inhibits the production of pro-inflammatory cytokines, up-regulates monocyte MHCII and CD23 and induces IgG and IgM synthesis in B cells, among other effects [47]. Its expression in lung is well correlated with mucus hyper secretion, which contributes to progression of airway allergic diseases [48-50]. Nonetheless, chronic inflammation is important in generating malignancy through the exposure of pro-inflammatory cytokines and sustained activation of signaling pathways such as NF-κB [45]. In this context, eHSP70 inhibition of IL-13 may contribute to drive cancer cells to a pro-inflammatory environment. Our results suggest that RAGE is the main receptor mediating the pro-inflammatory effects of eHSP70 in carcinoma A549 cell line.

Recent works have revealed that tumor cells do not act alone to promote tumorigenesis, cancer progression, angiogenesis, and metastasis, but instead collaborate with an activated microenvironment [51]. In this context, it seemed important to evaluate how the effects of RAGE activation by eHSP70 in A549 could modulate immune cells responses. Thus, to contextualize tumor biology, we studied the effects of A549 conditioned medium on the modulation of U937 monocyte gene transcription. In response to conditioned medium of A549 cells treated with eHSP70, monocytes produced a marked reduction of pro-inflammatory and increase in anti-inflammatory profile (Table 2, Fig. 6 B): expression of IL-6 mRNA was remarkably decreased in U937 and IL-13 was highly expressed in those conditions. Changes in TNF-α and IL-1β seem to decrease, although presented no significant difference; TGF-β also did not change expression levels, as in A549 cells. In U937 monocytes incubated with conditioned medium of RAGE-silenced A549 cells treated with eHSP70, cytokine expression returned to levels similar to observed in monocytes exposed to unstimulated A549 conditioned medium. It is known that IL-13 down-regulates macrophage activity, by inhibition of pro-inflammatory cytokines and chemokines [52]. Here, increased IL-13 expression by monocytes might regulate the reduction of mRNA levels TNF-α, IL-1β and IL-6, as part of an immunosuppressive response to compensate the pro-inflammatory state of tumor cells.

Tumor associated macrophages (TAM) secrete several factors that contribute to tumor growth and metastasis, while maintaining the immunosuppressive milieu [51, 53]. This cross-talking between tumor cells and their microenvironment determine tumor's outcome, that is, if it will be able to grow (and ultimately invade other tissues) or if it will be

terminated by the immune system. Here, monocyte cells expressed an immunosuppressed profile in response to modulators released by A549 cells stimulated with eHSP70. There are many candidate molecules which may be secreted by A549 and drive monocyte responses, as HMGB1, that can drive pro-tumoral activities of M2 macrophages [54], or TGF-β, which together with other factors can 'educate' TAMs and create a promising immunosuppressive micro-environment for tumor progression [55]. As the identification of the factors secreted by A549 after HSP70 stimulation was not the initial focus of our work, our study is limited to these data. Nonetheless, this seems to be an interesting analysis to be performed by future studies, as it may further contribute to a deeper understanding of the biological significance of the eHSP70-RAGE interaction.

It is known that eHSP70 can mediate pro-inflammatory cytokine production via the MyD88/IRAK/NF-κB signal transduction pathway through TLR and CD-14 interaction [7, 17, 56]. Our study was the first to show the relevancy of RAGE on NF-κB transcriptional activity by eHSP70. In some conditions, RAGE can act as co-receptor along with other receptors to interact with ligands, and its absence could impair the function of other receptors [57]. In other situations, RAGE itself, in monomeric or oligomeric form, can bind agonists to produce different responses [58]. Here, lack of RAGE prevented downstream signal transduction by eHSP70, showing the essentiality of this receptor to A549 responsiveness.

Conclusion

In conclusion, we show here that eHSP70 evokes important pro-inflammatory changes in A549 cells via RAGE. The incubation of human lung cancer cells with eHSP70 produced a binding interaction with RAGE, evidenced by co-immunoprecipitation and protein proximity assay. Besides, interaction between the extracellular protein and the receptor was able to trigger typical cellular effects of RAGE activation, such as ERK phosphorylation, activation of the NF-κB transcription factor, increased expression of RAGE and pro-inflammatory cytokines. This modulatory action of eHSP70 on A549 inflammatory response through RAGE was able to evoke important expression changes in monocyte, showing its relevance in influencing the interaction between cancer and immune system cell. In addition, and more importantly, RAGE has been shown to play a key role as a mediator of the cellular responses activated by eHSP70, because once the receptor has been silenced or blocked, the signaling effects evoked by extracellular protein were inhibited. Our present findings indicate that eHSP70 acts as a functional RAGE agonist, modulating inflammatory responses in cancer cell line (A549) and playing a key role in cross-talking between cancer and immune cells.

Abbreviations

Ab (antibody); APC (antigen-presenting cell); B2M (beta-2-microglobulin); CD-14 (protein encoded by the gene cluster of differentiation 14); CD23 (low-affinity" receptor for IgE); DAMP (damage associated molecular pattern); eHSP70 (extracellular heat-shock protein 70 kDa); ERK (extracellular signal–regulated kinases); GNB2L (Guanine nucleotide-binding protein beta-peptide 2-like 1); IgM (immunoglobulin M); IgG (immunoglobulin G); IL-1β (interleukin 1 beta); IL-6 (Interleukin 6); IL-13 (Interleukin 13); IP (immunoprecipitation); JNK (c-Jun N-terminal kinase); MAPK (mitogen-activate protein kinases); MHC (I, major histocompatibility complex I); MHC (II, major histocompatibility complex II); M2 (profile, macrophages polarizated to profile 2, anti-inflammatory); NF-κB (nuclear factor kappa B); RAGE (receptor for advanced glycation endproducts); RAGE-siRNA (siRNA specific for RAGE); Scrb-siRNA (siRNA scrambled sequence); TLR (Toll-like receptor); TGF-β (transforming growth factor beta); TNF-α (tumor necrosis factor alpha); WB (western blot).

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Disclosure Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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