Universidade Federal do Rio Grande do Sul
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Departamento de Bioquímica Professor Tuiskon Dick
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A relação com a sepse e a possível sinalização pela ligação ao receptor dos produtos finais de glicação avançada - RAGE

Marcelo Sartori Grunwald

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Aluno: Marcelo Sartori Grunwald

Orientador: Professor Dr. José Cláudio Fonseca Moreira

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Parte 1

Resumo: A expressão da HSP70 intracelular está associada a efeitos citoprotetores contra uma variada gama de estímulos estressores, tais como processos inflamatórios, estresse oxidativo, endotoxinas bacterianas, infecções e febre. Este efeito citoprotetor é principalmente atribuído à habilidade de as proteínas de choque térmico estabilizarem estruturas protéicas através de interações reversíveis. A HSP70 foi recentemente detectada no meio extracelular, e sua presença tem sido associada a situações patológicas, nas quais ela exerce efeitos modulatórios sobre células do sistema imunológico. Previamente, nós descrevemos a relação entre os níveis de HSP70 sérica, o estatus oxidante e o desfecho clínico de pacientes sépticos; o grupo de pacientes com maiores níveis pró-oxidantes e maiores níveis de HSP70 sérica também foi aquele que houve maior mortalidade. Afim de investigar a possível associação entre HSP70 oxidada e efeitos citoprotetores ou morte celular, macrófagos da linhagem RAW 264.7 foram incubados com HSP70 e HSP70 oxidada, e a produção de nitrito, proliferação celular, viabilidade celular, produção de espécies reativas de oxigênio, liberação de TNF-α e atividade fagocítica foram avaliadas. Também foram avaliadas as modificações estruturais causadas pela oxidação na HSP70 purificada. Observamos que a oxidação da HSP70 alterou a estrutura da proteína; e que os efeitos modulatórios da HSP70 oxidada sobre a linhagem de macrófagos RAW 264.7 foram diferentes dos efeitos modulatórios da HSP70 nativa. Os macrófagos tratados com HSP70 oxidada apresentaram menor proliferação, maior produção de espécies reativas de oxigênio, menor atividade fagocítica e menor liberação de TNF-α. Estes resultados indicam que a oxidação da HSP70 extracelular modifica suas propriedades sinalizadoras, causando alterações na modulação das funções e da viabilidade dos macrófagos.

Abstract: Expression of intracellular HSP70 is associated to cytoprotective effects against a wide range extent of stressful stimuli, such as inflammation, oxidative stress, hypoxia, endotoxins, infections and fever. This cytoprotective effect is mainly attributed to their ability to stabilize protein structures through chaperon-like reversible interactions. HSP70 was recently detected in the extracellular medium and its presence in serum is commonly associated with pathological situations, where it exerts modulatory effects on cells of the immune system. Previously, we have described the relationship between serum HSP70 levels, oxidant status and clinical outcome of septic patients; the group of patients with higher pro-oxidant status and higher serum HSP70 had also higher mortality. To investigate the possible association between oxidized HSP70 and cytoprotection or cell death, we incubated RAW 264.7 macrophages with oxidized HSP70 and evaluated nitrite production, cell proliferation, cell viability, reactive oxygen species production, TNF-α release and phagocytic activity. We also evaluated structural modifications caused by oxidation in purified HSP70. Oxidation of HSP70 altered its protein structure; besides, the modulatory effect of oxidized HSP70 on RAW265.7 cells was different from native HSP70. Macrophages treated with oxidized HSP70 presented lower proliferation, higher reactive oxygen species production, lower phagocytic activity and TNF-α release. These results indicate that oxidation of extracellular HSP70 modify its signaling properties, causing alterations on its modulatory effects on macrophage function and viability.

Lista de Abreviaturas

HSP70: heat shock protein 70

oxHSP70: heat shock protein 70 oxidada

IAA: iodoacetamida

ATP: adenosina trifosfato

SOD: superóxido dismutase

CAT: catalase

LPS: lipopolissacarídeo

SIRS: síndrome da resposta inflamatória sistêmica

HSR: resposta heat shock

PRRs: receptores de reconhecimento de padrões

TLR4: receptor tipo toll 4

TLR2: receptor tipo toll 2

RAGE: receptor dos produtos finais de glicação avançada

AGE: produto final de glicação avançada

PAMPs: padrões moleculares associados à patógenos

MyD88: molécula adaptadora fator de diferenciação mielóide 88

TNF-α: fator de necrose tumoral α

SIRS: síndrome da resposta inflamatória sistêmica

1. Introdução

1.1 Sepse – Caracterização do problema

Apesar dos avanços da medicina, a sepse é a principal causa de morte nas unidades de terapia intensiva e está entre as principais causas de morte nos EUA (Martin *et al.* 2003). Em torno de 2% a 11% das internações hospitalares e nas unidades de terapia intensiva são por esta doença (Angus & Wax 2001). A mortalidade varia na maioria dos estudos entre 20% e 80% (Friedman *et al.* 1998), sendo que no período de 1979 a 2000, houve um grande aumento na incidência, em torno de 8,7% ao ano. No Brasil, os aspectos epidemiológicos da sepse têm sido investigados, destacando-se o BASES Study (Brazilian Sepsis Epidemiological Study) – estudo de coorte multicêntrico e observacional realizado em cinco unidades de terapia intensiva públicas e privadas –, no qual se identificou uma densidade de incidência de sepse de 57,9 por 1000 pacientes-dia (95% IC 51,5-65,3). A taxa de letalidade de pacientes com SIRS (independente se devido a sepse ou outra causa), sepse, sepse grave e choque séptico foi 24,2%, 33,9%, 46,9%, e 52,2%, respectivamente. De forma ainda mais preocupante, o estudo multicêntrico PROGRESS– do qual fizeram parte sete unidades de tratamento intensivo (UTIs) nacionais –, revelou que as taxas de letalidade nas UTIs no Brasil foram maiores (56%) que aquelas de outros países em desenvolvimento (45%) e de países desenvolvidos (30%)(Siqueira-Batista *et al.* 2011).

A sepse é definida como síndrome da resposta inflamatória sistêmica (do inglês *systemic inflammatory response syndrome* – *SIRS*), como uma febre, taquipnéia, taquicardia ou leucocitose, causada por um organismo infeccioso, podendo ser uma bactéria, um vírus ou um fungo. Dependendo da graduação

da severidade da doença, ela pode ser classificada como sepse severa ou choque séptico. A sepse severa é caracterizada pelos sintomas da infecção em conjunto com a hiperperfusão ou disfunção de órgãos, como disfunção cardíaca, disfunção pulmonar aguda ou estado mental alterado (Morrell *et al.* 2009). O choque séptico é definido pela sepse severa em conjunto de uma tríade clínica: coagulação intravascular disseminada, hipoglicemia e insuficiência cardiovascular, que culmina com uma hipotensão vascular grave.

Tanto a sepse severa como o choque séptico podem ser causados por múltiplos fatores, incluindo patógenos microbianos endógenos (malária, por exemplo) e condições comórbidas (HIV). As bactérias Gram-positivas são responsáveis por 30 – 50% das associações de microorganismos com sepse severa e choque séptico (Annane *et al. 2005*), já as Gram-negativas representam 25 – 30% dos casos (Alberti *et al.* 2002). As bactérias com múltipla resistência a drogas, juntamente com fungos, representam 25%; sendo que vírus e parasitas estão relacionados com apenas 2 – 4% dos casos.

1.2 Sepse – Estado atual de conhecimento

A sepse é o resultado de um conjunto de complexas interações entre microorganismos infecciosos e as respostas imunológica, inflamatória e coagulatória do hospedeiro. Uma séries de eventos patológicos são responsáveis pela transição da sepse para sepse severa e para o choque séptico. A reação inicial à infecção é uma resposta neurohumoral, pró-inflamatória generalizada e anti-inflamatória, a qual começa com a ativação celular de monócitos, macrófagos e neutrófilos, que interagem com células endoteliais através de receptores de reconhecimento de patógenos (Beutler

2004). Após essa resposta inicial, ocorre a mobilização de substâncias do plasma como resultado da ativação celular e do rompimento endotelial. Essas substâncias do plasma incluem citocinas, como o fator de necrose tumoral e interleucinas; enzimas, como caspases e proteases; espécies reativas de oxigênio, óxido nítrico, ácido araquidônico, fatores de ativação de plaquetas e eicosanóides. A ativação do sistema complemento (Haeney 1998) juntamente com cascatas de coagulação amplificam essa complexa cadeia de eventos.

O endotélio vascular é o sítio predominante dessas interações, e, como resultado disso, ocorre injúria microvascular, trombose e uma perda da integridade endotelial, resultando em isquemia tecidual (Aird 2003). Esse rompimento endotelial difuso é responsável pela disfunção de vários órgãos e pela hipoxia de diversos tecidos, característicos da sepse severa e do choque séptico.

Sabe-se que os parâmetros oxidativos durante o desenvolvimento da sepse severa letal diferem dos parâmetros da sepse não-letal. Na sepse severa os níveis de grupamentos carbonila proteico encontram-se elevados, há um desequilíbrio na relação das enzimas superóxido dismutase/catalase, evidenciando um quadro exacerbado de estresse oxidativo (Andrades *et al.* 2005). Além disso, o soro de pacientes sépticos é capaz de induzir a ativação de macrófagos, que passam a produzir interleucina-10, citocina cuja função é inibir a ativação dos mesmos, mantendo o controle homeostático das reações imunes inatas e celulares. A ativação e o índex fagocitário dos macrófagos estão relacionados com a severidade da doença e com a mortalidade, sugerindo que os mecanismos anti-inflamatórios são predominantes em casos mais severos de sepse (Peck *et al.* 2009).

A análise do soro de pacientes com sepse severa letal mostrou uma relação entre mortalidade e a presença sérica da proteína de choque térmico de 70kDa – HSP70, porém os mecanismos e os motivos que estão envolvidos permanecem desconhecidos.

1.3 Resposta Anti-inflamatória

A complexidade da resposta anti-inflamatória requer que suas diversas funções sejam controladas coordenadamente em algumas situações mas independentemente em outras. Isso ocorre através de diferentes mecanismos que atuam em diferentes níveis, incluindo alterações na composição das células de defesa dos tecidos, alterações na responsividade ao estímulo inflamatório, regulação das rotas de sinalização e alterações nos padrões de expressão gênica (Medzhitov & Horng 2009). Assim, esses mecanismos podem ser divididos em célula-específicos, ou seja, operam a nível celular, regulando o seu recrutamento e ativação; sinal-específicos, categoria na qual são incluídos todos os mecanismos responsáveis pela transdução do sinal e, finalmente, os mecanismos gene-específicos, que operam a nível genético.

1.4 Macrófagos

Os macrófagos são células fagocitárias presente nos tecidos, derivados dos monócitos do sangue e que desempenham papéis importantes nas respostas imunes inata e adaptativa. Essas células podem ser ativadas por produtos bacterianos, como os lipolissacarídeos (LPS) ou pela parede celular microbiana. Um fato que vem sendo bem descrito na literatura é a capacidade que a HSP70 intracelular tem de ativar e influenciar a sobrevivência de células do sistema imunológico, principalmente de macrófagos (Hirvonen *et al.* 1996).

Macrófagos ativados destroem microrganismos fagocitados, principalmente pela síntese aumentada de espécies reativas de oxigênio e de óxido nítrico, assim como de enzimas lisossômicas, as quais são potentes agentes microbicidas que são produzidas dentro dos lisossomos dos macrófagos e destroem microrganismos ingeridos depois que os fagossomos se fundem com os lisossomos.

Além disso, os macrófagos ativados estimulam a inflamação aguda por meio da secreção de citocinas, principalmente TNF e IL-1, quimiocinas, e mediadores lipídicos de vida curta, tais como o fator de ativação de plaquetas (PAFs), prostaglandinas e leucotrienos. A ação coletiva dessas citocinas e dos mediadores lipídicos é produzir uma inflamação local que é rica em neutrófilos e recrutar mais monócitos, os quais se tornam macrófagos e fagocitam e destroem organismos infecciosos. As principais funções dos macrófagos no sistema imunológico estão resumidas na **figura 1**.

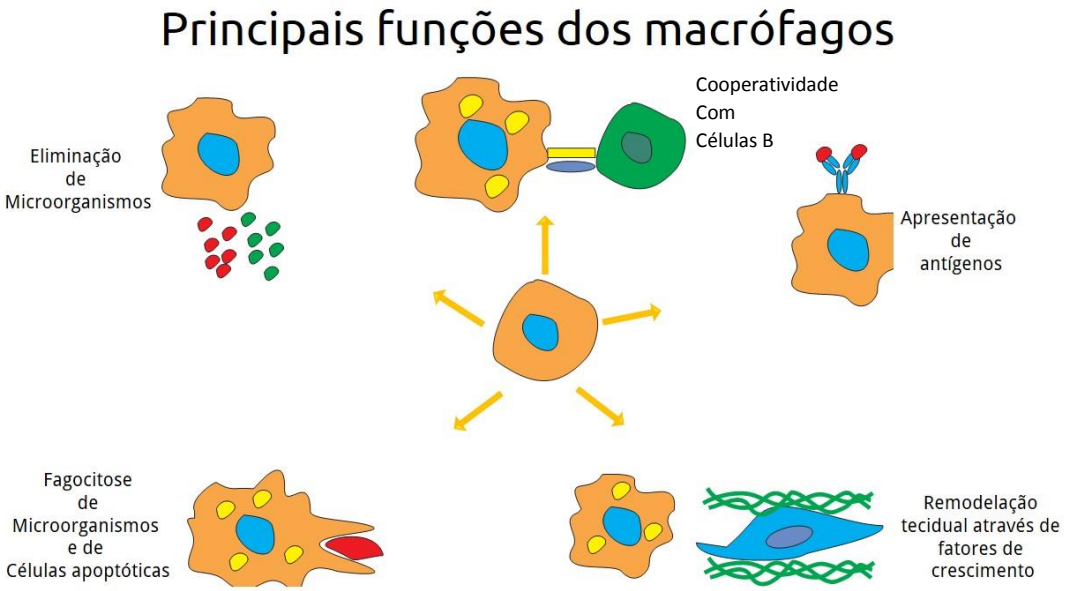

Figura 1

Principais funções dos macrófagos no sistema imunológico.

1.5 As proteínas de choque térmico

A resposta Heat Shock - HSR (do inglês Heat Shock Response) é um dos mais primitivos e conservados sistemas de defesa celular conhecidos (Morimoto *et al.* 1997). Virtualmente, todos os organismos, de procariotos à humanos, possuem a capacidade de montar a HSR. Essa resposta é definida pela rápida expressão um grupo específico de proteínas (conhecidas como Heat Shock Proteins – HSPs) quando as células, tecidos ou organismos encontram diversas formas de estresse ambiental. Apesar de ter sido descrita primeiramente na exposição de células de glândula salivar ao aumento da temperatura ambiental (Ritossa 1962), por isso o termo “heat shock”, sabe-se agora que essa resposta ocorre frente a outros tipos de estresse celular, como metais pesados, agentes oxidantes, e lesões de isquemia-reperfusão(Tang *et al.* 2007). Observações clínicas mostram que a HSR é induzida (caracterizada pela expressão de HSPs) em pacientes com choque séptico, trauma ou síndrome da angústia respiratória aguda (Kindas-Mugge *et al.* 1993, 1996).

As proteínas de choque térmico (do inglês, heat shock proteins – HSPs) são constituintes de uma família proteica extremamente conservada durante o curso da evolução, presentes em bactérias a mamíferos. As HSPs variam em tamanho, de 7 a 110 kDa, e podem ser encontradas em quaisquer compartimento celular, incluindo núcleo, citoplasma e mitocôndrias. As nomenclaturas mais comuns para as proteínas de choque térmico fazem uso do seu peso molecular, como por exemplo em HSP70, na qual 70 corresponde ao peso molecular em kDa.

A HSP70 tem uma seqüência de 700 resíduos de aminoácidos os quais constituem uma proteína de 70 kDa com três diferentes domínios: o domínio ligante de nucleotídeos (NBD), o domínio ligante de substrato ou de peptídeos (SBD) e uma porção C-terminal de função ainda não definida (**Figura 2**).

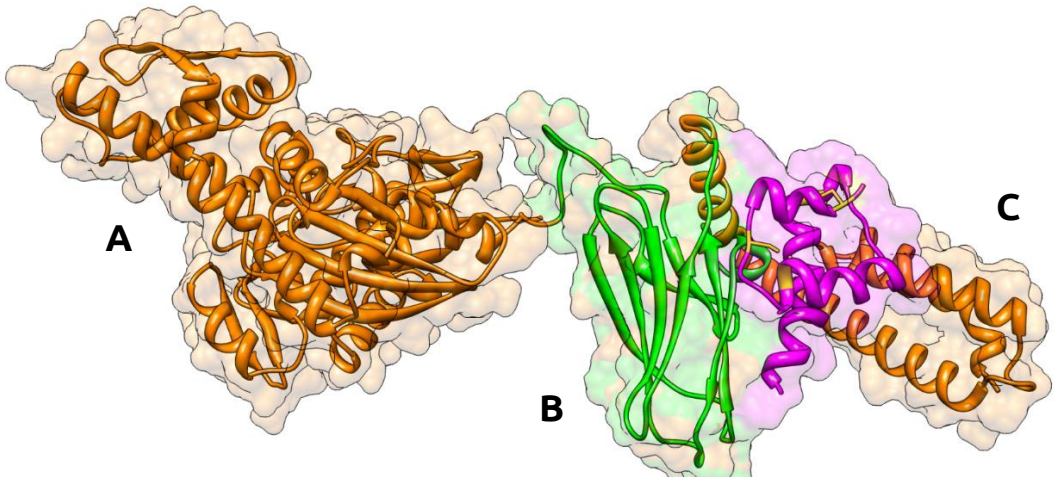

Figura 2

Estrutura molecular da HSP70, evidenciando seus três diferentes domínios. A) Domínio ligante de nucleotídeos (NBD). B) Domínio ligante de substrato (SBD). C) Porção C-terminal.

Algumas HSPs são expressas constitutivamente, como por exemplo, a HSP90, enquanto que outras, possuem sua expressão aumentada rapidamente em resposta a um estresse celular.

Classicamente, as HSPs funcionam como chaperonas moleculares dependentes de ATP, transportando diversas proteínas intracelulares em células sob estresse ou em condições normais, e auxiliam no dobramento e na estabilização de proteínas intracelulares danificadas. Além dessa função clássica, foi atribuído às HSPs um importante papel na proteção celular contra diversas formas de injúria. Por exemplo, a indução de HSP70 levou a proteção de células do epitélio respiratório contra danos oxidativos (Wong *et al.* 1997). A

superexpressão específica de HSP70 levou a proteção do epitélio respiratório contra a morte celular mediada pro hipoxia, peroxidação lipídica e depleção intracelular de ATP (Wong *et al.* 1998). Além disso, a indução da HSR e de HSPs se mostrou associada à proteção de animais contra a síndrome da angústia respiratória aguda, choque séptico e lesões da isquemia-reperfusão (Ryan *et al.* 1992, Villar *et al.* 1993, Yang & Lin 1999). O aumento da expressão da HSP70 intracelular resulta em efeitos protetores contra hipoxia, excesso de espécies reativas de oxigênio, endotoxinas bacterianas, infecções e febre (Tang *et al.* 2007). Esse efeito citoprotetor é principalmente atribuído à habilidade que as HSPs têm de estabilizar estruturas protéicas através de interações reversíveis do tipo chaperona (Ribeiro *et al.* 1994).

Juntos esses dados demonstram uma função fundamental da HSR e da HSP70: proteção a nível celular e tecidual contra processos patofisiológicos freqüentes nas unidades de terapia intensiva, sendo que a desregulação da resposta anti-inflamatória é uma característica comum a eles.

1.6 O papel das HSP70 extracelular

As HSPs encontram-se distribuídas em diversos compartimentos celulares, incluindo o citosol, núcleo e organelas membranosas, como a mitocôndria e o retículo endoplasmático; recentemente, elas têm sido encontradas no meio extra-celular, particularmente após o estabelecimento de diversas condições patológicas (Campisi *et al.* 2003). Estudos recentes mostraram que as HSPs podem ser produzidas e liberadas ativamente pelas células, possuindo a capacidade de interagir com outras células. Primeiramente, acreditava-se que essa liberação para o meio extracelular ocorria somente em células necróticas

após algum evento danoso. Entretanto, a secreção de HSP70 ocorre na ausência da morte celular, através de um mecanismo não-clássico. Aparentemente, essa proteína é translocada para a membrana celular e é então liberada por um processo associado à membrana, o qual pode ser exocitose ou evaginação inversa (Vega *et al.* 2008). Especificamente, a HSP70 é capaz de ativar macrófagos, células dendríticas e células natural-killer através de um processo mediado por receptores.

Recentemente, foi descrito um processo na qual a HSP70 é translocada para a membrana plasmática e liberada para o meio extracelular, onde ela exerce efeitos modulatórios sobre as células do sistema imunológico (Asea 2007, Asea, Kraeft, *et al.* 2000, Asea *et al.* 2002). Além disso, a HSP70 sérica está associada a condições patológicas como processos inflamatórios, infecções virais e bacterianas, e processos tumorigênicos (Multhoff 2007).

Em humanos, sabe-se que a HSP70 extracelular é liberada na circulação sistêmica pelo tecido cerebral e pelo tecido hepatoesplênico (Febbraio, Ott, *et al.* 2002, Lancaster *et al.* 2004), mas não pelo tecido muscular (Febbraio, Steensberg, *et al.* 2002). O exercício físico leva a uma indução da liberação da HSP70 extracelular, fato descrito primeiramente em um experimento no qual os participantes praticaram caminhada em esteira por 60 minutos a 70% da capacidade máxima de oxigênio (Walsh *et al.* 2001). Depois disso, o aumento na expressão da HSP70 tem sido observado após a prática de exercícios prolongados e parece ser dependente da duração e da intensidade do exercício (Fehrenbach *et al.* 2005). Níveis aumentados de HSP70 extracelular têm sido observados na circulação de pessoas sedentárias após 1 hora de exercício moderado e intenso, junto com um aumento na quimiotaxia de

neutrófilos e macrófagos e da capacidade microbicida dos netrófilos (Ortega *et al.* 2006, 2009), sugerindo que a HSP70 extracelular possui um importante papel na inflamação induzida pelo exercício e na modulação da resposta imunológica inata.

As HSPs extracelulares possuem a capacidade de iniciarem tanto a resposta imunológica inata quanto a resposta imunológica adquirida (Johnson & Fleshner 2006, Srivastava 2002). HSPs derivadas de patógenos ou HSPs derivadas das células do hospedeiro podem diretamente ativar ou aumentar a atividade de diversas células do sistema imunológico inato de produzir citocinas e quimiocinas (Tsan & Gao 2009). Essas proteínas possuem a capacidade de promover a maturação e migração das células apresentadoras de antígenos (APCs), e subsequente interação com as células T (APC-T), iniciando assim uma resposta imunológica adaptiva. As HSPs podem ainda ligar a peptídeos antigênicos derivados de tumores ou de vírus, tanto que elas têm sido aplicadas como adjuvantes no desenvolvimento de vacinas.

De acordo com a teoria “sinal de perigo” ou “sinal de alerta” (ou do inglês “danger signal theory”), as células do sistema imunológico são ativadas por sinais de alerta derivados de células sob estresse ou por proteínas danificadas (Matzinger 2002). É aceito que a HSP70 extracelular se encaixa no critério de um sinal de alerta, pois ela possui a capacidade de desencadear a produção de citocinas pró-inflamatórias pelas APCs e uma resposta imune antígeno específico. Além de apresentar antígenos a células T durante situações de “alerta” metabólico, estando também relacionadas com a patogênese de diversas doenças autoimunes (Chen & Cao 2010, Millar *et al.* 2003).

Em um de nossos trabalhos relacionados à HSP70, nós avaliamos a relação entre o imunoconteúdo da HSP70 sérica e o estado oxidante na sepse. Pacientes com sepse severa foram monitorados por 28 dias após serem diagnosticados ou até o desfecho clínico. O soro desses pacientes apresentou elevados níveis de HSP70. Analisando os parâmetros oxidativos, foi possível observar que aqueles pacientes com pronunciado dano oxidativo também apresentaram maiores níveis de HSP70 sérica; enquanto que os pacientes sépticos com parâmetros oxidativos semelhantes aos controles apresentaram menores níveis de HSP70 sérica. Pacientes com maiores níveis de HSP70 no soro também apresentaram maior taxa de mortalidade, levando-nos à conclusão de que os níveis de HSP70 no soro estão sendo modulados de acordo com o estado oxidante do paciente, e de que níveis elevados de HSP70 no soro estão associados à elevada mortalidade na sepse (Gelain *et al.* 2011).

Sabemos que o soro de pacientes sépticos é capaz de induzir a ativação de macrófagos e a produção de interleucina-10; também sabemos que o índice fagocítico desses macrófagos está correlacionado com a severidade e com a mortalidade da doença (Peck *et al.* 2009). Modelos murinos de sepse letal são caracterizados por apresentarem elevados níveis de carbonilação proteica e por apresentarem um desbalanço no sistema enzimático SOD/CAT quando comparados com o grupo não-letal, indicando que o estresse oxidativo é exacerbado no modelo letal (Andrades *et al.* 2005). Assim, é bastante provável que diversas proteínas sejam oxidadas por espécies reativas e, consequentemente, tenham suas funções alteradas.

Porém, os trabalhos realizados até o momento não elucidam completamente os mecanismos ativados pela HSP70, sendo que eles diferem de acordo com o

contexto e com o momento patológico; além disso, os mecanismos celulares responsáveis pelo reconhecimento da HSP70 extracelular também permanecem parcialmente conhecidos. Na maioria dos processos inflamatórios graves, ocorre uma perda no balanço entre elementos oxidantes e as defesas antioxidantes do organismo, caracterizando uma situação de estresse oxidativo. Sob essas condições, muitas proteínas sofrem a ação de radicais livres, como o peróxido de hidrogênio (H_20_2), sendo oxidadas, alterando ou perdendo parcial ou totalmente suas funções. A HSP70 está entre elas; em teoria, as cisteínas dessa proteína podem formar ligações dissulfeto com outras HSP70 ou até mesmo com outras proteínas. Além disso, a ligação de peptídeos antigênicos pela HSP70 é mais efetiva sob condições oxidantes quando comparada com condições redutoras; sendo que este fato é provavelmente causado pelo ganho de estruturas secundárias (maior proporção de estruturas alfa hélices e beta-pregueadas em relação às estruturas randômicas) em meio oxidante (Callahan *et al.* 2002). Juntos estes dados sugerem que esta proteína tenha um papel específico no que diz respeito ao fluxo de informações dentro do sistema imunológico durante uma situação de estresse.

Considerando que nós demonstramos previamente que a sepse está associada com o desbalanço REDOX e com o estresse oxidativo, nosso objetivo é investigar a possível relação entre a HSP70 previamente oxidada e a ativação de macrófagos.

1.7 Sinalização celular por RAGE e TLR4

Recentemente foi demonstrado que a HSP70 extracelular realiza uma ligação de alta afinidade com a membrana plasmática das APCs (células apresentadoras de antígeno), induz um rápido fluxo da [Ca^{2+}] intracelular, ativa o fator de transcrição NF-Kβ, causa um aumento na expressão das citocinas pró-inflamatórias TNF-α, IL-1β e IL-6, por um processo dependente de CD-14 (Asea, Kraeft, *et al.* 2000, Asea *et al.* 2002, He *et al.* 2000), utilizando tanto o receptor Toll-like 2 quanto o receptor Toll-like 4.

A primeira linha de defesa contra agentes infecciosos, incluindo infecções virais e bacterianas, são as células do sistema imunológico inato, as quais apresentam receptores de reconhecimento de padrões (PRRs). Os PRRs tais como os Toll-like e CD14 são expressos como proteínas solúveis ou como proteínas de membrana que reconhecem estruturas molecular invariantes, chamadas de padrões moleculares associados ao patógeno (PAMPs).

Dentre as células do sistema imunológico que apresentam a capacidade de ligação à HSP70, encontram-se as células natural-killer (NK) (Gross *et al.* 2003), APCs dendríticas (Asea *et al.* 2002), macrófagos e monócitos periféricos (Asea, Kabingu, *et al.* 2000, Asea, Kraeft, *et al.* 2000), e linfócitos B (Arnold-Schild *et al.* 1999). Sabe-se, atualmente, que essa interação é mediada pelos receptores TLR2 e TLR4 com seu cofator CD14 (Asea *et al.* 2002), o receptor scavenger CD36 (Delneste *et al.* 2002), e a molécula co-estimuladora CD40 (Becker *et al.* 2002).

A ligação da HSP70 com TLR2 e TLR4 está associada à liberação e translocação nuclear do NF-kB. A ativação desse fator de transcrição é

dependente de MyD88 quando apenas TLR2 ou apenas TLR4 reconhecem e ligam HSP70, e é independente de MyD88 quando a ligação e sinalização de TLR2 e TLR4 ocorre sinergisticamente. Além da ativação de NF-kB, essa rota de sinalização é caracterizada pela liberação de TNF-α, IL-1β, IL-6 e IL-12. Já a interação CD40-HSP70 parece ser mais importante nos linfócitos B, nas quais a ligação a transdução da sinalização ocorre pela fosforilação de p38, a qual pode levar a uma liberação de TNF-α e IFN-γ (Asea 2003).

Apesar de importantes, esses dados acerca da ligação e da sinalização desencadeada pela HSP70 extracelular não respondem todas as perguntas. Sabemos que células imunocompetentes têm a capacidade de ligar à HSP70 e que esta ligação resulta na sinalização do tipo chaperoquina (termo cunhado para atribuir características de citocinas a chaperonas, especialmente à HSP70), bastante importante na sobrevivência do hospedeiro à patógenos microbianos, mas não esclarece a relação dessa proteína com outros processos patológicos e com o agravamento destes (Asea 2003, 2008).

Recentemente, foi demonstrado através de um ELISA screening a ligação da HSP70 com o receptor dos produtos finais de glicação avançada (RAGE) (Ruan *et al.* 2010). O RAGE é um receptor da superfamília das imunoglobulinas descoberto em 1992 como um ligante de AGEs (produtos de glicação avançada), um grupo de proteínas e lipídeos glicosilados não enzimaticamente e acumulados como um resultado do processo do envelhecimento ou de processos inflamatórios, como a diabetes (Neeper *et al.* 1992). O RAGE apresenta a capacidade de se ligar a um vasto repertório de moléculas, tanto solúveis como associadas a células, envolvidas na resposta do organismo hospedeiro aos danos teciduais, processos infecciosos e inflamatórios. A

interação de um ligante com RAGE leva à indução da ativação de múltiplas rotas de sinalização celular que variam de acordo com o agonista, tipo celular e microambiente; mediando assim, diversas respostas celulares.

2. Objetivos

2.1 Objetivo geral

A presente dissertação tem como objetivo evidenciar através de ensaio clínico, o efeito do estado pró-oxidativo sobre a relação positiva existente entre os níveis séricos de HSP70 extracelular e um pior desfecho clínico de pacientes com sepse severa. Por meio de ensaios in vitro e in silico subsequentes, foram descritos os efeitos da HSP70 oxidada sobre a modulação da ativação de macrófagos e a interação dessa proteína com o receptor dos produtos finais de glicação avançada – RAGE.

2.2 Objetivos específicos

(i) Mostrar a correlação entre os níveis séricos de HSP70 extracelular, os níveis de estresse oxidativo e o desfecho clínico de pacientes com sepse severa;

(ii) Mostrar as alterações estruturais ocasionadas pela ação do agente oxidante peróxido de hidrogênio sobre a HSP70;

(iii) Estudar os efeitos modulatórios da HSP70 sobre a ativação de macrófagos RAW 264.7;

(iv) Demonstrar a possibilidade de ligação entre HSP70 e RAGE.

Parte 2

3. Resultados

Os resultados estão apresentados na forma de artigos publicados ou já submetidos ainda sem resposta, dispostos por capítulos.

Capítulo 1

Artigo publicado na revista “Shock”

“Serum heat shock protein 70 levels, oxidant status, and mortality in sepsis”

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SERUM HEAT SHOCK PROTEIN 70 LEVELS, OXIDANT STATUS, AND MORTALITY IN SEPSIS

Daniel Pens Gelain,* Matheus Augusto de Bittencourt Pasquali,* Clarissa M. Comim,† Marcelo Sartori Grunwald,* Cristiane Ritter,† Cristiane Damiani Tomasi,† Sarah Cascaes Alves,† Joao Quevedo,† Felipe Dal-Pizzol,† and José Cláudio Fonseca Moreira*

**Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul; and †Laboratory of Experimental Physiopathology and National Institute of Translational Science and Technology in Medicine, Posgraduation Program in Programa de Pós-Graduação em Ciências da Saúde, Unidade Acadêmica de Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, Santa Catarina, Brazil*

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ABSTRACT—Animal studies as well as prospective randomized clinical trials associated sepsis with redox imbalance and oxidative stress, but other studies failed to establish a correlation between antioxidant-based therapies and improvement of sepsis condition. This is also true for studies on the role of the chaperone heat shock protein 70 (HSP70), which is increased in serum during sepsis. Heat shock protein 70 is affected at several levels by oxidative stress, but this relationship has never been studied in sepsis. Here, we evaluated the relationship between serum HSP70 immunocontent and oxidant status in sepsis. Patients with severe sepsis were followed up for 28 days after diagnosis, or until death. Up to a maximum of 12 h after sepsis diagnosis, serum was collected for determination of HSP70 immunocontent by Western blot and evaluation of oxidative parameters (TRAP [total radical-trapping antioxidant parameter], TBARSs [thiobarbituric acid–reactive substances], and carbonyl levels). Serum of sepsis patients presented enhanced HSP70 levels. Analysis of oxidative parameters revealed that septic patients with pronounced oxidative damage in serum had also increased HSP70 serum levels. Sepsis patients in whom serum oxidative stress markers were not different from control presented normal serum HSP70. Analysis of septic patients according to survival outcome also indicated that patients with increased HSP70 serum levels presented increased mortality. We concluded that serum HSP70 levels are modulated according to the patient oxidant status, and increased serum HSP70 is associated to mortality in sepsis.

KEYWORDS—Sepsis, oxidative stress, HSP70, serum

INTRODUCTION

Sepsis is an inflammation-induced syndrome resulting from a complex interaction between host and infectious agents. Sepsis is considered severe when associated with acute organ dysfunction, which accounts for the main cause underlying sepsis-induced death. Severe sepsis has become a leading cause of mortality in critical illness and a major public health burden throughout the world (1). The rate of severe sepsis ranges between 6% and 14% among critical-care admissions, with a hospital mortality rate between 27% and 59% (2).

Heat shock proteins (HSPs) are upregulated in cells subjected to stressful stimuli, including inflammation and oxidative stress (3). Upregulation of intracellular HSP results in protective effects against hypoxia, excess oxygen radicals, endotoxin, infections, and fever (4). This cytoprotective effect is attributed to their ability to stabilize protein structures through chaperone-like reversible interactions (5). Heat shock protein 70, a 70-kd member of the HSP superfamily, has been recently observed to exert extracellular effects in cells of the immune system (6–8). In humans, serum HSP70 is associated to stress conditions as inflammation, bacterial or viral infections, and oncological diseases (9). However, very little is known on the role and function of extracellular HSP70.

Sepsis has been associated with redox imbalance and oxidative stress (10). Animal studies as well as prospective randomized clinical trials have been providing increasing evidence in support of antioxidant therapies in sepsis (11, 12). Some studies, however, failed to establish a correlation between antioxidant-based therapies and improvement of sepsis condition (13). Previous works attempted to establish a correlation with either HSP70 or oxidative stress with sepsis, but there are no works studying the relationship between both parameters in sepsis. Here, we studied the relation between oxidative stress and modulation of HSP70 in the serum of septic patients and aimed to establish an association with clinical outcome.

METHODS

Subjects and serum samples

This study was approved by the local ethics committee. Fifteen patients admitted to a general intensive care unit with severe sepsis according to the Consensus Conference of the American College of Chest Physicians and Society of Critical Care Medicine (1992), 18 years or older, without chronic hepatic or renal insufficiency, without cancer or immunodeficiency, were included in the study. These patients were followed up for 28 days after sepsis diagnosis or until death. Up to a maximum of 12 h after sepsis diagnosis, blood was drawn into dry tubes, and the serum was stored at −80°C until the

Address reprint requests to Daniel Pens Gelain, PhD, Rua Ramiro Barcelos, 2600-anexo, CEP 90035-003, Porto Alegre, RS, Brazil. E-mail: dgelain@yahoo.com.br.

D.P.G., M.A.B.P., and C.C. contributed equally to this work.

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determination of HSP70 and oxidative parameters. All patients' data and analyses were obtained under informed consent before their inclusion in the study. The group of sepsis had eight patients with severe sepsis and seven with septic shock. The origin of infection was pneumonia in eight patients, intra-abdominal infection in five patients, pyelonephritis in one patient, and catheter-related infection in one patient. The control group consisted of 24 normal, healthy subjects (15 males, 9 females; aged 45 ± 18 years) in whom blood was collected for routine checkups.

Heat shock protein 70

Heat shock protein 70 serum detection was performed by Western blot analysis using a mouse monoclonal anti-HSP70 (Sigma, St Louis, Mo) at 1:1,000 dilution range and anti–mouse immunoglobulin G peroxidase-linked secondary antibody (1:5,000 dilution range) suitable for enhanced chemiluminescence detection (ECL Plus kit, Rockford, Ill). Densitometric analysis of the films was performed with ImageQuant software. Blots were developed to be linear in the range used for densitometry.

Oxidative stress parameters

Nonenzymatic antioxidant potential of serum samples was determined by the total radical-trapping antioxidant parameter (TRAP) assay (14). Serum lipoperoxidation levels were estimated by quantifying levels of thiobarbituric acid–reactive substances (TBARSs) (15), whereas the oxidative damage to serum proteins was assessed by the determination of carbonyl group levels in samples through reaction with dinitrophenylhydrazine (16).

Statistical analysis

The results are expressed as means ± SE; differences among multiple groups were compared by one-way ANOVA followed by Tukey post hoc test. Statistical significance was assigned to $P < 0.05$.

RESULTS

The group of sepsis had eight patients with severe sepsis and seven with septic shock. The origin of infection was pneumonia in eight patients, intra-abdominal infection in five patients, pyelonephritis in one patient, and catheter-related infection in one patient. In Table 1, we show age, Acute Physiology andM Chronic Health Evaluation II (APACHE II), Sequential Organ Failure Assessment (SOFA) D1, and SOFA D3 scores of survivor and nonsurvivor patients. These patients presented altered values for oxidative parameters in serum. As depicted in Table 2, TRAP assay indicated an increase in nonenzymatic antioxidant reactivity in serum, whereas TBARSs and carbonyl assays showed increased lipoperoxidation and protein oxidative damage in septic serum. To better understand this apparent divergence between TRAP and TBARSs/carbonyl results, we analyzed the results from each patient individually. Total radical-trapping antioxidant parameter results indicated the existence of two subgroups of septic patients according to the oxidant status in serum; eight patients had antioxidant TRAP values in relation to control subjects, with a mean significantly below control (15.71%), whereas seven patients presented pro-oxidant TRAP values, with a mean significantly greater than control (138.11%). Thiobarbituric acid–reactive substance and carbonyl levels were also decreased in patients with antioxidant TRAP values, whereas the group of patients with pro-oxidant status of TRAP presented significant increases in both parameters, which reinforced the indication that sepsis patients may present two distinct profiles of oxidant status. Figure 1 shows the dual distribution of TRAP values of serum from septic patients in relation to control; individual values greater than control

TABLE 1. **APACHE II, SOFA D1, and SOFA D3 scores of sepsis patients**

	Patients	
	Nonsurvivors	Survivors
Age, y	57.4 (14.6)	58.4 (17.9)
APACHE II	15.2 (8.4)*	11.2 (7.1)
SOFA D1	3.2 (1.3)	1.9 (1.1)*
SOFA D3	2.6 (2.4)	(1.4)*

Values are presented as mean (SD).
*Significant differences (one-way ANOVA followed by Newman-Keuls post hoc, $P < 0.05$).

TABLE 2. **Serum oxidative parameters of sepsis patients**

	TRAP, % control	TBARSs, nmol/mg protein	Carbonyl, nmol/mg protein
Control (n = 24)	100 ± 1.92	0.145 ± 0.003	1.35 ± 0.28
Sepsis (n = 15)	72.86 ± 16.81*	0.394 ± 0.050†	3.14 ± 0.38‡
Antioxidant (n = 8)	15.71 ± 3.11§	0.233 ± 0.053‡	2.02 ± 0.28
Pro-oxidant (n = 7)	138.11 ± 8.34‡	0.588 ± 0.041‖	4.43 ± 0.32‡
Survivors (n = 8)	48.12 ± 21.01*	0.340 ± 0.020†	2.40 ± 1.66
Nonsurvivors (n = 7)	106.14 ± 23.65	0.280 ± 0.011‡	3.40 ± 2.20‡

Serum samples from 24 healthy subjects (control) and 15 sepsis patients were used to measure the nonenzymatic antioxidant potential by TRAP analysis, lipoperoxidation by TBARS quantification, and protein damage by carbonyl quantification. The values depicted for TRAP represent the chemiluminescence induced by the free radical generator AAPH in the samples (converted to % of control) and were normalized by the protein content of the sample. Thiobarbituric acid–reactive substances and carbonyl serum levels were quantified in the same samples. Antioxidant, pro-oxidant, survivors, and nonsurvivors groups refer to different subsets of the group of sepsis patients. Control group consisted of 24 normal, healthy subjects (15 males, 9 females, aged 45 ± 18 years).
Values represent mean ± SEM.
*Lower than control.
†Higher than ‡group.
‡Higher than control.
§Lower than *group.
‖Higher than †group (one-way ANOVA followed by Newman-Keuls post hoc, $P < 0.05$).

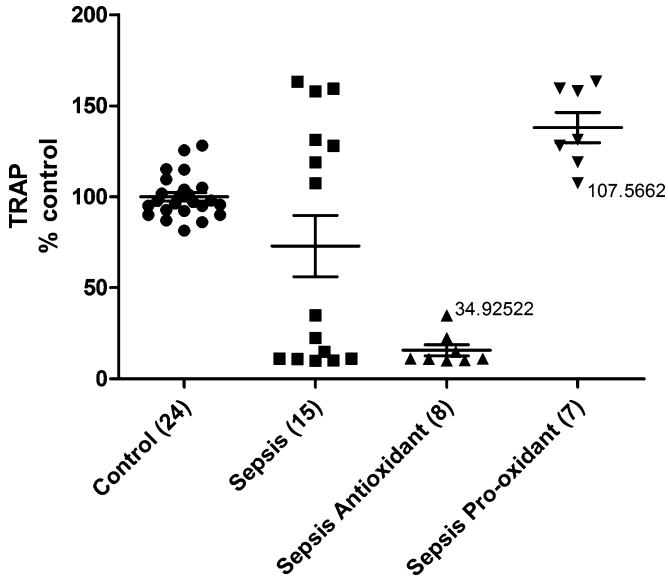

FIG. 1. **Total radical-trapping antioxidant parameter values of serum samples from septic patients**. Distribution of individual TRAP values of control and sepsis patients shown in Table 2 with the subgroups of antioxidant and pro-oxidant sepsis patients with respective cutoff values. Control group consisted of 24 normal, healthy subjects (15 males, 9 females, aged 45 ± 18 years).

TABLE 3. **APACHE II, SOFA D1, SOFA D3 scores, and type and origin of sepsis at the pro-oxidant and antioxidant groups of patients**

	Patients	
	Pro-oxidant	Antioxidant
Age, mean (SD), y	57.2 (13.9)	57.9 (20.1)
APACHE II, mean (SD)	13.8 (9.2)	12.1 (8.7)
SOFA D1, mean (SD)	3.1 (2.0)	2.2 (1.5)
SOFA D3, mean (SD)	2.6 (2.3)	1.9 (1.4)
Severe sepsis, no. patients	3	5
Septic shock, no. patients	4	3
Pneumonia, no. patients	5	3
Intra-abdominal infection, no. patients	2	3
Pyelonephritis, no. patients	0	1
Catheter-related infection, no. patients	1	0

Values represent mean (SD) for APACHE II, SOFA D1, and SOFA D3 scores, and number of patients for type of sepsis and origin of infection. No significant differences were detected among groups.

(100%) were grouped as pro-oxidant sepsis, whereas values less than control were grouped as antioxidant sepsis. We plotted in Table 3 APACHE II, SOFA D1, and SOFA D3 scores for pro-oxidant and antioxidant groups of sepsis patients, along with origin of infection.

We next analyzed the serum immunocontent of HSP70 of septic patients, and we observed significant increased values for serum HSP70 immunocontent compared with control (Fig. 2A). Considering the well-known relationship between oxidative stress and induction of HSP70 expression, we plotted the values for serum HSP70 immunocontent of septic patients according to their TRAP-based antioxidant or pro-oxidant serum status. Interestingly, we observed a very distinct pattern for serum HSP70 immunocontent between antioxidant and pro-oxidant patients. The subgroup of septic antioxidant patients presented serum HSP70 levels not different from those of control subjects, whereas the subgroup of septic pro-oxidant patients had 30-fold increased serum HSP70 compared with control. Altogether, these data indicate a relationship among serum oxidant status and modulation of extracellular HSP70 in sepsis patients.

Oxidative stress parameters and HSP70 levels in serum were also analyzed according to the survival outcome of septic patients, to identify a possible relationship between sepsis severity and oxidant-dependent modulation of extracellular HSP70. Compared with control, sepsis survivors had a small decrease in TRAP values, whereas nonsurvivors presented a small although significant increase (Table 2). Thiobarbituric acid–reactive substance levels were increased in both survivors and nonsurvivors, whereas carbonyl levels were increased only in nonsurvivor patients. Serum HSP70 immunocontent was also significantly increased in nonsurvivor patients (Fig. 2B). Altogether, these results suggest that serum HSP70 levels in sepsis patients are related to serum oxidant status and that oxidative stress and increased HSP70 in serum may be also related to sepsis severity and outcome.

DISCUSSION

The molecular mechanisms underlying sepsis are complex and relate different levels of metabolic and signal coordination; for such reason, comprehensive studies relating different aspects of sepsis-induced perturbations at cellular and molecular levels are more urged than ever. This is evidenced by the differential outcomes of biochemical studies focusing on isolated aspects of sepsis molecular mechanisms. For instance, many animal studies as well as prospective randomized clinical trials associated sepsis with redox imbalance and oxidative stress, but other studies failed to establish a correlation between antioxidant-based therapies and improvement of sepsis condition (11, 17, 18). This is also true for studies on the role of HSP70 in sepsis. Animal and cellular models corroborated the existence of a relationship between HSP70 and sepsis, but clinical observations failed to establish a direct correlation between HSP70 modulation and protection against septic shock (19).

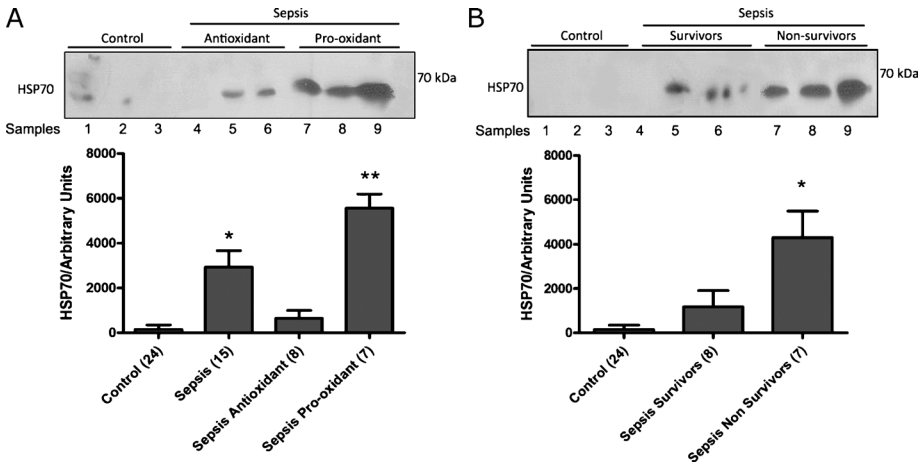

FIG. 2. **Representative immunoblots of HSP70 immunocontent of serum samples from sepsis patients**. A, Heat shock protein70 gel immunoblot showing three representative samples of control subjects and six of sepsis patients (three from antioxidant patients and three from pro-oxidant patients). Bars represent mean ± SEM of all patients; samples contained equal amounts of protein. B, Representative gel showing HSP70 immunoblots of control patients (three samples), sepsis survivors (three samples), and sepsis nonsurvivors (three samples). The graph shows replotted HSP70 mean ± SEM according to survival outcome. *Different from control, $P < 0.05$; **different from control ($P < 0.001$) and from *group ($P < 0.05$) according to one-way ANOVA followed by Newman-Keuls post hoc, $P < 0.05$.

There is a more or less generalized effort to establish a correlation among increased HSP70 expression and protection against septic shock in the literature, probably because of the usual cytoprotective effects of this protein. So far, most works focused on modulation of intracellular HSP70 in blood cells (19–22), but the outcomes are controversial. Results of different works suggest that an attenuation of cytokine liberation caused by inducible HSP70 expression could have beneficial effects in cases of systemic inflammation (19). On the other hand, cellular levels of HSP70 were shown to be markedly decreased in critically ill patients of sepsis (21). A potential role for extracellular HSP70 in sepsis and other conditions has been addressed only recently, and results are still scarce. In children with septic shock, a significant increase in serum HSP70 levels was reported (23), and similar observations were made earlier with patients from severe trauma (24, 25). Administration of exogenous HSP70 was observed to protect rats from endotoxic shock and modify the response of myeloid cells to lipopolysaccharide (26), but a role for extracellular HSP70 in clinical sepsis has not been established yet.

Our group has been studying the role of oxidative stress in sepsis, and we believe that redox signaling exerts an important influence in the regulation of sepsis response. Our data confirmed that sepsis patients have increased serum levels of HSP70. But, most interestingly, we also observed here that serum HSP70 is modulated according to the serum oxidant status, which vary among different patients. Based on the analysis of serum oxidative stress parameters (TRAP, lipid peroxidation, and protein carbonyl levels), septic patients may be classified into two opposite phenotypes, one presenting a general antioxidant profile and the other being pro-oxidant. This is an important observation as many biochemical parameters with clinical relevance may be subjected to oxidant-dependent regulatory processes, which could lead one to data misinterpretation when analyzing sepsis patients as a homogenous group. To our knowledge, the correlation between HSP70 levels and sepsis has never been studied based on oxidative parameters.

Heat shock protein 70 levels in different organs, cells, and fluids may undergo several types of regulation, which may include active release to extracellular medium in response to different mechanisms (27). In sepsis, it is possible that increased HSP70 levels are a result of cytokine action stimulating gene expression in blood cells and that the extent of oxidative stress may contribute to serum HSP70 levels modulation by causing HSP70 active secretion or lytic release by necrotic cell death. This might also explain the different observations reported by studies correlating HSP70 expression in different tissues or cells and sepsis outcome. Sepsis affects many physiological processes and the function of different organs to an extent that varies according to the origin of sepsis, severity, and history of each patient. Because oxidative stress and HSP70 expression, activation, and release are also affected and modulated at several degrees by infection, inflammation, water deprivation, and oxygen supply, it is not surprising that HSP70 release is regulated according to the oxidant status during sepsis.

Diverse mechanisms may account for the modulation of extracellular levels of HSP70. Physiologically, necrotic cell lysis from injury or infection is known to release HSPs into the extracellular compartment where they activate antigen-presenting cell–mediated defense (28). Active release of HSP70 to the extracellular environment was also observed in different cells, via exosomal export originated from trans-Golgi and endoplasmic reticulum–derived vesicles (29). Once at the extracellular space, HSP70 is believed to act as an immunostimulatory signal, as it was reported to activate Toll-like receptors 2 and 4 (7). Besides, a plethora of immune responses was reported to follow HSP70 immunostimulation, such as increased release of TNF-α, IL-1β, IL-6, IL-12, granulocyte-macrophage colony-stimulating factor, nitric oxide, macrophage inflammatory protein 1, monocyte chemotactic protein 1, and RANTES (6). Most of these data were acquired with the inducible isoform of HSP70 (i.e., HSP72), and it is believed that this is the predominant isoform in active release to the extracellular space (29). Here, we used a pan-HSP70 antibody that detects both the constitutive (HSP73) and the inducible isoforms, which contribute to the total pool of extracellular HSP70.

The patient's oxidant status may influence both HSP70 modulation during sepsis and its activity toward immune system regulation, which could account for many of the differences in the outcome of such patients. This could also explain why different works present different correlations between serum or plasma HSP70 levels and sepsis outcome. It is possible that variations in individual antioxidant defense systems (enzymes plus nonenzymatic antioxidants at cellular and systemic levels) may account to the differences in serum HSP70 levels and that combination of oxidative stress and extracellular HSP70 action is responsible for various deleterious effects observed in the course of sepsis. However, oxidative stress may not be the sole factor influencing HSP70 expression, activity, and release. Differences in sepsis outcome possibly related to HSP70 function and oxidative stress may also reflect differences in such other factors.

In conclusion, we report that serum HSP70 levels are modulated according to the oxidant status during sepsis, and increased serum HSP70 seems to be also associated to mortality. Although serum oxidative stress was observed to be correlated to HSP70 modulation, markers of oxidant damage in serum (i.e., TRAP, TBARSs, and carbonyl levels) showed variable degrees of association to sepsis outcome.

REFERENCES

1. Sands KE, Bates DW, Lanken PN, Graman PS, Hibberd PL, Kahn KL, Parsonnet J, Panzer R, Orav EJ, Snydman DR, et al.: Epidemiology of sepsis syndrome in 8 academic medical centers. *JAMA* 278:234–240, 1997.
2. Brun-Buisson C: The epidemiology of the systemic inflammatory response. *Intensive Care Med* 26(Suppl 1):S64–S74, 2000.
3. Ziegler TR, Ogden LG, Singleton KD, Luo M, Fernandez-Estivariz C, Griffith DP, Galloway JR, Wischmeyer PE: Parenteral glutamine increases serum heat shock protein 70 in critically ill patients. *Intensive Care Med* 31:1079–1086, 2005.
4. Tang D, Kang R, Xiao W, Wang H, Calderwood SK, Xiao X: The anti-inflammatory effects of heat shock protein 72 involve inhibition of high-mobility-group box 1 release and proinflammatory function in macrophages. *J Immunol* 179:1236–1244, 2007.
5. Ribeiro SP, Villar J, Downey GP, Edelson JD, Slutsky AS: Sodium arsenite induces heat shock protein-72 kilodalton expression in the lungs and protects rats against sepsis. *Crit Care Med* 22:922–929, 1994.

6. Asea A: Mechanisms of HSP72 release. *J Biosci* 32:579–584, 2007.
7. Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK: HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 6:435–442, 2000.
8. Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK: Novel signal transduction pathway utilized by extracellular HSP70: role of Toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 277: 15028–15034, 2002.
9. Multhoff G: Heat shock protein 70 (HSP70): membrane location, export and immunological relevance. *Methods* 43:229–237, 2007.
10. Halliwell B, Gutteridge JMC: *Free Radicals in Biology and Medicine*. Oxford: Oxford University Press, 2007.
11. Andrades ME, Ritter C, Dal-Pizzol F: The role of free radicals in sepsis development. *Front Biosci (Elite Ed)* 1:277–287, 2009.
12. Dal-Pizzol F: Alternative activated macrophage: a new key for systemic inflammatory response syndrome and sepsis treatment? *Crit Care Med* 32: 1971–1972, 2004.
13. Dare AJ, Phillips AR, Hickey AJ, Mittal A, Loveday B, Thompson N, Windsor JA: A systematic review of experimental treatments for mitochondrial dysfunction in sepsis and multiple organ dysfunction syndrome. *Free Radic Biol Med* 47:1517–1525, 2009.
14. Silva EG, Behr GA, Zanotto-Filho A, Lorenzi R, Pasquali MA, Ravazolo LG, Bordignon CL Jr, Silva FA, Aboy AL, Bassani VL, et al.: Antioxidant activities and free radical scavenging potential of *Bauhinia microstachya* (RADDI) MACBR. (Caesalpinaceae) extracts linked to their polyphenol content. *Biol Pharm Bull* 30:1488–1496, 2007.
15. Draper HH, Hadley M: Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol* 186:421–431, 1990.
16. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER: Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 186:464–478, 1990.
17. Rinaldi S, Landucci F, De Gaudio AR: Antioxidant therapy in critically septic patients. *Curr Drug Targets* 10:872–880, 2009.
18. Berger MM, Chiolero RL: Antioxidant supplementation in sepsis and systemic inflammatory response syndrome. *Crit Care Med* 35:S584–S590, 2007.
19. Bruemmer-Smith S, Stuber F, Schroeder S: Protective functions of intracellular heat-shock protein (HSP) 70-expression in patients with severe sepsis. *Intensive Care Med* 27:1835–1841, 2001.
20. Weiss YG, Bouwman A, Gehan B, Schears G, Raj N, Deutschman CS: Cecal ligation and double puncture impairs heat shock protein 70 (HSP-70) expression in the lungs of rats. *Shock* 13:19–23, 2000.
21. Schroeder S, Bischoff J, Lehmann LE, Hering R, von Spiegel T, Putensen C, Hoeft A, Stuber F: Endotoxin inhibits heat shock protein 70 (HSP70) expression in peripheral blood mononuclear cells of patients with severe sepsis. *Intensive Care Med* 25:52–57, 1999.
22. Hashiguchi N, Ogura H, Tanaka H, Koh T, Nakamori Y, Noborio M, Shiozaki T, Nishino M, Kuwagata Y, Shimazu T, et al.: Enhanced expression of heat shock proteins in activated polymorphonuclear leukocytes in patients with sepsis. *J Trauma* 51:1104–1109, 2001.
23. Wheeler DS, Fisher LE Jr, Catravas JD, Jacobs BR, Carcillo JA, Wong HR: Extracellular hsp70 levels in children with septic shock. *Pediatr Crit Care Med* 6:308–311, 2005.
24. Pittet JF, Lee H, Morabito D, Howard MB, Welch WJ, Mackersie RC: Serum levels of HSP72 measured early after trauma correlate with survival. *J Trauma* 52:611–617, discussion 617; 2002.
25. Pockley AG, Shepherd J, Corton JM: Detection of heat shock protein 70 (HSP70) and anti- HSP70 antibodies in the serum of normal individuals. *Immunol Invest* 27:367–377, 1998.
26. Rozhkova E, Yurinskaya M, Zatsepina O, Garbuz D, Karpov V, Surkov S, Murashev A, Ostrov V, Margulis B, Evgen'ev M, et al.: Exogenous mammalian extracellular HSP70 reduces endotoxin manifestations at the cellular and organism levels. *Ann N Y Acad Sci* 1197:94–107, 2010.
27. Kuo HT, Chen HW, Hsiao HH, Chen HC: Heat shock response protects human peritoneal mesothelial cells from dialysate-induced oxidative stress and mitochondrial injury. *Nephrol Dial Transplant* 24:1799–1809, 2009.
28. Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK: Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int Immunol* 12:1539–1546, 2000.
29. Radons J, Multhoff G: Immunostimulatory functions of membrane-bound and exported heat shock protein 70. *Exerc Immunol Rev* 11:17–33, 2005.

Capítulo 2

Artigo submetido e em processo de revisão na revista “Molecular and Cellular Biochemistry”

“The oxidation of HSP70 is associated with protein impairment and lack of macrophage activation”

Dear Mr. Grunwald,

We are in receipt of your manuscript titled "The oxidation of HSP70 is associated with protein impairment and lack of macrophage activation" and thank you for having submitted it for eventual publication in Molecular and Cellular Biochemistry.

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Title: The oxidation of HSP70 is associated with protein impairment and lack of macrophage activation

Running Title: Oxidized HSP70 lacks macrophage activation

Authors: Marcelo Sartori Grunwald[1], André Simões Pires[1], Juciano Gasparotto[1], Daniel Pens Gelain[1], Diogo Ribeiro Demartini[2], Cinthia Maria Schöler[3], Paulo Ivo Homem de Bittencourt Jr.[3], José Cláudio Fonseca Moreira[1].

[1] Department of Biochemistry, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

[2] Center of Biotechnology, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

[3] Department of Physiology, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

Corresponding Author Address: Ramiro Barcelos Street, 2600. Biochemistry Department - Porto Alegre/RS, Brazil ZIP code 90035-003 Phone number 55 51 3308 5578 marcelo.sartori@yahoo.com.br

Abstract: Expression of intracellular HSP70 is associated to cytoprotective effects against a wide range extent of stressful stimuli, such as inflammation, oxidative stress, hypoxia, endotoxins, infections and fever. This cytoprotective effect is mainly attributed to their ability to stabilize protein structures through chaperon-like reversible interactions. HSP70 was recently detected in the extracellular medium and its presence in serum is commonly associated with pathological situations, where it exerts modulatory effects on cells of the immune system. Previously, we have described the relationship between serum HSP70 levels, oxidant status and clinical outcome of septic patients; the group of patients with higher pro-oxidant status and higher serum HSP70 had also higher mortality. To investigate the possible association between oxidized HSP70 and cytoprotection or cell death, we incubated RAW 264.7 macrophages with oxidized HSP70 and evaluated nitrite production, cell proliferation, cell viability, reactive oxygen species production, TNF-α release and phagocytic activity. We also evaluated structural modifications caused by oxidation in purified HSP70. Oxidation of HSP70 altered its protein structure; besides, the modulatory effect of oxidized HSP70 on RAW265.7 cells was different from native HSP70. Macrophages treated with oxidized HSP70 presented lower proliferation, higher reactive oxygen species production, lower phagocytic activity and TNF-α release. These results indicate that oxidation of extracellular HSP70 modify its signaling properties, causing alterations on its modulatory effects on macrophage function and viability.

Keywords: HSP70; inflammation; oxidative modifications; macrophage activation

Introduction

Heat shock proteins (HSPs) were first described in 1962 when Ferrucio Ritossa accidentally exposed salivary gland cells to a heat shock [1]. They function as ATP dependent molecular chaperones, transporting intracellular proteins in cells under stress and assisting the correct folding and stabilization of damaged proteins. Since then, these proteins have been associated with many other stressful stimuli, including inflammation and oxidative stress [2], gaining a new cytoprotective function. Up-regulation of intracellular HSP70 results in protective effects against hypoxia, excess oxygen radicals, endotoxin, infections and fever [3]. This cytoprotective effect is attributed to their ability to stabilize protein structures through chaperon-like reversible interactions [4]. Members of the heat-shock superfamily have a highly conserved structure, being present from bacteria till mammals. HSP70 has an amino acid sequence constituted by 700 residues that form a 70 kDa protein with three different domains: the nucleotide binding domain (NBD), the substrate binding domain (SBD) and a c-terminal portion with yet unknown function. HSPs are distributed in diverse cell compartments, including cytosol, nucleus and membrane organelles. Recently, HSP70 has been observed to be translocated to the plasma membrane and released to extracellular environments, where they exert modulatory effects in cells of the immune system [5-7]; in addition, serum HSP70 is associated with stress conditions as inflammation, bacterial or viral infections, and oncological diseases [8].

In humans, extracellular HSP70 (eHSP70) is known to be released into the systemic circulation by the brain and hepatosplanchnic tissue [9, 10], but not by the contracting muscles [11], although physical exercise induces eHSP70 release, as first described in an experiment where subjects followed a 60 minutes treadmill at 70% of maximal oxygen uptake [12]. After that, the up-regulation of eHSP70 has been observed after prolonged exercise, which appears to rely on the duration and intensity of the exercise [13]. Increased levels of eHSP70 have been reported in the circulation of sedentary people after 1 hour of moderate and intensive exercise along with increased neutrophil microbicide capacity and chemotaxis [14, 15], suggesting that eHSP70 plays an important role in the exercise-induced inflammation and modulation of the innate immune response.

Extracellular HSPs can initiate both innate and adaptive immunity [16, 17]. HSP proteins derived from pathogens or the host cells can directly activate or enhance the activity of various innate immune cells to produce cytokines and chemokines [18]. These proteins promote maturation, migration of antigen-presenting cells (APC) and subsequent APC–T cell interaction, thus initiating the adaptive immune response. HSP can also bind antigenic peptides derived from tumor or virus, and has been applied to vaccine development as adjuvants. According to the ‘‘danger signal’’ theory, immune cells are activated by alarm signals derived from stress or damaged self-proteins [19]. It is accepted that extracellular HSP fits the criteria for danger signals as they can promote inflammatory cytokine production of APC and initiate antigen-specific immune response, present antigens to T cells in a ‘‘danger’’ context, and have been linked to the pathogenesis of several autoimmune diseases [20, 21].

In a previous work of our group we evaluated the relationship between serum HSP70 immunocontent and oxidant status in sepsis. Patients with severe sepsis were followed up for 28 days after diagnosis, or until death. The serum of these septic patients presented augmented HSP70 levels. Analysis of oxidative parameters revealed that septic patients with pronounced oxidative damage in serum had also increased HSP70 levels, while septic patients with control-like serum oxidative parameters had lower HSP70 levels. Furthermore, an analysis of septic patients according to survival outcome indicated that those patients with higher HSP70 serum levels presented increased

mortality, leading to the conclusion that the serum HSP70 levels are being modulated according to the patient oxidant status, and increased serum HSP70 levels is associated to mortality in sepsis [22].

It is known that the serum of septic patients is able to induce the activation of macrophages and the production of IL-10; also, the phagocytic index of these macrophages were correlated with the severity and mortality of the disease [23]. Murine models of lethal sepsis have higher protein carbonyl levels and an imbalance in SOD / CAT (superoxide dismutase / catalase) when compared with the non-lethal group, indicating that oxidative stress is exacerbated in the lethal model [24]. Thus, it is likely that several proteins related to anti-inflammatory response undergo oxidation by reactive species and, consequently, their functions are changed.

Considering that we had previously demonstrated that sepsis is associated with redox imbalance and oxidative stress, the aim of this work was to investigate a possible relationship between previously oxidized HSP70 and the activation of macrophage cells.

Methods

Oxidation of HSP70

Purified bovine HSP70 was purchased from Sigma-Aldrich® (H9776). HSP70 was treated with different concentrations (1, 5, 10, 25, 50 and 100 mM) of the oxidizing agent hydrogen peroxide (H_2O_2) for 1 hour at 37ºC under agitation. HSP70 was added so that the final concentration was 1 µg of protein per 1 mL of medium.

Polyacrylamide gel electrophoresis

We added 4 µg of protein to 12 µl of H_2O_2 or Milli-Q water (for control), incubated at 37ºC for 1h then rest at -20ºC for 20 minutes. Samples were mixed with 16 µl of non-denaturing buffer or denaturing buffer and loaded into a 12% polyacrylamide gel. Electrophoresis followed for approximately 1h. Gels were stained by Coomassie Blue and digitalized.

For the alkylation experiment, 4 µg of HSP70 were previously treated with 10 mM of iodoacetamide for 30 minutes in the absence of light. After that, samples were lyophilized to remove the alkylating agent and resuspended in Milli-Q water. Samples were treated with H_2O_2 for 1h at 37ºC and rested at -20ºC for 20 minutes. A non-denaturing buffer was utilized. Electrophoresis followed for approximately 1h. Gels were stained by Comassie Blue and digitalized.

Cell culture line macrophage RAW 264.7

Exponential growing macrophage-derived cell line RAW 264.7, obtained from the Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, Brazil) were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM of glutamine, 0.28 µg/µL of gentamicin and 250 µg of amphotericin B, in a humidified atmosphere of 5% of CO_2 in air at 37 °C. Cell medium was replaced each 2 days and the cells were sub-cultured once they reached 90% confluence. These cells are grown in plastic bottles and scrapped when they reach 70 - 90% confluence, counted and plated. Cells were seeded in 96-well plates for DCFH-DA, nitrite production, SRB and MTT assays; in 24-well plates for phagocytosis assay and in 6-well plates for TNF-α release assay.

Nitrite assay

The accumulation of NO_2^-, a stable end product of NO formation, in conditioned media was measured as an indicator of NO production. Cells were seeded in a 96 well plate and then treated with oxidized HSP70 ($_{ox}$HSP70) or non-oxidized HSP70 (HSP70). 100 µL cell-free conditioned medium was transferred and incubated with 100 µL of Griess reagent at 37ºC for 15 minutes and the absorbance measured in a microplate reader at 540 nm [25].

SRB assay

Cells were seeded in a 96 well microplate and then treated with $_{ox}$HSP70 or HSP70. After removal of the medium, cells were fixed with 10% trichloroacetic acid for 1 hour at 4ºC. Following by staining for 30 minutes with 0.4% (wt/vol) sulforhodamine B (SRB) dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base [tris (hydroxymethyl) aminomethane] for determination of optical density in a computer-interfaced, 96-well microtiter plate reader at 515 nm [26].

MTT assay

Cell viability was measured via MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. RAW 264.7 macrophages were seeded in a 96 well microplate and then treated with or not with HSP70 or $_{ox}$HSP70 for 24 hours. Medium was removed and cells were washed with PBS. MTT (0, 05 mg/mL) was added for 1 hour at 37ºC. Cells were again washed and DMSO was added for 15 minutes in the absence of light. Absorbance was read at 560 nm and 630 nm [27].

DCFH-DA assay

Changes in reactive species production were detected by DCFH-DA oxidation-based method. Cells were seeded in a 96 well microplate for 24 hours. After removal of the medium, the plate was washed with PBS and 100 µl of DCFH (100 µM) diluted in 1 % medium was added in each well. The plate was then incubated for 1 hour at 37ºC. The medium with DCFH was removed and the plate was again washed with PBS. HSP70 or $_{ox}$HSP70 were added, diluted in medium, and the fluorescence was monitored by 90 minutes in the microplate reader, with excitation at 485 nm and emission at 532 nm at 37ºC [28].

Phagocytic Activity

The modulation of macrophages phagocytic activity was assessed by the zimosan particles assay. Zimosan (1,25 mg) was incubated under agitation with neutral red dye (200mg/mL, dissolved in DMSO) for 10 minutes. This preparation was resuspended in 20 volumes of PBS, centrifuged and washed. The resulting pellet was resuspended in 2,5 mL of PBS. 50 µL of this suspension was mixed with 650 mL of RPMI 1640 medium. Cells seeded in 24 well plates were incubated with 2mM of PMA (phorbol 12-myristate 13-acetate) or ethanol. After 30 minutes, the treatments ($_{ox}$HSP70 or HSP70) were added to the plate for 30 minutes in an incubator at 37ºC. After that, 50 µL of the preparation of neutral red stained zimosan was added in each well of the plate. The process of phagocytosis lasted 30 minutes and then was interrupted by adding cold PBS. The phagocytic macrophages as well as the particles were counted in a digital microscope IX-81 (Olympus) utilizing the program Cell. The phagocytic index was calculated based on the Hishikawa index [29].

ELISA indirect assay for TNF-α release

The induction of TNF- α release to the incubation medium was quantified by ELISA indirect assay. Cells were seeded in 6 well plates and treated for 24 hours. After that, 200 µl of the medium was transferred to a 96 wells ELISA plate for 36 hours for protein adhesion. Following removal of the medium, the plate was washed with TTBS 1x and 100 µ of 1:10.000 antibody anti-TNF-α solution was added for 24 hours at 4ºC. After washing, the plate was incubated with a solution containing the secondary antibody (anti-rabbit 1:10.000) for 3 hours at room temperature, washed again with TTBS 1x and added the revelation solution for 30 minutes. The reaction was stopped with sulfuric acid and the absorbance was read at 450 nm in a microplate reader.

Results

In order to elucidate the role of oxidation in the possible structural modifications HSP70 may undergo, we treated purified HSP70 with different concentrations of hydrogen peroxide and submitted it to a polyacrylamide gel electrophoresis. In the presence of a reducing agent, the protein showed as a major band of 70 kDa, as expected. But we can observe a decrease in the intensity of this band as the concentration of hydrogen peroxide increases. The eletrophoretic analysis also shows a 140-kDa band that probably is the dimerized form of HSP70. This band showed the same behavior as the main 70-kDa, presenting a decrease in its intensity as the hydrogen peroxide concentration is increased [Figure 1].

In an oxidizing environment, proteins with cysteine residues tend to form inter and intramolecular disulfide bonds, which are reversed by a reducing agent. To eliminate this interfering condition, we utilized a sample buffer without ß-mercaptoethanol [Figure 2]. Here, the predominant band is the 70 kDa one, which seems to fade in intensity as the H_2O_2 concentration increases. The 140 kDa presents the same pattern. In addition, there is a 90 kDa band that was evidenced in these conditions which also presented the pattern of decreased intensity according the increase in H_2O_2 concentration. Although all the bands decrease, it is not as smooth as it is in the reducing gel.

If the non-reducing gel has a different pattern than the reducing one, could it be due to the disulfide bonds or the interactions caused by the pro-oxidizing environment? Moreover, are these interactions important to the correct folding of the protein? When pre-treated with an alkylating agent and then treated with hydrogen peroxide, the major 70 kDa band appeared to be split into two minor bands [Figure 3]. Besides, additional bands in the range of 60 kDa were visualized, suggesting that the protein may not be completely and correctly assembled.

These same treatments were used to investigate the cellular response that oxHSP70 could trigger in macrophages. RAW 264.7 cells were incubated with oxHSP70 during 30, 60, 120 minutes and 24 hours and then nitrite production and cell viability were evaluated. We observed increased nitrite production in the pre-oxidized HSP70 treated cells (10 mM hydrogen peroxide) at 30 minutes (with H_2O_2 10 mM) and after 24 h (with H_2O_2 25 mM) of incubation; no differences were observed in other periods of incubation [Figure 4]. To assess cell survival, we used the SRB incorporation assay as a parameter of cell number, and no significant differences were observed at 30, 60 and 120 minutes of exposition. On the other hand, after 24 hours of exposition, we observed that the HSP70 oxidized with 5, 25, 50 and 100 mM of hydrogen peroxide induced a decrease in SRB incorporation compared to control ($P < 0,05$), indicating a decrease in the number of cells [Figure 5].

To further explore the effect of oxHSP70 in the number of cells, we decided to evaluate the effect of oxSHP70 in a parameter of cell viability. For this purpose, we performed a MTT reduction assay to evaluate cell viability after treatment HSP70 or $_{ox}$HSP70 for the same period of time. MTT reduction in cells treated with HSP70 was not different to control (not treated cells), while cells treated with $_{ox}$HSP70 (10, 25, 50 and 100 mM of H_2O_2) had decreased MTT reduction compared to HSP70-treated cells and control ($P < 0,05$) [Figure 6], suggesting oxHSP70 affects cell viability.

To evaluate the effect of oxHSP70 on the production of reactive oxygen species in RAW 264.7 cells, we performed the same treatments described above and monitored the oxidation of DCFH by the real-time DCFH-DA oxidation assay during 90 minutes. We observed that HSP70 previously oxidized with 25, 50 and 100 mM of hydrogen peroxide induced higher levels of reactive oxygen species when compared to HSP70-treated cells and control ($P < 0,05$) [Figure 7].

We next evaluated effect of oxHSP70 on the modulation of phagocytosis RAW 264.7 cells were treated during 30 minutes with oxHSP70 or HSP70 the phagocytic activity was assessed by the zimosan-based assay. As expected we observed that HSP70 induced an increase in the score of Hishikawa index when compared to control; oxHSP70 was not able to induce the same effect of normal HSP70, indicating that oxidation of HSP70 affects its modulatory effect on phagocytosis ($P < 0,05$) [Figure 8].

Another classical parameter of macrophage activation, the secretion of the cytokine TNF-α, was assessed. RAW 264.7 macrophages were treated with HSP70 or $_{ox}$HSP70 for 24 hours and the levels of TNF-α were evaluated by indirect ELISA. As expected, HSP70 induced a significant increase in TNF-α release by RAW 264.7 cells, while $_{ox}$HSP70 failed to induce this effect ($P < 0,05$) [Figure 9]. This result also indicates that oxidation of HSP70 induces a loss of the extracellular signaling function of this protein.

Discussion

Many years have passed since HSP70 has been discovered; nowadays researches show that its functions and roles are much wider than the original chaperone activity described. Recently, these proteins have been identified in the serum after diverse forms of stress (heavy exercise, inflammatory process, septic shock and trauma) [30-32]; first it was thought that its presence was caused by the necrotic disruption of cell membrane and consequently extravasation of cytosolic proteins, among them HSP70. After that, it was showed that HSP70 could be actively secreted from the intracellular environment to the extracellular space [33], leading to the belief that they could have a deeper signaling role during pathogenic processes.

In a recent study of our group [22], we have shown the correlation between the clinical outcome of septic patients and the presence of HSP70 in their serum. More than that, the patients which died and had more oxidative stress levels in their serum also had increased HSP70 serum levels. Here, we submitted purified HSP70 to oxidation and evaluated structural changes in the protein and the effects of oxHSP70 on cell viability and immunomodulatory properties. When submitted to an oxidative agent (hydrogen peroxide), HSP70 showed some alterations on the electrophoretic profile with a reducing sample buffer; the major protein band of 70 kDa and the 140 kDa band (the probable dimer) decreased in intensity as the gradient levels of oxidation increases, suggesting that the oxidative environment is damaging the protein and changing their tertiary structure [Figure 1]. In non-reducing conditions, electrophoresis of both normal and oxidized HSP70 evidenced a 90 kDa band, which is not apparent in the gel under reducing conditions. Although we do not know what process caused the appearance of this 90 kDa in both electrophoretic

separations, it is very likely that this process is a redox-related modification, such as disulfide bond formation, since this band is decreased in HSP70 submitted to higher levels of H_2O_2. Thus, we suggest that this 90 kDa band visualized in the gel under reducing conditions is an intermediate, redox-modified form of HSP70 [Figure2].

As formation of intramolecular or intermolecular disulfide bonds is one of the most notable modifications of proteins containing cysteine residues, we blocked the possible sites for disulfide formation by pre-treating HSP70 with an alkylating agent, iodoacetamide (IAA); so, when the protein is incubated with hydrogen peroxide the disulfide bonds cannot be formed. The electrophoretic profile formed by running HSP70 in this condition shows a splitting of the 70 kDa band, suggesting that this major band was actually formed by bands of very close molecular weight [Figure 3].

If extracellular HSP70 plays a role in inflammatory processes, as these are commonly associated with oxidative unbalance and stress, what are the effects of a possible oxidation of oxHSP70 on macrophages? To solve this question we treated RAW 264.7 cells by 30, 60, 120 and 24 hours with HSP70 pretreated or not with hydrogen peroxide. We did not observe any significative differences between the production of nitrite by cells treated with $_{ox}$HSP70 or HSP70; still, the cell proliferation quantified through the SRB assay did not change in short times but after 24 hours of exposition we observe an interesting effect, where pre-oxidized HSP70 diminished the proliferation of the cells as compared to non-treated cells and cells treated with non-oxidized HSP70. Also interesting was the observation that the phagocytic activity of the macrophages was diminished in cells treated with pre-oxidized HSP70 as compared to the cells treated with non-oxidized HSP70, suggesting that $_{ox}$HSP70 did not activate macrophages with the same efficacy as the non-oxidized HSP70 [Figure 8].

HSP70 has the ability to induce macrophage activation; one of the parameters observed when this event occurs is the production of inflammatory cytokines, such as IL-8, IL-10 and TNF-α. We treated RAW 264.7 macrophages with HSP70 or $_{ox}$HSP70 for 24 hours and analyzed the content of TNF-α in the incubation medium. The groups treated with $_{ox}$HSP70 had similar TNF-α levels to the control group; besides, TNF-α release by cells treated with normal HSP70 were increased compared to both $_{ox}$HSP70-treated and control cells, suggesting that $_{ox}$HSP70 failed to activate macrophages in a classical way. It is known that TNF-α plays an important role in the proliferation, apoptosis and differentiation of macrophages. Long-term survival of macrophages is dependent on autocrine signaling by TNF-α [34]. Because TNF-α mediates many of the pathological effects of LPS-TLR4 in conditions such as septic shock, it is suggested that prolonged macrophage survival mediated by TNF-α plays an important role in sepsis [35]; this can be related to our findings which shown that after 24 hours of treatment with $_{ox}$HSP70, RAW 264.7 macrophages produced lower levels of TNF-α [Figure 6] and had lower cell viability [Figure 9].

In a general way, HSP70 can be released to the extracellular space as a “danger signal” to the system, as it can be recognized by TLR and trigger a pro-inflammatory signaling pathway. In this context, we could infer that the oxidation and subsequently modification and impairment of HSP70 can be related to a lack of this “danger signal” when necessary. Moreover, $_{ox}$HSP70 could be exerting its effects by activating other receptors than TLR, such as the receptor for advanced end-glycation products (RAGE), a key pattern recognition receptor capable of binding a diverse repertoire of soluble and cell-associated molecules involved in the host response to tissue injury, infection and inflammation. In a recent study [36] it was shown that HSP70 is a possible novel ligand for RAGE. Ligation of RAGE induces the activation of multiple signaling pathways that may vary depending on the ligand, cell and tissue microenvironment, and thus mediates diverse cellular responses, such as the one observed by $_{ox}$HSP70.

Nevertheless, it is important to note that the immune effects of HSP vary depending on several factors including the specific HSP family (HSP60, HSP70, HSP90), the cellular source of the HSP (normal, cancerous, viral-infected, bacterial), cellular location (intracellular, cell surface, serum) and the physiological circumstances modulating HSP expression (oxidative stress, bacterial infection, viral infection, psychological stress, physical stress, whole organism vs. localized cellular stress). Thus, the specific physiological context of these proteins greatly impacts their function, preventing any global statements about the *in vivo*, immunological functions of HSP70 [16].

Taking into account the results presented in this paper and in our previous findings, we suggest that in a pro-oxidant situation, common to pro-inflammatory pathologic processes, HSP70 could undergo structural changes which could lead to functional impairment, as we observed before in septic patients [22]. In the present paper we showed that oxidized HSP70 induced a decrease in RAW 264.7 macrophages number after 24 hours treatment, a decrease in phagocytic activity and a decrease in TNF-α production. We believe that this may be correlated with the worst outcome observed in our previous work.

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References

1. Ritossa, F., *A new puffing pattern induced by temperature shock and DNP in drosophila.* Cellular and Molecular Life Sciences 1962. **18**(12).
2. Ziegler, T.R., et al., *Parenteral glutamine increases serum heat shock protein 70 in critically ill patients.* Intensive Care Med, 2005. **31**(8): p. 1079-86.
3. Tang, D., et al., *The anti-inflammatory effects of heat shock protein 72 involve inhibition of high-mobility-group box 1 release and proinflammatory function in macrophages.* J Immunol, 2007. **179**(2): p. 1236-44.
4. Ribeiro, S.P., et al., *Sodium arsenite induces heat shock protein-72 kilodalton expression in the lungs and protects rats against sepsis.* Crit Care Med, 1994. **22**(6): p. 922-9.
5. Asea, A., et al., *HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine.* Nat Med, 2000. **6**(4): p. 435-42.
6. Asea, A., et al., *Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4.* J Biol Chem, 2002. **277**(17): p. 15028-34.
7. Asea, A., *Hsp72 release: mechanisms and methodologies.* Methods, 2007. **43**(3): p. 194-8.
8. Multhoff, G., *Heat shock protein 70 (Hsp70): membrane location, export and immunological relevance.* Methods, 2007. **43**(3): p. 229-37.
9. Febbraio, M.A., et al., *Exercise induces hepatosplanchnic release of heat shock protein 72 in humans.* J Physiol, 2002. **544**(Pt 3): p. 957-62.
10. Lancaster, G.I., et al., *Exercise induces the release of heat shock protein 72 from the human brain in vivo.* Cell Stress Chaperones, 2004. **9**(3): p. 276-80.

11. Febbraio, M.A., et al., *Reduced glycogen availability is associated with an elevation in HSP72 in contracting human skeletal muscle.* J Physiol, 2002. **538**(Pt 3): p. 911-7.
12. Walsh, R.C., et al., *Exercise increases serum Hsp72 in humans.* Cell Stress Chaperones, 2001. **6**(4): p. 386-93.
13. Fehrenbach, E., et al., *Exercise intensity and duration affect blood soluble HSP72.* Int J Sports Med, 2005. **26**(7): p. 552-7.
14. Ortega, E., et al., *Role of Hsp72 and norepinephrine in the moderate exercise-induced stimulation of neutrophils' microbicide capacity.* Eur J Appl Physiol, 2006. **98**(3): p. 250-5.
15. Ortega, E., et al., *The effect of stress-inducible extracellular Hsp72 on human neutrophil chemotaxis: a role during acute intense exercise.* Stress, 2009. **12**(3): p. 240-9.
16. Johnson, J.D. and M. Fleshner, *Releasing signals, secretory pathways, and immune function of endogenous extracellular heat shock protein 72.* J Leukoc Biol, 2006. **79**(3): p. 425-34.
17. Srivastava, P., *Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses.* Annu Rev Immunol, 2002. **20**: p. 395-425.
18. Tsan, M.F. and B. Gao, *Heat shock proteins and immune system.* J Leukoc Biol, 2009. **85**(6): p. 905-10.
19. Matzinger, P., *The danger model: a renewed sense of self.* Science, 2002. **296**(5566): p. 301-5.
20. Millar, D.G., et al., *Hsp70 promotes antigen-presenting cell function and converts T-cell tolerance to autoimmunity in vivo.* Nat Med, 2003. **9**(12): p. 1469-76.
21. Chen, T. and X. Cao, *Stress for maintaining memory: HSP70 as a mobile messenger for innate and adaptive immunity.* Eur J Immunol, 2010. **40**(6): p. 1541-4.
22. Gelain, D.P., et al., *Serum heat shock protein 70 levels, oxidant status, and mortality in sepsis.* Shock, 2011. **35**(5): p. 466-70.
23. Peck, G., et al., *Serum-induced macrophage activation is related to the severity of septic shock.* Inflamm Res, 2009. **58**(2): p. 89-93.
24. Andrades, M., et al., *Oxidative parameters differences during non-lethal and lethal sepsis development.* J Surg Res, 2005. **125**(1): p. 68-72.
25. Li, Y.H., et al., *Activation of macrophage nuclear factor-kappa B and induction of inducible nitric oxide synthase by LPS.* Respir Res, 2002. **3**: p. 23.
26. Skehan, P., et al., *New colorimetric cytotoxicity assay for anticancer-drug screening.* J Natl Cancer Inst, 1990. **82**(13): p. 1107-12.
27. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.* J Immunol Methods, 1983. **65**(1-2): p. 55-63.
28. Tarpey, M.M., D.A. Wink, and M.B. Grisham, *Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations.* Am J Physiol Regul Integr Comp Physiol, 2004. **286**(3): p. R431-44.
29. Hishikawa, T., et al., *Calcium transients during Fc receptor-mediated and nonspecific phagocytosis by murine peritoneal macrophages.* J Cell Biol, 1991. **115**(1): p. 59-66.
30. Kindas-Mugge, I., G. Steiner, and J.S. Smolen, *Similar frequency of autoantibodies against 70-kD class heat-shock proteins in healthy subjects and systemic lupus erythematosus patients.* Clin Exp Immunol, 1993. **92**(1): p. 46-50.

31. Trautinger, F., et al., *Stress proteins in the cellular response to ultraviolet radiation.* J Photochem Photobiol B, 1996. **35**(3): p. 141-8.
32. Trautinger, F., et al., *Increased expression of the 72-kDa heat shock protein and reduced sunburn cell formation in human skin after local hyperthermia.* J Invest Dermatol, 1996. **107**(3): p. 442-3.
33. Vega, V.L., et al., *Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages.* J Immunol, 2008. **180**(6): p. 4299-307.
34. Lombardo, E., et al., *TLR4-mediated survival of macrophages is MyD88 dependent and requires TNF-alpha autocrine signalling.* J Immunol, 2007. **178**(6): p. 3731-9.
35. Conte, D., et al., *Inhibitor of apoptosis protein cIAP2 is essential for lipopolysaccharide-induced macrophage survival.* Mol Cell Biol, 2006. **26**(2): p. 699-708.
36. Ruan, B.H., et al., *Complement C3a, CpG oligos, and DNA/C3a complex stimulate IFN-alpha production in a receptor for advanced glycation end product-dependent manner.* J Immunol, 2010. **185**(7): p. 4213-22.

Figure Captions

Figure 1. a) Polyacrylamide gel electrophoresis of HSP70 after 1 hour of exposition or not to different concentrations of hydrogen peroxide at 37ºC under agitation. Samples were mixed in reducing buffer containing ß-mercaptoethanol. b) Densitometry of the 70 kDa band. c) Densitometry of the 140 kDa band. Representative image of three independent experiments. No statistics applied

Figure 2. a) Polyacrylamide gel electrophoresis of HSP70 after 1 hour of exposition or not to different concentrations of hydrogen peroxide at 37ºC under agitation. Samples were mixed in a non-reducing buffer to preserve oxidative modifications (without ß-mercaptoethanol). b) Densitometry of the 70 kDa band. c) Densitometry of the 90 kDa band. d) Densitometry of the 140 kDa band. Representative image of three independent experiments. No statistics applied

Figure 3. a) Polyacrylamide gel electrophoresis of HSP70 pre-treated or not with iodoacetamide for 1 hour then exposed for 1 hour to different concentrations of hydrogen peroxide at 37ºC under agitation. Samples were mixed in a non-reducing buffer to preserve alkylating and oxidizing modifications (without ß-mercaptoethanol). a) Densitometry of the 70 kDa band. c) Densitometry of the 140 kDa band. Representative image of three independent experiments. No statistics applied

Figure 4. Nitrite production by RAW 264.7 macrophages after 30, 60, 120 minutes and 24 hours of exposition or not to HSP70 (non-oxidized) and to oxHSP70 (oxidized HSP70 by different concentrations of H_2O_2 for 1 hour at 37ºC). Experiments were triplicated and data is present as mean (SD). One way ANOVA was performed followed by Tukey's post-hoc test. No significant differences between treatments were observed. $P < 0,05$

Figure 5. Measurement of RAW 264.7 macrophages cell proliferation after 30, 60, 120 minutes and 24 hours of exposition or not to HSP70 (non-oxidized) and to oxHSP70 (oxidized HSP70 by different concentrations of H_2O_2 for 1 hour at 37ºC). Experiments were triplicated and data is presented as mean (SD). One way ANOVA was performed followed by Tukey's post-hoc test. HSP70 oxidized by 5, 25, 50 and 100 mM of H_2O_2 differed significatively from control (non-treated) and from HSP70 non-oxidized. $P < 0,05$

Figure 6. Measurement of RAW 264.7 macrophages cell viability after 24 hours of exposition or not to HSP70 (non-oxidized) and to oxHSP70 (oxidized HSP70 by different concentrations of H_2O_2 for 1 hour at 37ºC). Experiments were triplicated and data is presented as mean (SD). 24 hours SRB placed by side for comparison. One way ANOVA was performed followed by Tukey's post-hoc test. All $_{ox}$HSP70 treatments showed lower cell viability when compared to control but only HSP70 oxidized by 10, 25, 50 and 100 mM of H_2O_2 differed significatively from control (non-treated) and from HSP70 non-oxidized. $P < 0,05$

Figure 7. Detection of changes in reactive species production on RAW 264.7 macrophages by DCFH-DA method during 90 minutes of exposition or not to HSP70 (non-oxidized) and to $_{ox}$HSP70 (oxidized HSP70 by different concentrations of H_2O_2 for 1 hour at 37ºC). Experiments were triplicated and data is presented as mean (SD). One way ANOVA was performed followed by Tukey's post-hoc test. HSP70 oxidized by 25, 50 and 100 mM of H2O2 differed significatively from control (non-treated) and from HSP70 non-oxidized, showing higher levels of reactive species production. $P < 0,05$. a) Kinetic of changes in reactive species production. b) Bar representation of the differential changes in reactive species production

Figure 8. a) Modulation of phagocytic activity of RAW 264.7 macrophages exposed or not to HSP70 (non-oxidized) and to $_{ox}$HSP70 (oxidized HSP70 by different concentrations of H_2O_2 for 1 hour at 37ºC). Experiments were triplicated and data is presented as mean (SD). Two way ANOVA was performed. HSP70 non-oxidized differed significatively from control (non-treated) and from $_{ox}$HSP70, showing a higher phagocytic index. $P < 0,05$. B) Representative images of the treatments effect on macrophage phagocytosis

Figure 9. Modulation of RAW 264.7 macrophages TNF-α production after exposition or not to HSP70 (non-oxidized) and to oxHSP70 (oxidized HSP70 by different concentrations of H_2O_2 for 1 hour at 37ºC). Experiments were triplicated and data is presented as mean (SD). One way ANOVA was performed followed by Tukey's post-hoc test. HSP70 non-oxidized differed significatively from control (non-treated) and from oxHSP70, showing a higher TNF-α production. $P < 0,05$

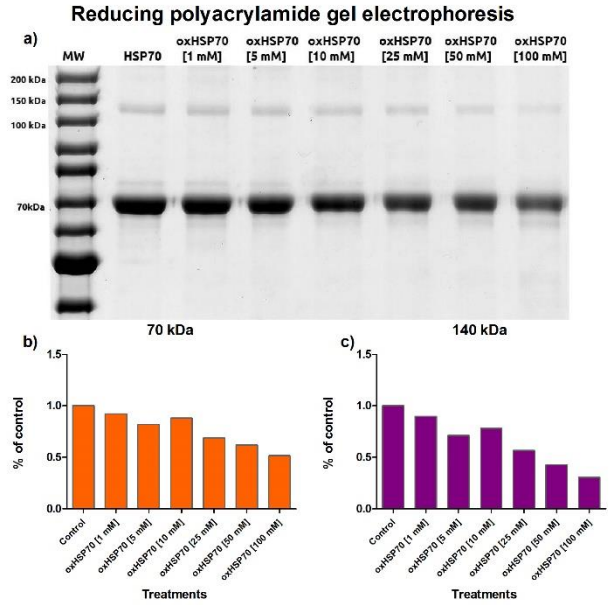
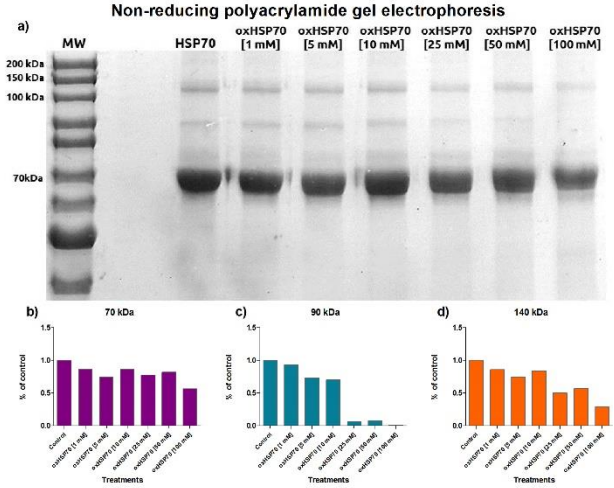


Fig. 1



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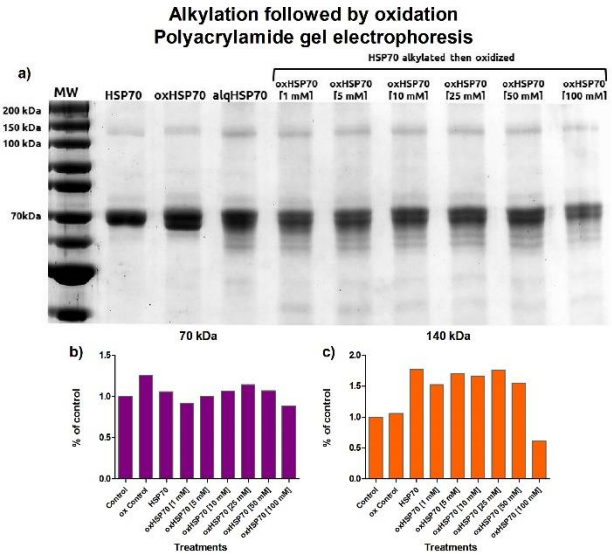

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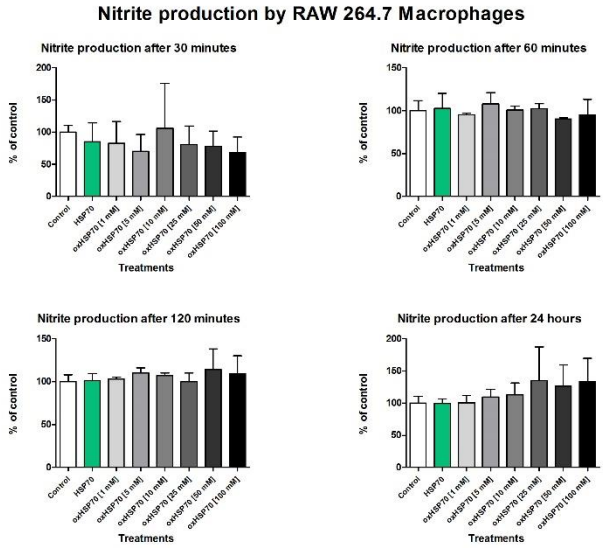

Fig. 4

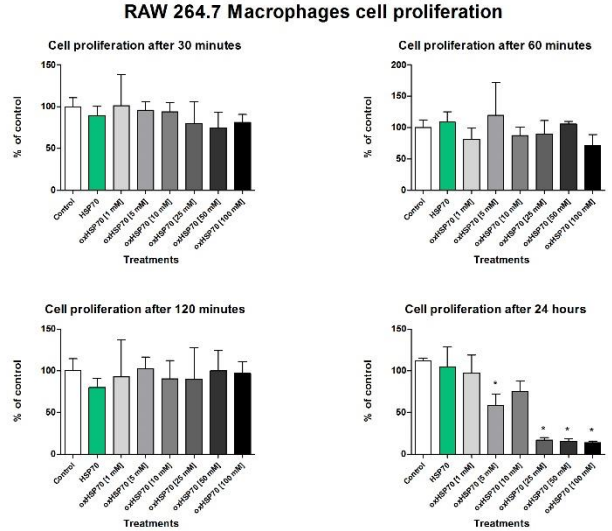

Fig. 5

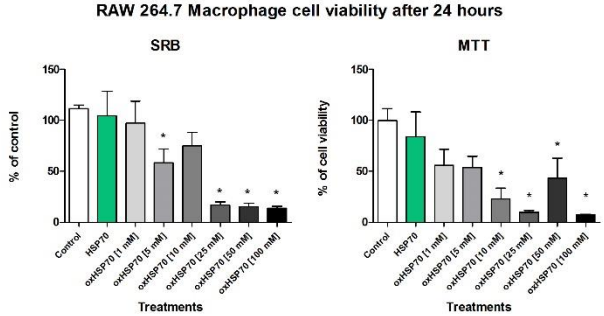

Fig. 6

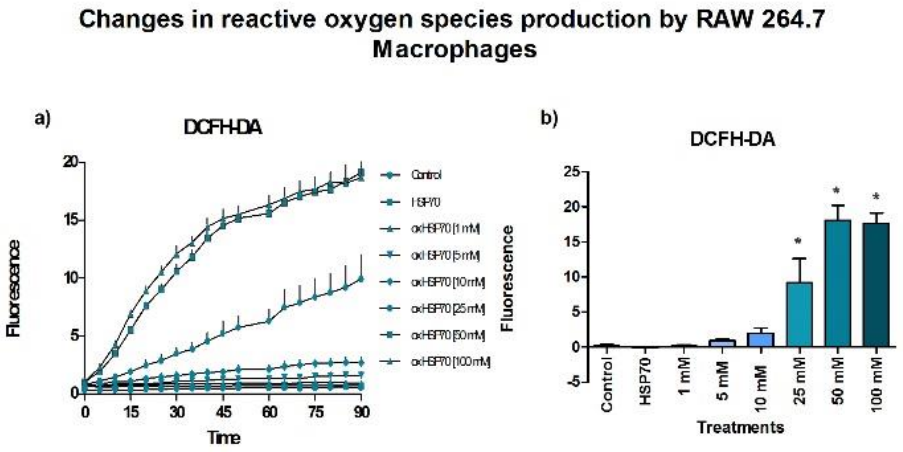

Fig. 7

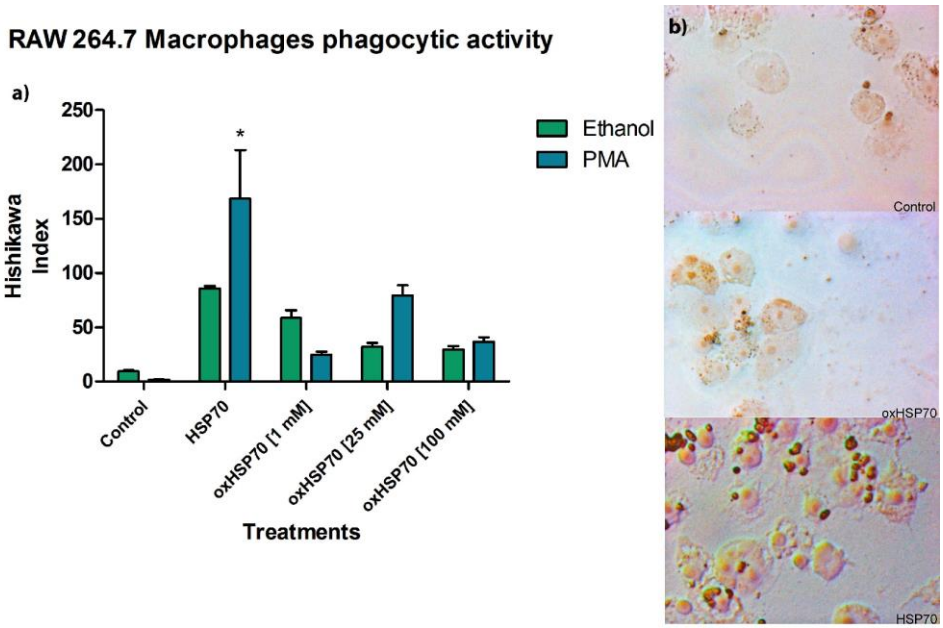

Fig. 8

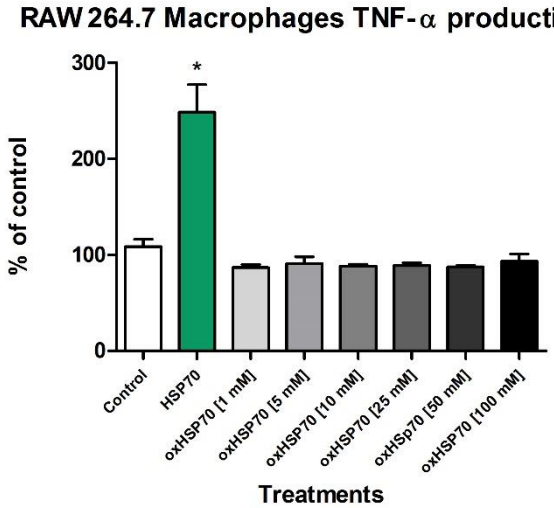

Fig. 9

Capítulo 3

Artigo submetido e em processo de revisão na revista “Journal of Molecular Modelling”

“The possible interaction between HSP70 and RAGE”

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Journal of Molecular Modeling

The possible interaction between HSP70 and RAGE

Marcelo Sartori Grunwald[1], Rodrigo Ligabue Braun[2], Hugo Verli[2], Juciano Gasparotto[1], Daniel Pens Gelain[1], José Cláudio Fonseca Moreira[1]

[1] Department of Biochemistry, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

[2] Center of Biotechnology, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

Correspondig author: Marcelo Sartori Grunwald

Corresponding Author Address: Rua Ramiro Barcelos, 2600. Anexo Dpto de Bioquímica - Bairro Santana, Porto Alegre - RS - CEP 90035-003 Phone number 55 51 3308 5578 marcelo.sartori@yahoo.com.br

Abstract

Extracellular HSP70 is known to be recognized by receptors located on the plasma membrane, such as TLR4, TLR2, CD14 and CD40, activating the transcription factor NF-KB and, consequently, leading to the release of proinflammatory cytokines, enhance the phagocytic activity of innate immune cells and to stimulate antigen-specific responses. Despite of that fact, the exact nature of HSP70 receptors it is still unknown and neither have all of its receptors been described. In regard of this matter, we tried, in the present study, to describe a theoretical possibility of binding between the HSP70 substrate binding domain and the receptor for advanced end glycation products through molecular docking and molecular dynamics simulation. The analysis of the RMSD, the radius of gyration and the distance between the center of mass of the two proteins components of the docking system showed that the system becomes stable throughout the simulation course. Comparative analyses of the interface of interaction of the initial and final frames of the molecular dynamics simulation gave us some insights about the amino acid residues interacting and the types of interaction between them. Finally, these results could be correlated with previous findings of our group (data not published yet), where oxidized HSP70 was responsible for a differential modulation of macrophage activation, that could be the result of a signaling pathway triggered by RAGE binding.

Keywords: HSP70; RAGE; signaling pathway; molecular docking

Introduction

Recently it was shown that the extracellular HSP70 binds with high affinity to the plasma membrane of antigen presenting cells (APCs), eliciting a rapid intracellular Ca^{+2} flux and activating the transcription factor NF-kB; these events are followed by a significant release of proinflammatory cytokines such as TNF-α, IL-1β e IL-6, in the APCs, through Toll-like receptor 2, Toll-like 4 and CD-14 dependent process. Amongst the immune system cells that present binding capacity with HSP70, there are the natural-killer cells (NK) (1), APCs (2), macrophages and peripheral monocytes (3, 4), and B lymphocytes (5). Nowadays, it is known that this interaction and the posterior signal transduction cascade are mediated by TLR2 and TLR4 with their cofactor CD14 (2), the scavenger receptor CD36 (6) and the co-stimulatory molecule CD40 (7).

Binding of HSP70 to TLR2 and TLR4 is associated with the release and nuclear translocation of NF-kB, which activation may be MyD88 dependent or MyD88 independent. Besides NF-kB activation, this signaling pathway is characterized by the release of TNF-α, IL1β, IL6 and IL12. The CD40-HSP70 interaction seems to be more important on B-lymphocytes, where the phosphorylation of p38 with subsequent release of TNF-α and IFN- γ.

Regardless of the importance of these data it is still unknown the exact nature of the surface bound receptor for HSP70. We do know that this interaction is important because it results in the chaperokine-induced transduction of signals important for the survival of the host against pathogens (8). Recently, it was shown by an ELISA screening-like assay that HSP70 is a possible novel ligand for the receptor of advanced end-glycation products (RAGE) (9).

RAGE presents the ability to bind a vast array of molecules, both soluble and membrane bound, involved in the host response against tissue damage, infectious and inflammatory processes. Ligation of RAGE induces the activation of multiple signaling pathways that may vary depending on the ligand, cell and tissue microenvironment, and thus mediates diverse cellular responses.

Here we develop the hypothesis of the binding between HSP70 and RAGE through a side by side comparison of the immunocontent of signaling phosphatases of RAW 264.7 macrophages after 1 hour treatment with HSP70 and well known RAGE agonists, and through the molecular dynamics analysis of the docking solution between HSP70 and RAGE. We conclude that our results corroborate with and fortify the hypothesis of the new interaction proposed.

Methods

Cell Culture line macrophage RAW 264.7

Exponential growing macrophage-derived cell line RAW 264.7, obtained from the Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, Brazil) were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM of glutamine, 0.28 μg/μL of gentamicin and 250 μg of amphotericin B, in a humidified atmosphere of 5% of CO_2 in air at 37 °C. Cell medium was replaced each 2 days and the cells were sub-cultured once they reached 90% confluence. These cells are grown in plastic bottles and scrapped when they reach 70 - 90% confluence, counted and plated.

Treatments

Cells were seeded in 6-wells plate and after 24 hours at the incubator (5% of CO_2 at 37ºC) the medium was changed and the following treatments were added: HSP70 1μg/mL, s100B 1μg/mL, Glycated albumin 1μg/mL and HMGB1 1μg/mL. Cells were incubated with these treatments for 1 hour.

Western Blotting analysis

To perform western blotting experiments, RAW 264.7 macrophage cells were lysed in Laemmli-sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol) and equal amounts of cell protein (30 μg/well) were fractionated by SDS-PAGE and electro-blotted onto nitrocellulose membranes. Protein loading and electro-blotting efficiency were verified through Ponceau S staining, and the membrane was blocked in Tween-Tris buffered saline (TTBS: 100 mM Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 5% albumin. Membranes were incubated overnight at 4ºC with each antibody separately in TTBS, at different working dilutions as suggested by the manufacturers, and then washed with TTBS. Anti-rabbit IgG peroxidase-linked secondary antibody was incubated with the membranes for additional 1 h (1:5000 dilution range), washed again and the immunoreactivity was detected by enhanced chemiluminescence using ECL Plus kit. Densitometric analysis of the films was performed with ImageJ software. Blots were developed to be linear in the range used for densitometry. All results were expressed as a relative ratio between the enzyme immunocontent and the β-actin internal control immunocontent.

Molecular Modelling and Docking

The molecular model of HSP70 was built using the bovine amino acid sequence (Accession: AAA73914.1 GI: 497938), the PDB entries 3C7N and 3DPO as known related structures (10, 11) and the software *MODELLER v9.8* (12). The best model of fifty was chosen based on the parameters given by the server PDBsum (*http://www.ebi.ac.uk/pdbsum/*) and its PROCHECK plots (13, 14). Our model comprises the nucleotide binding domain (NBD), the substrate binding domain (SBD) and C-terminal domain. All models were visualized, colored, rotated and aligned on *UCSF CHIMERA.*

The molecular docking of HSP70 and the receptor for advanced end glycation products (RAGE) was carried out with PIPER on ClusPro 2.0 (15, 16). We used the PDB entry 3CJJ (17) as a model for the receptor. This structure comprises the variable domain (Ig-V) and the first constant domain (Ig-C1) of the receptor.

Molecular Dynamics calculations

The free HSP70 and the best docking solution for the HSP70-RAGE complex were subjected to MD simulations with GROMACS 4 suite (18) using GROMOS96 53a1 force field (19). The systems were solvated in triclinic boxes using periodic boundary conditions and SPC water models (20). Counterions were added to neutralize the systems. The MD protocol was built based on previous studies (21, 22). The Lincs method (23) was applied to constrain covalent bond lengths, allowing an integration step of 2 fs after an initial energy minimization using Steepest Descents algorithm. Electrostatic interactions were calculated with Particle Mesh Ewald method (24). Temperature and pressure were kept constant by coupling proteins, ions, and solvent to external temperature and pressure baths with coupling constants of $\tau = 0.1$ and 0.5 ps (25), respectively. The dielectric constant was treated as $\varepsilon = 1$, and the reference temperature was adjusted to 310 K. The systems were slowly heated from 50 to 310 K, allowing a progressive thermalization of the molecular systems. The simulation was then performed to 50 ns, with no restraint, while a reference value of 3.5 Å between heavy atoms was considered for a hydrogen-bond, and a cutoff angle of 30° was used between hydrogen-donor-acceptor (18).

Results

In order to investigate the possible interaction of HSP70 and RAGE, we first performed a western blotting analyses of important signaling enzymes located downstream in the RAGE signaling pathway, after interaction with a ligand. When RAW 264.7 macrophages were incubated with HSP70 for 1 hour, the immunocontent of the phosphorylated form of the enzymes ERK ½ was present as it was in the well-known RAGE agonists: S100B, Glycated albumin and HMGB1 (**Figure 1**).

The molecular dynamics run of the Heat Shock Protein 70 (HSP70) showed through analysis of the atom-positional root mean square deviation (RMSD) of all atoms that the protein reaches stability 5 ns after the beginning of the simulation (**Figure 2a**) and the radius of gyration of the protein shows some extent of compaction, that stabilizes after 35 ns of simulation (**Figure 2b)**, as it can be observed through the superimposition of the initial and final frames of the trajectory of the molecular dynamics (**Figure 3**).

The molecular dynamics run of the docking solution between HSP70´s SPB and RAGE showed through analysis of the atom-positional root mean square deviation (RMSD) of all atoms that the system becomes stable after 25 ns of simulation (**Figure 4a).** The radius of gyration of the system is constant through the simulation course (**Figure 4b**) and the distance between the center of mass of the two proteins components of the docking system reaches stability after 30 ns of the simulation period (**Figure 4c**), as it can be observed through the superimposition of the initial and final frames of the trajectory of the molecular dynamics run of the system (**Figure 5**).

Analyzing the interface of interaction from the first frame of the molecular dynamics run of the docking solution with PDBsum (protein-protein interaction) showed that the contact surface for RAGE is 578 A^2 with 14 interacting residues, and for HSP70´s SBD is 602 A^2 with 9 interacting residues. The interactions between these residues are mainly composed by non-bonded contacts (43) with some hydrogen bonds (5) (**Figure 6c**). The interface of interaction from the last frame of the molecular dynamics run of the docking solution with PDMsum (protein-protein interaction) showed that the contact surface for RAGE is 693 A^2 with 14 interacting residues and for HSP70´s SBD is 699 A^2 with 12 interacting residues. The interactions between these residues are composed mainly by non-bonded contacts (54) with some hydrogen bonds (7) (**Figure 6d**).

Discussion

Many years have passed since HSP70 has been discovered; nowadays researches show that its functions and roles are much wider than the original chaperone activity described. Recently, these proteins have been identified in the serum after diverse forms of stress (heavy exercise, inflammatory process, septic shock and trauma) (26–28); first it was thought that its presence was caused by the necrotic disruption of cell membrane and consequently extravasation of cytosolic proteins, among them HSP70. After that, it was showed that HSP70 could be actively secreted from the intracellular environment to the extracellular space (29), leading to the belief that they could have a deeper signaling role during pathogenic processes.

In a recent study of our group (30), we have shown the correlation between the clinical outcome of septic patients and the presence of HSP70 in their serum. More than that, the patients which died and had more oxidative stress levels in their serum also had increased HSP70 serum levels. Our last experiments (data not published yet) showed that macrophages treated with pre-oxidized HSP70 had a different pattern of activation when compared to macrophages treated with HSP70. Those macrophages presented lower cell proliferation and viability, higher reactive oxygen species production, lower levels of TNF-α release and lower phagocytic activity. These aspects of activation are very similar to the parameters showed by macrophages upon RAGE ligand binding.

After demonstrating through a clinical trial the relationship between extracellular HSP70, augmented oxidative damage and mortality in sepsis; through an *in vitro* experiment the relationship between oxidized HSP70 and the different pattern of macrophage activation, we tried here to demonstrate through an *in silico* experiment the possible interaction between HSP70 and RAGE, the theoretical mechanism of interaction by which the *in vitro* effects are occurring.

Conclusion

Taken together, these results suggest that the interaction between the substrate binding domain of HSP70 and the receptor for advanced end-glycation products showed via molecular docking and refined by molecular dynamics is possible as the docked system is very stable through the course of 50 ns of simulation and showed a significant amount of interacting residues in the protein-protein interface.

Figure captions

Fig 1. Comparison of the cell signaling triggered by known RAGE ligands and HSP70. The phosphorylated form of ERK ½ was detected by Western Blotting. Experiment was performed in triplicate. No statistics applied

Fig 2. Time-dependence of structural parameters from molecular dynamics (MD) simulations. a) Atom-positional root mean square deviation (RMSD) of all atoms of the free HSP70. b) Radius of gyration (Rg) of free HSP70

Fig 3. Superimposition of the initial (colored in magenta) and final (colored in orange) frames of the molecular dynamic run of the HSP70 model. Models were colored by secondary structure succession. N-terminal portion is indicated by the letter N and the C-terminal is indicated by the letter C

Fig 4. Time-dependence of structural parameters from molecular dynamics (MD) simulations. a) Atom-positional root mean square deviation (RMSD) of all atoms of the docking solution between the peptide binding domain (PBD) of HSP70 and the advanced end glycation products (RAGE). b) Radius of gyration (Rg) of the docking solution and of the components of the system separately. c) Distance between the center of mass of the two components of the system

Fig 5. Superimposition of the initial and final frames of the molecular dynamic simulation of the docking between the peptide-binding domain of HSP70 and the receptor for advanced end-glycation products. RAGE initial frame structure is colored in orange, HSP70 PBD initial frame structure is colored in green. Both final frames structures are colored in blue

Fig 6. Extracts from the protein–protein interaction diagrams in PDBsum for PDB resulting from the initial and final frames of the molecular dynamics run of the docking solution between HSP70´s SBD and RAGE. (a) Thumbnail image of the 3D structural initial frame model of the docking solution, RAGE is colored in magenta (chain A) and HSP70´s SBD is colored in red (chain B). (b) Thumbnail image of the 3D structural final frame model of the docking solution, RAGE is colored in magenta (chain A) and HSP70´s SBD is colored in red (chain B). (c) Detail of the individual residue–residue interactions across this interface from the initial frame of the molecular dynamics run of the docking solution. (d) Detail of the individual residue–residue interactions across this interface from the final frame of the molecular dynamics run of the docking solution. The color of the interactions is as above

References

1. **Gross C, Hansch D, Gastpar R, Multhoff G**. 2003. Interaction of heat shock protein 70 peptide with NK cells involves the NK receptor CD94. Biological chemistry **384**:267–79.

2. **Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK**. 2002. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem, 2002/02/12 ed. **277**:15028–15034.

3. **Asea A, Kabingu E, Stevenson MA, Calderwood SK**. 2000. HSP70 peptidembearing and peptide-negative preparations act as chaperokines. Cell Stress Chaperones, 2001/02/24 ed. **5**:425–431.

4. **Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK**. 2000. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nature medicine **6**:435–42.

5. **Arnold-Schild D, Hanau D, Spehner D, Schmid C, Rammensee HG, De la Salle H, Schild H**. 1999. Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. Journal of immunology (Baltimore, Md. : 1950) **162**:3757–60.

6. **Delneste Y, Magistrelli G, Gauchat J, Haeuw J, Aubry J, Nakamura K, Kawakami-Honda N, Goetsch L, Sawamura T, Bonnefoy J, Jeannin P**. 2002. Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. Immunity **17**:353–62.

7. **Becker T, Hartl F-U, Wieland F**. 2002. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. The Journal of cell biology **158**:1277–85.

8. **Asea A**. 2003. Chaperokine-induced signal transduction pathways. Exerc Immunol Rev, 2003/12/23 ed. **9**:25–33.

9. **Ruan BH, Li X, Winkler AR, Cunningham KM, Kuai J, Greco RM, Nocka KH, Fitz LJ, Wright JF, Pittman DD, Tan XY, Paulsen JE, Lin LL, Winkler DG**. 2010. Complement C3a, CpG oligos, and DNA/C3a complex stimulate IFN-alpha production in a receptor for advanced glycation end product-dependent manner. J Immunol, 2010/09/08 ed. **185**:4213–4222.

10. **Schuermann JP, Jiang J, Cuellar J, Llorca O, Wang L, Gimenez LE, Jin S, Taylor AB, Demeler B, Morano KA, Hart PJ, Valpuesta JM, Lafer EM, Sousa R**. 2008. Structure of the Hsp110:Hsc70 nucleotide exchange machine. Molecular cell **31**:232–43.

11. **Liebscher M, Roujeinikova A**. 2009. Allosteric coupling between the lid and interdomain linker in DnaK revealed by inhibitor binding studies. Journal of bacteriology **191**:1456–62.

12. **Sánchez R, Sali A**. 2000. Comparative protein structure modeling. Introduction and practical examples with modeller. Methods in molecular biology (Clifton, N.J.) **143**:97–129.

13. **Laskowski RA, MacArthur MW, Moss DS, Thornton JM**. 1993. PROCHECK: a program to check the stereochemical quality of protein structures. Journal of Applied Crystallography **26**:283–291.

14. **Laskowski RA**. 2009. PDBsum new things. Nucleic acids research **37**:D355–9.

15. **Kozakov D, Brenke R, Comeau SR, Vajda S**. 2006. PIPER: an FFT-based protein docking program with pairwise potentials. Proteins **65**:392–406.

16. **Comeau SR, Gatchell DW, Vajda S, Camacho CJ**. 2004. ClusPro: a fully automated algorithm for protein-protein docking. Nucleic acids research **32**:W96–9.

17. **Koch M, Chitayat S, Dattilo BM, Schiefner A, Diez J, Chazin WJ, Fritz G**. 2010. Structural basis for ligand recognition and activation of RAGE. Structure (London, England : 1993) **18**:1342–52.

18. **Hess B, Kutzner C, Van Der Spoel D, Lindahl E**. 2008. GROMACS 4: Algorithms for Highly Efficient, Load-Balanced, and Scalable Molecular Simulation. Journal of Chemical Theory and Computation **4**:435–447.

19. **Oostenbrink C, Villa A, Mark AE, Van Gunsteren WF**. 2004. A biomolecular force field based on the free enthalpy of hydration and solvation: the GROMOS force-field parameter sets 53A5 and 53A6. Journal of Computational Chemistry **25**:1656–1676.

20. **Berendsen HJC, Grigera JR, Straatsma TP**. 1987. The missing term in effective pair potentials. Journal of Physical Chemistry **91**:6269–6271.

21. **Verli H, Guimarães JA**. 2004. Molecular dynamics simulation of a decasaccharide fragment of heparin in aqueous solution. Carbohydrate Research **339**:281–290.

22. **Pol-Fachin L, Franco Becker C, Almeida Guimarães J, Verli H**. 2011. Effects of glycosylation on heparin binding and antithrombin activation by heparin. Proteins **79**:2735–45.

23. **Hess B, Bekker H, Berendsen HJC, Fraaije JGEM**. 1997. LINCS: A linear constraint solver for molecular simulations. Journal of Computational Chemistry **18**:1463–1472.

24. **Darden T, York D, Pedersen L**. 1993. Particle mesh Ewald: An N·log(N) method for Ewald sums in large systems. The Journal of Chemical Physics **98**:10089.

25. **Berendsen HJC, Postma JPM, Van Gunsteren WF, DiNola A, Haak JR**. 1984. Molecular dynamics with coupling to an external bath. The Journal of Chemical Physics **81**:3684–3690.

26. **Kindas-Mugge I, Steiner G, Smolen JS**. 1993. Similar frequency of autoantibodies against 70-kD class heat-shock proteins in healthy subjects and systemic lupus erythematosus patients. Clin Exp Immunol, 1993/04/01 ed. **92**:46–50.

27. **Trautinger F, Kindas-Mugge I, Knobler RM, Honigsmann H**. 1996. Stress proteins in the cellular response to ultraviolet radiation. J Photochem Photobiol B, 1996/09/01 ed. **35**:141–148.

28. **Trautinger F, Knobler RM, Honigsmann H, Mayr W, Kindas-Mugge I**. 1996. Increased expression of the 72-kDa heat shock protein and reduced sunburn cell formation in human skin after local hyperthermia. J Invest Dermatol, 1996/09/01 ed. **107**:442–443.

29. **Vega VL, Rodriguez-Silva M, Frey T, Gehrmann M, Diaz JC, Steinem C, Multhoff G, Arispe N, De Maio A**. 2008. Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages. J Immunol, 2008/03/07 ed. **180**:4299–4307.

30. **Gelain DP, De Bittencourt Pasquali MA, C MC, Grunwald MS, Ritter C, Tomasi CD, Alves SC, Quevedo J, Dal-Pizzol F, Moreira JC**. 2011. Serum heat shock protein 70 levels, oxidant status, and mortality in sepsis. Shock, 2011/02/19 ed. **35**:466–470.

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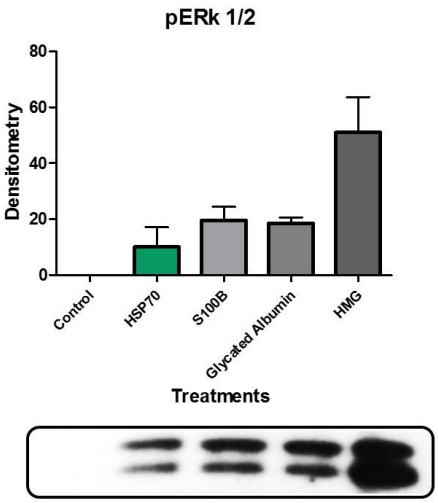

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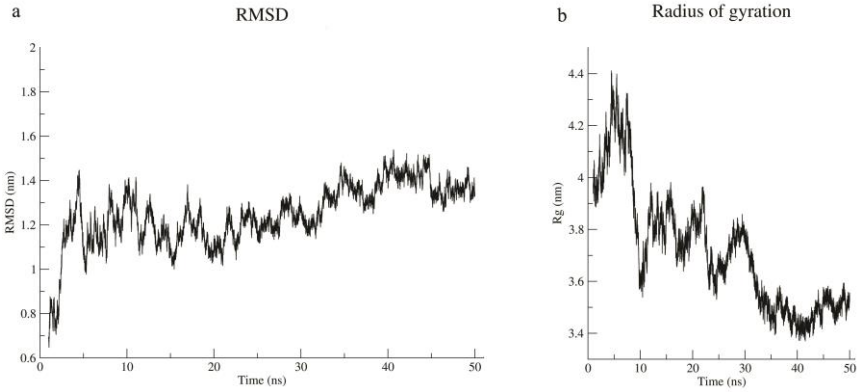

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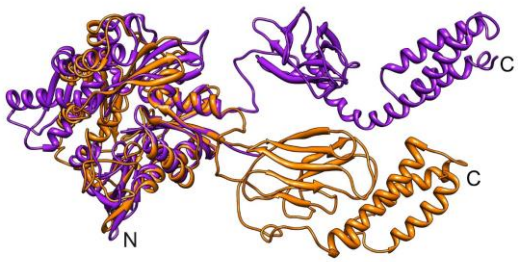

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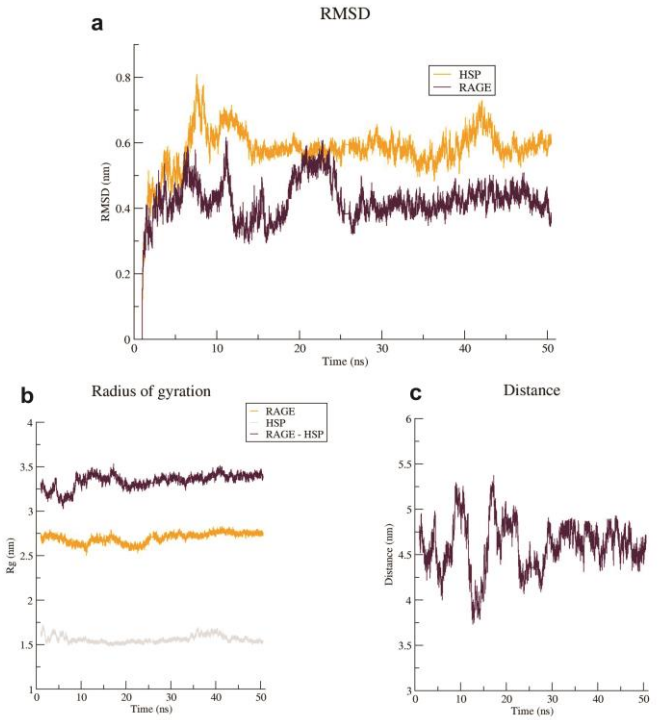

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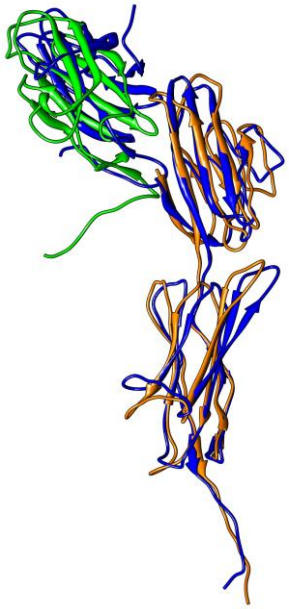

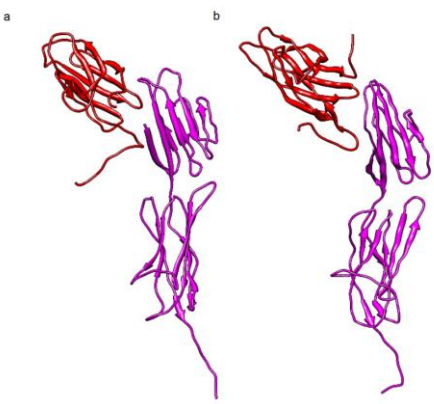

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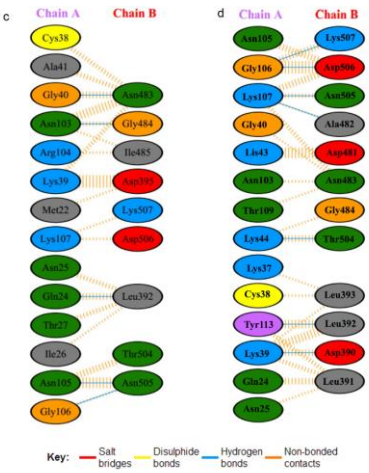

Fig. 6

4. Discussão

Muitos anos se passaram desde que a HSP70 foi descoberta; os estudos atuais nos mostram que suas funções e seus papéis são muito maiores do que a atividade de chaperona primeiramente descrita. Recentemente, essas proteínas têm sido observadas presentes no soro após diversas formas de estresse (exercício físico intenso, processos inflamatórios, choque séptico e trauma); em um primeiro momento, pensou-se que sua presença era causada pela disrupção necrótica da membrana celular de células em processo de necrose com consequente extravasamento do conteúdo proteico citosólico, incluindo a HSP70. Logo após, foi demonstrado que a HSP70 poderia ser ativamente secretada de um ambiente intracelular para um ambiente extracelular, levando à hipótese de que essa proteína poderia ter um papel muito mais complexo e importante na sinalização celular durante processos patogênicos.

No **capítulo 1** nós demonstramos a relação entre a presença da HSP70 no soro de pacientes com sepse, os níveis séricos de estresse oxidativo e o desfecho clínico. Os mecanismos moleculares que estão por trás da sepse são complexos e relacionam-se com diferentes níveis de coordenação metabólica; por isso, faz-se importante estudar e caracterizar as perturbações celulares e moleculares decorrentes da sepse. Fato este, que é evidenciado pelas diferentes conclusões dos estudos bioquímicos realizados acerca deste assunto quando tentam atribuir funções e elucidar mecanismos baseados em aspectos isolados. Temos como exemplo, diversos estudos animais e ensaios clínicos que associam a sepse com o desbalanço redox e estresse oxidativo; enquanto que outros estudos falham ao estabelecer uma correlação entre

terapias baseadas em antioxidantes e a melhora na condição séptica (Andrades *et al.* 2009, Berger & Chioléro 2007, Rinaldi *et al.* 2009). Esse fato é também verdadeiro em relação aos estudos sobre as funções HSP70 na sepse. Modelos animais e celulares corroboram com a existência de uma relação entre HSP70 e sepse, porém, observações clínicas não obtiveram êxito em estabelecer uma correlação direta entre a modulação da HSP70 e um efeitor protetor contra o choque séptico (Bruemmer-Smith *et al.* 2001).

Existe um empenho consensualizado na literatura em estabelecer uma correlação entre o aumento na expressão da HSP70 e proteção contra choque séptico. Até pouco tempo, a maioria das pesquisas tinham como foco demonstrar a modulação da HSP70 intracelular e estabelecer relações com processos patológicos, porém, os resultados apresentados foram controversos; além disso, pouquíssimos estudos avaliam o papel da HSP70 extracelular. Em crianças com choque séptico, um aumento nos níveis séricos de HSP70 foi observado (Wheeler *et al.* 2005). Além disso, a administração de HSP70 exógena em ratos, levou a uma proteção aumentada contra o choque endotóxico (Rozhkova *et al.* 2010).

Com base no fato de que a sinalização redox exerce uma importanta influência na regulação da resposta do sistema imunológico contra a sepse, nós demonstramos que os níveis séricos de HSP70 são modulados de acordo com o status oxidativo do soro, o qual varia de acordo com o paciente. Assim, é possível que a amplitude do estresse oxidativo possa contribuir com a modulação dos níveis séricos de HSP70, através de um aumento na expressão e da liberação da HSP70 para o meio extracelular e da liberação por células necróticas. Uma vez no espaço extracelular, a HSP70 funciona como uma das

proteína enquadradas na teoria “danger signal”, realizando uma sinalização imunoestimulatória, ativando receptores TLR2 e TLR4, aumentando a liberação de TNF-α e citocinas pró-inflamatórias, espécies reativas de oxigênio e óxido nítrico, principalmente de macrófagos.

Concluindo, nós demonstramos no **capítulo 1** que os níveis séricos de HSP70 são modulados de acordo com o estado oxidativo durante a sepse, e, que os níveis elevados de HSP70 no soro estão associados à mortalidade dos pacientes.

Tomando como base os dados resultantes do estudo clínico realizado no **capítulo 1**; nós procuramos no **capítulo 2** estabelecer e elucidar os mecanismos por trás da relação existente entre o estado redox, HSP70 e os efeitos decorrentes. Para isso, nós submetemos a HSP70 purificada a um agente oxidante e avaliamos as mudanças estruturais na proteína e os efeitos da oxHP70 na viabilidade celular e nas propriedades imunomodulatórias.

Quando submetida a um agente oxidante (peróxido de hidrogênio), a HSP70 apresentou algumas alterações no perfil eletroforético com tampão de amostra redutor, a banda majoritária da proteína de 70 kDa e a banda de 140 kDa (peso molecular do provável dímero) diminuíram em intensidade conforme o gradiente do agente oxidativo aumentou, sugerindo que o ambiente oxidativo está danificando a proteína e, possivelmente, alterando em algum nível sua estrutura. Em condições não-redutoras, a eletroforese da HSP70 e da oxHSP70 evidenciou uma banda de aproximadamente 90 kDa, que não é aparente no gel sob condições redutoras.Apesar de não podermos estabelecer exatamente qual o processo causou o surgimento dessa banda, é bastante

provável que seja um mecanismo redox dependente, tal como a formação ou redução de ligações dissulfeto, visto que essa banda também diminui em intensidade quando a HSP70 é submetida a concentrações maiores de H_2O_2. Assim, essa banda pode corresponder a uma forma intermediária, redox-modificada da HSP70.

A formação de ligações dissulfeto intramoleculares e intermoleculares sob condições redox é uma das modificações mais marcantes das proteínas que contêm resíduos de cisteína. Para avaliar o papel das ligações dissulfeto e/ou das interações entres os resíduos de cisteína, nós bloqueamos os possíveis sítios de formação de ligações dissulfeto com o pré-tratamento da HSP70 com o agente alquilante iodoacetamida; assim, quando a proteína for incubada com o agente oxidante, as ligações dissulfeto seriam impedidas de acontecer. O perfil eletroforético resultante nessas condições mostrou uma leve divisão da banda majoritária de 70 kDa, sugerindo que esta banda era formada por bandas de peso molecular muito próximo, e, que este processo pode estar relacionado à correta interação entre os resíduos de cisteína.

É válido mencionar que realizamos diversas tentativas de quantificar e qualificar as modificações estruturais ocasionadas pelo tratamento da HSP70 com um agente oxidativo através de espectrometria de massas, tanto por MALDI-TOFF quanto por Q-TOF. Nossos esforços não foram exitosos, provavelmente devido a um procedimento inadequado no que diz respeito ao preparo das amostras ou a uma inadequada abordagem de avaliação dos resultados obtidos.

Se a HSP70 extracelular está envolvida com processos inflamatórios, e estes são associados com estresse oxidativo e desbalanço redox, nós nos perguntamos quais seriam os efeitos *in vitro* da oxidação da HSP70 sobre a modulação da ativação de células do sistema imunológico? Para responder essa questão, macrófagos da linhagem RAW 264.7 foram tratados por 30, 60, 120 minutos, e 24 horas com a HSP70 pré-tratada ou não com agente oxidante e avaliamos a produção de nitrito e proliferação e viabilidade celular. Em relação à produção de nitrito, não foram observadas diferenças significativas; porém, a proliferação celular quantificada pela técnica de SRB mostrou que os macrófagos tratados com oxHSP70 apresentam menor proliferação do que os não tratados e do que os tratados com HSP70 após 24 horas de tratamento.

Outro fato interessante também foi observado: a atividade fagocítica dos macrófagos estava diminuída nas células tratadas com oxHSP70 quando comparadas com as não tratadas e com o controle, sugerindo que a oxHSP70 não ativou os macrófagos RAW 264.7 com a mesma eficácia que a HSP70.

Sabe-se que a HSP70 têm a habilidade de induzir a ativação de macrófagos; um dos parâmetros observados quando este evento ocorre é a produção de citocinas inflamatórias, tais como IL8, IL10 e TNF-α. Macrófagos RAW 264.7 foram tratados com HSP70 ou oxHSP70 por 24 horas e o conteúdo de TNF-α no meio de incubação foi analisado. Os grupos tratados com oxHSP70 apresentaram níveis de TNF-α semelhantes aos do grupo controle; além disso, a liberação de TNF-α pelas células tratadas foi maior quando comparada a tanto com os grupos tratados com oxHSP70 quanto com o grupo controle, sugerindo que a oxHSP70 não foi capaz de ativar os macrófagos RAW 264.7 pela via clássica.

Sabe-se que a citocina TNF-α têm um importante papel na proliferação, apoptose e diferenciação de macrófagos. A sobrevivência a longo prazo dos macrófagos é dependente da sinalização autócrina por TNF-α (Lombardo *et al.* 2007). Devido ao fato de a TNF-α mediar muitos dos efeitos patológicos da interação LPS-TLR4 em condições patológicas, como na sepse por exemplo, é sugerido que a sobrevivência prolongada dos macrófagos mediada por TNF-α têm um importante papel no curso da sepse (Conte *et al.* 2006); assim, esse fato pode ser relacionado com nossos resultados que mostram que após 24 horas de tratamento com oxHSP70 os macrófagos da linhagem RAW 264.7 produziram menores níveis de TNF-α e também tiveram menor viabilidade e proliferação celular.

De um modo geral, a HSP70 pode ser liberada no meio extracelular com um "sinal de perigo" (teoria "danger signal") para o sistema, pois ela pode ser reconhecida pelos receptores TLR2 e TLR4 e desencadear uma rota de sinalização pró-inflamatória. Nesse contexto, nós podemos inferir que a oxidação e subsequente modificação com prejuízo da estrutura proteica da HSP70 pode ser relacionado à perda desse "sinal de perigo" quando ele se faz necessário. Além disso, a oxHSP70 poderia exercer seus efeitos pela ativação de outros receptores além dos TLR2, TLR4 e CD40, como o receptor dos produtos finais de glicação avançada (RAGE), um receptor de padrões capaz de ligar a um vasto repertório de proteínas solúveis e também associadas à membrana envolvidas na resposta do hospedeiro à injúria tecidual, infecção e inflamação. Em um estudo recente (Ruan *et al.* 2010), foi demonstrado através de um ELISA screening que a HSP70 é um dos novos possíveis ligantes de RAGE. A ligação de RAGE induz a ativação de múltiplas rotas de sinalização

celular que podem variar de acordo com o agonista, tipo celular e microambiente tecidual, e assim é o mediador de diversas respostas celulares, tais como a observada após o tratamento com oxHSP70 nos macrófagos.

Ainda assim, é importante salientar que os efeitos imunológicos das HSPs podem variar dependendo de diversos fatores, incluindo a família da proteína de choque térmico (HSP60, HSP70, HSP90), a fonte celular de HSP (normal, cancerosa, infecção-viral ou bacteriana), a localização celular (intracelular, extracelular ou associada à membrana) e ainda as circunstâncias fisiológicas modulando a expressão de HSP (estresse oxidativo, infecção bacteriana, infecção viral, estresse físico, etc.). Assim, o contexto fisiológico específico no qual essas proteínas estão inseridas tem um grande impacto na sua função, impedindo qualquer afirmação global sobre as funções imunológicas in vivo da HSP70 (Johnson & Fleshner 2006).

Levando em consideração os resultados apresentados no **capítulo 1 e no capítulo 2**, é sugerido que em uma situação pró-oxidante, comum a todos os processos patológicos, a HSP70 pode sofrer modificações estruturais as quais podem levar à perda da funcionalidade da proteína, como a observada nos pacientes sépticos. No **capítulo 2** foi demonstrado que a HSP70 oxidada induz um decréscimo na viabilidade e na proliferação de macrófagos RAW 264.7 após 24 horas de tratamento e um decréscimo na produção de TNF-α. Assim, acredita-se que essa relação possa estar correlacionada com o desfecho clínico observado anteriormente. Além destes efeitos, os macrófagos tratados com oxHSP70 apresentaram outras características que podem ser resumidas, de uma forma geral, na tabela abaixo (**Tabela 1**).

	HSP70	oxHSP70
ROS	Níveis semelhantes ao controle	Níveis elevados
NO	Níveis semelhantes ao controle	Níveis semelhantes ao controle
FAGOCITOSE	Níveis elevados	Níveis diminuídos
VIABILIDADE CELULAR	Níveis semelhantes ao controle	Níveis diminuídos
PROLIFERAÇÃO CELULAR	Níveis semelhantes ao controle	Níveis diminuídos
LIBERAÇÃO DE TNF-A	Níveis elevados	Níveis diminuídos

Tabela 1. Resumo comparativo dos efeitos modulatórios sobre a ativação de macrófagos tratados com HSP70 e oxHSP70.

Apesar de os efeitos celulares da HSP70 extracelular serem descritos através da interação com TLR-2, TLR-4 e CD40 em macrófagos, os efeitos exercidos e observados pela oxHSP70 não podem ser explicados, em sua totalidade, através dessa interação, como exposto por Asea et al, 2002, trabalho no qual o autor aponta que a sinalização desencadeada pela interação da HSP70 com um receptor de membrana ainda não está completamente elucidada, e, nem todos os receptores partipantes dessa interação estão descritos. Em 2010, Ruan et al, publicou um trabalho no qual é demonstrada a interação por afinidade de ligação entre HSP70 e RAGE, através de um ensaio de *screening* para novos ligantes através de *ELISA*. Como podemos analisar na **figura 3,** quando comparadas lado a lado, as rotas de sinalização celular ativadas pela ligação de um agonista ao RAGE e ao TLR são compartilhadas em diversos pontos, sendo bastante difícil a caracterização de qual rota está ativa somente pela identificação de seus componentes *downstream.*

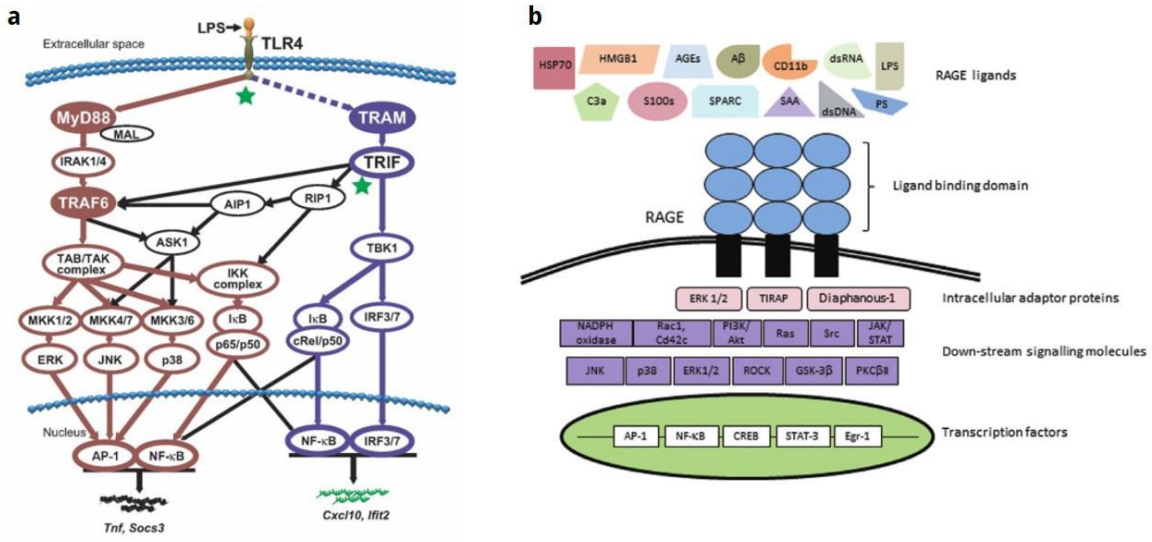

Figura 3

Comparação das vias de sinalização celular ativadas por TLR4 (a) e por RAGE (b). Figuras retiradas de (Selvarajoo *et al.* 2008) e de (Ruan *et al.* 2010), respectivamente.

Considerando as novas informações acerca da interação HSP70 e RAGE, e os dados de ativação de macrófagos observados no **capítulo 2**, nós estabelecemos no **capítulo 3** o objetivo de identificar essa interação através de docking molecular refinado por dinâmica molecular, tentando caracterizá-la, fortalecendo e corroborando com a idéia.

Quando tratados com diferentes agonistas de RAGE, macrófagos RAW 264.7 apresentam a mesma resposta de sinalização celular ativada pelo tratamento com HSP70. Além disso, quando submetidos a docking molecular, o domínio ligante de substrato da HSP70 e o receptor dos produtos finais de glicação avançada apresentam resultados plausíveis; resultados estes, que quando refinados através de cálculos de dinâmica molecular fortalecem ainda mais a

possibilidade da interação ocorrer conforme proposto. Destes resultados podemos ressaltar a estabilidade do sistema no decorrer da trajetória de simulação e a distância entre os dois componentes. Analisando a interface de interação do primeiro frame da trajetória da dinâmica molecular gerada pelo servidor PDBsum pode-se perceber que a área de contato para o RAGE é de 578 $Å^2$ com 14 resíduos interagindo, e para o SBD da HSP70 é de 602 $Å^2$ com 9 resíduos interagindo. As interações entre estes resíduos são compostas principalmente por ligações do tipo *non-bonded contacts* (43) com algumas ligações de hidrogênio (5). Já a interface de interação do ultimo frame da trajetória da dinâmica molecular mostra que a superfície de interação para o Rage é de 693 $Å^2$ com 14 resíduos interagindo, e para o SBD da HSP70 é de 699 $Å^2$ com 12 resíduos. As interações entre estes resíduoes são principalmente do tipo *non-bonded contacts* (54) com algumas ligações de hidrogênio (7).

5. Conclusões

Os resultados dispostos nessa dissertação foram publicados em revistas científicas internacionais (**capítulo 1**) ou encontram-se submetidos e em processo de revisão em revistas científicas internacionais (**capítulos 2 e 3)**.

Podemos sumarizar o corpo de resultados encontrados em:

i) Foi possível estabelecer uma correlação entre a quantificação da HSP70 extracelular no soro de pacientes sépticos, os níveis de estresse oxidativo e o desfecho clínico;

ii) Foram caracterizadas algumas das possíveis modificações estruturais decorrentes da oxidação da HSP70 em ambiente extracelular;

iii) Foram observados diferentes efeitos modulatórios da HSP70 extracelular oxidada sobre a ativação de macrófagos RAW 264.7;

iv) Com a utilização de ferramentas de docking molecular, refinado por dinâmica molecular, foi possível caracterizar um teórica interação HSP70 e RAGE

Sendo assim estes achados representam grande passo no processo de elucidação dos mecanismos agravantes da sepse, da relação da HSP70 extracelular com processos inflamatórios e com as células do sistema imunológico, e também da novíssima interação entre HSP70 e RAGE, contribuindo para a caracterização desse novo mecanismo de transdução de sinalização celular.

6. Referências Bibliográficas

AIRD, W.C. 2003. The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. Blood 101:3765-77.

ALBERTI, C., BRUN-BUISSON, C., BURCHARDI, H., MARTIN, C., GOODMAN, S., ARTIGAS, A., SICIGNANO, A., PALAZZO, M., MORENO, R., BOULMÉ, R., LEPAGE, E. & LE GALL, R. 2002. Epidemiology of sepsis and infection in ICU patients from an international multicentre cohort study. Intensive care medicine 28:108-21.

ANDRADES, M., RITTER, C., MOREIRA, J.C.F. & DAL-PIZZOL, F. 2005. Oxidative parameters differences during non-lethal and lethal sepsis development. The Journal of surgical research 125:68-72.

ANDRADES, M.E., RITTER, C. & DAL-PIZZOL, F. 2009. The role of free radicals in sepsis development. Front Biosci (Elite Ed) 1:277-287.

ANGUS, D.C. & WAX, R.S. 2001. Epidemiology of sepsis: an update. Critical care medicine 29:S109-16.

ANNANE, D., BELLISSANT, E. & CAVAILLON, J.-M. 2005. Septic shock. Lancet 365:63-78.

ARNOLD-SCHILD, D., HANAU, D., SPEHNER, D., SCHMID, C., RAMMENSEE, H.G., DE LA SALLE, H. & SCHILD, H. 1999. Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. Journal of immunology (Baltimore, Md. : 1950) 162:3757-60.

ASEA, A. 2003. Chaperokine-induced signal transduction pathways. Exerc Immunol Rev 9:25-33.

ASEA, A. 2007. Hsp72 release: mechanisms and methodologies. Methods 43:194-198.

ASEA, A. 2008. Hsp70: a chaperokine. Novartis Found Symp 291:173-183,221-224.

ASEA, A., KABINGU, E., STEVENSON, M.A. & CALDERWOOD, S.K. 2000. HSP70 peptidembearing and peptide-negative preparations act as chaperokines. Cell Stress Chaperones 5:425-431.

ASEA, A., KRAEFT, S.K., KURT-JONES, E.A., STEVENSON, M.A., CHEN, L.B., FINBERG, R.W., KOO, G.C. & CALDERWOOD, S.K. 2000. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nature medicine 6:435-42.

ASEA, A., REHLI, M., KABINGU, E., BOCH, J.A., BARE, O., AURON, P.E., STEVENSON, M.A. & CALDERWOOD, S.K. 2002. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem 277:15028-15034.

BECKER, T., HARTL, F.-U. & WIELAND, F. 2002. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. The Journal of cell biology 158:1277-85.

BERGER, M.M. & CHIOLÉRO, R.L. 2007. Antioxidant supplementation in sepsis and systemic inflammatory response syndrome. Critical care medicine 35:S584-90.

BEUTLER, B. 2004. Inferences, questions and possibilities in Toll-like receptor signalling. Nature 430:257-63.

BRUEMMER-SMITH, S., STÜBER, F. & SCHROEDER, S. 2001. Protective functions of intracellular heat-shock protein (HSP) 70-expression in patients with severe sepsis. Intensive care medicine 27:1835-41.

CALLAHAN, M.K., CHAILLOT, D., JACQUIN, C., CLARK, P.R. & MÉNORET, A. 2002. Differential acquisition of antigenic peptides by Hsp70 and Hsc70 under oxidative conditions. The Journal of biological chemistry 277:33604-9.

CAMPISI, J., LEEM, T.H. & FLESHNER, M. 2003. Stress-induced extracellular Hsp72 is a functionally significant danger signal to the immune system. Cell stress & chaperones 8:272-86.

CHEN, T. & CAO, X. 2010. Stress for maintaining memory: HSP70 as a mobile messenger for innate and adaptive immunity. Eur J Immunol 40:1541-1544.

CONTE, D., HOLCIK, M., LEFEBVRE, C.A., LACASSE, E., PICKETTS, D.J., WRIGHT, K.E. & KORNELUK, R.G. 2006. Inhibitor of apoptosis protein cIAP2 is essential for lipopolysaccharide-induced macrophage survival. Mol Cell Biol 26:699-708.

DELNESTE, Y., MAGISTRELLI, G., GAUCHAT, J., HAEUW, J., AUBRY, J., NAKAMURA, K., KAWAKAMI-HONDA, N., GOETSCH, L., SAWAMURA, T., BONNEFOY, J. & JEANNIN, P. 2002. Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. Immunity 17:353-62.

FEBBRAIO, M.A., OTT, P., NIELSEN, H.B., STEENSBERG, A., KELLER, C., KRUSTRUP, P., SECHER, N.H. & PEDERSEN, B.K. 2002. Exercise induces hepatosplanchnic release of heat shock protein 72 in humans. J Physiol 544:957-962.

FEBBRAIO, M.A., STEENSBERG, A., WALSH, R., KOUKOULAS, I., VAN HALL, G., SALTIN, B. & PEDERSEN, B.K. 2002. Reduced glycogen availability is associated with an elevation in HSP72 in contracting human skeletal muscle. J Physiol 538:911-917.

FEHRENBACH, E., NIESS, A.M., VOELKER, K., NORTHOFF, H. & MOOREN, F.C. 2005. Exercise intensity and duration affect blood soluble HSP72. Int J Sports Med 26:552-557.

FRIEDMAN, G., SILVA, E. & VINCENT, J.L. 1998. Has the mortality of septic shock changed with time. Critical care medicine 26:2078-86.

GELAIN, D.P., DE BITTENCOURT PASQUALI, M.A., C, M.C., GRUNWALD, M.S., RITTER, C., TOMASI, C.D., ALVES, S.C., QUEVEDO, J., DAL-PIZZOL, F. & MOREIRA, J.C. 2011. Serum heat shock protein 70 levels, oxidant status, and mortality in sepsis. Shock 35:466-470.

GROSS, C., HANSCH, D., GASTPAR, R. & MULTHOFF, G. 2003. Interaction of heat shock protein 70 peptide with NK cells involves the NK receptor CD94. Biological chemistry 384:267-79.

HAENEY, M.R. 1998. The role of the complement cascade in sepsis. The Journal of antimicrobial chemotherapy 41 Suppl A:41-6.

HE, H., CHEN, C., XIE, Y., ASEA, A. & CALDERWOOD, S.K. 2000. HSP70 and heat shock factor 1 cooperate to repress Ras-induced transcriptional activation of the c-fos gene. Cell Stress Chaperones 5:406-411.

HIRVONEN, M.R., BRÜNE, B. & LAPETINA, E.G. 1996. Heat shock proteins and macrophage resistance to the toxic effects of nitric oxide. The Biochemical journal 315 (Pt 3:845-9.

JOHNSON, J.D. & FLESHNER, M. 2006. Releasing signals , secretory pathways , and immune function of endogenous extracellular heat shock protein 72. 79:425-434.

KINDAS-MUGGE, I., HAMMERLE, A.H., FROHLICH, I., OISMULLER, C., MICKSCHE, M. & TRAUTINGER, F. 1993. Granulocytes of critically ill patients spontaneously express the 72 kD heat shock protein. Circ Shock 39:247-252.

KINDAS-MUGGE, I., POHL, W.R., ZAVADOVA, E., KOHN, H.D., FITZAL, S., KUMMER, F. & MICKSCHE, M. 1996. Alveolar macrophages of patients with adult respiratory distress syndrome express high levels of heat shock protein 72 mRNA. Shock 5:184-189.

LANCASTER, G.I., MOLLER, K., NIELSEN, B., SECHER, N.H., FEBBRAIO, M.A. & NYBO, L. 2004. Exercise induces the release of heat shock protein 72 from the human brain in vivo. Cell Stress Chaperones 9:276-280.

LOMBARDO, E., ALVAREZ-BARRIENTOS, A., MAROTO, B., BOSCA, L. & KNAUS, U.G. 2007. TLR4-mediated survival of macrophages is MyD88 dependent and requires TNF-alpha autocrine signalling. J Immunol 178:3731-3739.

MARTIN, G.S., MANNINO, D.M., EATON, S. & MOSS, M. 2003. The epidemiology of sepsis in the United States from 1979 through 2000. The New England journal of medicine 348:1546-54.

MATZINGER, P. 2002. The danger model: a renewed sense of self. Science 296:301-305.

MEDZHITOV, R. & HORNG, T. 2009. Transcriptional control of the inflammatory response. Nature reviews. Immunology 9:692-703.

MILLAR, D.G., GARZA, K.M., ODERMATT, B., ELFORD, A.R., ONO, N., LI, Z. & OHASHI, P.S. 2003. Hsp70 promotes antigen-presenting cell function and converts T-cell tolerance to autoimmunity in vivo. Nat Med 9:1469-1476.

MORIMOTO, R.I., KLINE, M.P., BIMSTON, D.N. & COTTO, J.J. 1997. The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. Essays in biochemistry 32:17-29.

MORRELL, M.R., MICEK, S.T. & KOLLEF, M.H. 2009. The management of severe sepsis and septic shock. Infectious disease clinics of North America 23:485-501.

MULTHOFF, G. 2007. Heat shock protein 70 (Hsp70): membrane location, export and immunological relevance. Methods 43:229-237.

NEEPER, M., SCHMIDT, A.M., BRETT, J., YAN, S.D., WANG, F., PAN, Y.C., ELLISTON, K., STERN, D. & SHAW, A. 1992. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. The Journal of biological chemistry 267:14998-5004.

ORTEGA, E., GIRALDO, E., HINCHADO, M.D., MARTINEZ, M., IBANEZ, S., CIDONCHA, A., COLLAZOS, M.E. & GARCIA, J.J. 2006. Role of Hsp72 and norepinephrine in the moderate exercise-induced stimulation of neutrophils' microbicide capacity. Eur J Appl Physiol 98:250-255.

ORTEGA, E., HINCHADO, M.D., MARTIN-CORDERO, L. & ASEA, A. 2009. The effect of stress-inducible extracellular Hsp72 on human neutrophil chemotaxis: a role during acute intense exercise. Stress 12:240-249.

PECK, G., ANDRADES, M., LORENZI, R., DA COSTA, M., PETRONILHO, F., MOREIRA, J.C.F., DAL-PIZZOL, F. & RITTER, C. 2009. Serum-induced macrophage activation is related to the severity of septic shock. Inflammation research : official journal of the European Histamine Research Society ... [et al.] 58:89-93.

RIBEIRO, S.P., VILLAR, J., DOWNEY, G.P., EDELSON, J.D. & SLUTSKY, A.S. 1994. Sodium arsenite induces heat shock protein-72 kilodalton expression in the lungs and protects rats against sepsis. Crit Care Med 22:922-929.

RINALDI, S., LANDUCCI, F. & DE GAUDIO, A.R. 2009. Antioxidant therapy in critically septic patients. Current drug targets 10:872-80.

RITOSSA, F. 1962. A new puffing pattern induced by temperature shock and DNP in drosophila. Cellular and Molecular Life Sciences 18.

ROZHKOVA, E., YURINSKAYA, M., ZATSEPINA, O., GARBUZ, D., KARPOV, V., SURKOV, S., MURASHEV, A., OSTROV, V., MARGULIS, B., EVGEN'EV, M. & VINOKUROV, M. 2010. Exogenous mammalian extracellular HSP70 reduces endotoxin manifestations at the cellular and organism levels. Annals of the New York Academy of Sciences 1197:94-107.

RUAN, B.H., LI, X., WINKLER, A.R., CUNNINGHAM, K.M., KUAI, J., GRECO, R.M., NOCKA, K.H., FITZ, L.J., WRIGHT, J.F., PITTMAN, D.D., TAN, X.Y., PAULSEN, J.E., LIN, L.L. & WINKLER, D.G. 2010. Complement C3a, CpG oligos, and DNA/C3a complex stimulate IFN-alpha production in a receptor for advanced glycation end product-dependent manner. J Immunol 185:4213-4222.

RYAN, A.J., FLANAGAN, S.W., MOSELEY, P.L. & GISOLFI, C. V. 1992. Acute heat stress protects rats against endotoxin shock. Journal of applied physiology (Bethesda, Md. : 1985) 73:1517-22.

SELVARAJOO, K., TAKADA, Y., GOHDA, J., HELMY, M., AKIRA, S., TOMITA, M., TSUCHIYA, M., INOUE, J.-I. & MATSUO, K. 2008. Signaling flux redistribution at toll-like receptor pathway junctions. PloS one 3:e3430.

SIQUEIRA-BATISTA, R., GOMES, A.P., CALIXTO-LIMA, L., VITORINO, R.R., PEREZ, M.C.A., MENDONÇA, E.G. DE, OLIVEIRA, M.G. DE A. & GELLER, M. 2011. Sepse: atualidades e perspectivas. Revista Brasileira de Terapia Intensiva 23:207-216.

SRIVASTAVA, P. 2002. Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. Annu Rev Immunol 20:395-425.

TANG, D., KANG, R., XIAO, W., WANG, H., CALDERWOOD, S.K. & XIAO, X. 2007. The anti-inflammatory effects of heat shock protein 72 involve inhibition of high-mobility-group box 1 release and proinflammatory function in macrophages. J Immunol 179:1236-1244.

TSAN, M.-F. & GAO, B. 2009. Heat shock proteins and immune system. Journal of leukocyte biology 85:905-10.

VEGA, V.L., RODRIGUEZ-SILVA, M., FREY, T., GEHRMANN, M., DIAZ, J.C., STEINEM, C., MULTHOFF, G., ARISPE, N. & DE MAIO, A. 2008. Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages. J Immunol 180:4299-4307.

VILLAR, J., EDELSON, J.D., POST, M., MULLEN, J.B. & SLUTSKY, A.S. 1993. Induction of heat stress proteins is associated with decreased mortality in an animal model of acute lung injury. The American review of respiratory disease 147:177-81.

WALSH, R.C., KOUKOULAS, I., GARNHAM, A., MOSELEY, P.L., HARGREAVES, M. & FEBBRAIO, M.A. 2001. Exercise increases serum Hsp72 in humans. Cell Stress Chaperones 6:386-393.

WHEELER, D.S., FISHER, L.E., CATRAVAS, J.D., JACOBS, B.R., CARCILLO, J.A. & WONG, H.R. 2005. Extracellular hsp70 levels in children with septic shock. Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies 6:308-11.

WONG, H.R., MENENDEZ, I.Y., RYAN, M.A., DENENBERG, A.G. & WISPÉ, J.R. 1998. Increased expression of heat shock protein-70 protects A549 cells against hyperoxia. The American journal of physiology 275:L836-41.

WONG, H.R., RYAN, M., MENENDEZ, I.Y., DENENBERG, A. & WISPÉ, J.R. 1997. Heat shock protein induction protects human respiratory epithelium against nitric oxide-mediated cytotoxicity. Shock (Augusta, Ga.) 8:213-8.

YANG, Y.L. & LIN, M.T. 1999. Heat shock protein expression protects against cerebral ischemia and monoamine overload in rat heatstroke. The American journal of physiology 276:H1961-7.

7. Lista de Figuras e Tabelas