UNIVERSIDADE ESTADUAL DE CAMPINAS
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KELLY LIMA CALISTO DA SILVA

EFEITOS DA ATORVASTATINA E DA DIACEREÍNA SOBRE A SOBREVIVÊNCIA E A RESISTÊNCIA À INSULINA EM RATOS SÉPTICOS

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"EFEITOS DA ATORVASTATINA E DA DIACEREÍNA SOBRE A SOBREVIVÊNCIA E A RESISTÊNCIA À INSULINA EM RATOS SÉPTICOS"

Kelly Lima Calisto da Silva

Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas, para obtenção do título de Doutor em Clínica Médica, Área de Concentração Ciências Básicas. Sob orientação do Prof. Dr. Mario José Abdalla Saad.

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Akt	Proteína quinase B
AP-1	Ativador da proteína 1
ATF6	Fator 6 ativador de transcrição
BASES	Estudo brasileiro epidemiológico de sepse
c-Jun	Substrato da quinase JNK
CLP	Ligadura e punção cecal *(cecal ligation and puncture)*
DATASUS	Banco de dados do Sistema Único de Saúde
DM-2	Diabetes mellitus tipo 2
eIF2α	Fator de iniciação da tradução eucariótico 2 alfa
ELISA	Ensaio imunoenzimático (*Enzime-Linked Immunosorbernt Assay)*
GLUT4	Transportador de glicose 4
IKK	Complexo quinase do inibidor do NF-κB (IkappaB kinase)
IL-1β	Interleucina 1 beta
IL-6	Interleucina 6
iNOS	Óxido nítrico sintase induzido
IR	Receptor da insulina
IRE	Enzima ativada por inositol
IRS1/2	Substrato do receptor de insulina 1/2
ITT	Teste de tolerância à insulina (*Insulin Tolerance Test)*
JNK	Proteína quinase da c-Jun ou c-Jun NH2-terminal quinase
KITT	Constante de decaimento de glicose
LPS	Lipopolissacarídeo *(lipopolysaccharide)*
NFκB	Fator de transcrição nuclear (*nuclear factor kappa B)*
NO	Óxido nítrico

PERK	Quinase do retículo endoplasmático semelhante à proteína quinase do pâncreas.
PI3K	Fosfatidilinositol 3-quinase
PPAR-γ	Receptor gama ativado por proliferador de peroxissomo
RE	Retículo endoplasmático
RI	Resistência à insulina
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
SH2	Domínios protéicos com homologia a Src2
SUS	Sistema Único de Saúde
TNF-α	Fator de necrose tumoral alfa
TLR4	Receptor toll like 4 (*Toll like receptor 4)*
UPR	Resposta à proteína não enovelada

LISTA DE ILUSTRAÇÕES

RESUMO

Na sepse o sistema imune torna-se hiperativo, levando a excessiva produção de mediadores pró-inflamatórios. Tanto componentes bacterianos, como o LPS, quanto citocinas pro-inflamatórias resultantes da resposta imune, podem ativar mecanismos intracelulares associados à resistência à insulina, como a via IKKβ/NFκB e a via da JNK. Hiperglicemia e resistência à insulina ocorrem durante a sepse, como consequência dos efeitos metabólicos da excessiva produção de mediadores pró-inflamatórios. Sabe-se que a resistência à insulina pode agravar ainda mais o quadro da sepse, todavia a manutenção da normoglicemia com a insulinoterapia reduz os índices de morbidade e mortalidade. Por esta razão, o objetivo do presente estudo foi investigar o efeito de fármacos (atorvastatina e diacereína) sobre a sobrevivência, sinalização inflamatória e, paralelamente, a sua ação sobre a via de sinalização da insulina em ratos com sepse induzida por peritonite. Nossos dados demonstram que os tratamentos com atorvastatina e diacereína aumentam a sobrevida, com um efeito benéfico sobre a sensibilidade à insulina, melhorando a sinalização da insulina dos animais sépticos. Ademais, os tratamentos reduziram a ativação de JNK e IKK e consequente expressão de citocinas pró-inflamatórias, e em paralelo reduziram estresse do retículo endoplasmático induzido pela sepse. Com isso, podemos sugerir que a restauração da sinalização da insulina demonstrada pela reativação da via PI3K/Akt induzida pelos tratamentos, desempenhou um papel fundamental no aumento da sobrevida na sepse. Neste contexto, acreditamos que a melhora na via de sinalização da insulina, induzida pelos tratamentos com atorvastatina e diacereína, em paralelo com uma atenuação da inflamação nos tecidos, pode ajudar a predizer a eficácia destes tratamentos na sepse.

ABSTRACT

During the onset of sepsis, the inflammatory system becomes hyperactive, and the persistent activating stimuli induce cells to produce excessive amounts of cytokines and mediators that lead to tissue damage. Both bacterial components and proinflammatory cytokines can directly activate pro-inflammatory pathways as IKKβ/NFκB and JNK that seem to be associated to disruption on insulin signaling. Hyperglycemia and insulin resistance occur during sepsis, as a consequence of the metabolic effects of stress hormone and cytokine production. Studies indicate that insulin resistance may aggravate sepsis. Furthermore, it was demonstrated that the maintenance of normoglycemia with insulin therapy reduces morbidity and mortality rates in sepsis. The aim of the present study was to investigate the effect of drugs (atorvastatin and diacerhein) on survival, inflammatory signaling and insulin signaling pathway in rat model of CLP-induced sepsis. Our data demonstrate that atorvastatin and diacerhein treatment improves survival in septic rats and this improvement is accompanied by a marked improvement in insulin sensitivity. Sepsis induced an increase in JNK and IKK/NF-κB activation, and blunted insulin-induced insulin signaling in liver, muscle and adipose tissue; atorvastatin and diacerhein reversed all these alterations in parallel with a decrease sepsis-induced endoplasmic stress reticulum and circulating levels proinflammatory cytokines. The improvement insulin signaling pathway through PI3K/Akt induced by treatments with atorvastatin and diacerhein, in parallel with a decrease in tissue inflammation, may play a central role in regulation of insulin signaling and survival in sepsis insult.

INTRODUÇÃO

1.1 Sepse

Sepse é o flagelo dos tempos modernos, uma das principais causas de morte no mundo (Linde-Zwirble e Angus, 2004).

Apesar de o termo sepse estar ligado à terapia intensiva moderna, o conceito médico é bem mais antigo. A palavra *sepsis* foi introduzida pela primeira vez por Hipócrates (cerca de 460-370 a.C.) e deriva da palavra grega *sipsi* que significa "putrefação" (Majno, 1991). Em meados do século 19, o obstetra húngaro Ignaz Semmelweis desenvolveu a primeira visão moderna da sepse, ao constatar que a incidência de febre puerperal poderia ser reduzida pela desinfecção das mãos. (Lane, Blum *et al.*, 2010). Atualmente, a sepse é definida como a síndrome de resposta inflamatória sistêmica decorrente de processo infeccioso.

A sepse é vista como o maior desafio clínico atual devido a sua alta prevalência e mortalidade (Wheeler e Bernard, 1999), registrando-se, todos os dias, mais de 1.400 óbitos por sepse no mundo (Levy, Dellinger *et al.*, 2010). Além de ser a principal causa de óbitos em UTIs (Vincent, Sakr *et al.*, 2006), a sepse é também a principal causa de morte, depois das doenças cardiovasculares e câncer (Tracey, 2005).

A incidência da sepse tem aumentado de forma preocupante. Mais de 18 milhões de casos são registrados a cada ano (Angus e Wax, 2001). Nos Estados Unidos são registrados mais de 750 mil casos de sepse por ano e estima-se que a mortalidade chegue a 30%, (Armstrong e Cohen, 1999; Cohen, 2003) o que representa a décima causa de morte nesse país (Cohen, 2003; Puskarich, Marchick *et al.*, 2009). No Reino Unido, são registrados mais de 21 mil casos por ano e estima-se que a mortalidade atinja 50% dos casos (Harrison, Welch *et al.*, 2006).

No Brasil, os números não são diferentes, estudos apontam que em 2004 a incidência de sepse, sepse grave e choque em UTI era de 46,9%, 27,3% e 23%,

respectivamente, e a mortalidade nestes pacientes foi 33,9%, 46,9% e 52,2%, respectivamente. Esta pesquisa constatou ainda que a mortalidade em 28 dias de internação em UTI era de 47% (Silva, Pedro Mde *et al.*, 2004). Em 2007, segundo dados do DATASUS, ocorreram 57.084 internações por sepse no SUS sendo que 41,79% destes pacientes vieram a óbito, o que representa um aumento de 4% na taxa de mortalidade em relação a 2005 (Datasus., 2007).

Apesar dos constantes avanços científicos, a manutenção de elevada mortalidade nos pacientes com sepse não reflete para um desfecho próximo ou exitoso na busca de soluções para esse mal.

Do ponto de vista fisiopatológico, a sepse é uma condição aguda decorrente da desregulação do sistema imune e está associada a eventos pró-trombóticos e anti-fibrinolíticos. O reconhecimento de bactérias por células do sistema imune com capacidade fagocítica, como macrófagos e monócitos, estimulam a síntese de citocinas pró-inflamatórias como IL-1β, IL-6 e TNF-α por essas células. A liberação destes mediadores na corrente sanguínea irá promover a ativação de neutrófilos, linfócitos, aumento da regulação da apresentação de moléculas de adesão, maior expressão de iNOS (óxido nítrico sintase induzida) e de proteínas de fase aguda (Annane, Bellissant *et al.*, 2005; Kortgen, Hofmann *et al.*, 2006).

A resposta do hospedeiro à infecção é indispensável para a sobrevivência do organismo, entretanto, a incapacidade do organismo de conter a resposta inflamatória leva a lesões orgânicas e os mecanismos inicialmente recrutados para combater a infecção induzem a falência de múltiplos órgãos e morte (Astiz e Rackow, 1998).

A partir destes conceitos sobre a sepse e de seus elevados índices de prevalências e mortalidade, consegue-se perceber que a compreensão plena dos mecanismos fisiopatológicos da doença é de extrema importância. E é por isso que, nas

últimas décadas, muito empenho por parte da comunidade científica tem sido destinado ao desenvolvimento de intervenções farmacológicas capazes de reverter estes índices.

1.2 Inflamação e Resistência à Insulina

Durante a sepse, a ativação intensa e persistente do sistema imune leva a liberação sistêmica de mediadores inflamatórios e a quebra da homeostase. Quando a homeostase não pode ser restaurada, esses mediadores inflamatórios induzem á danos teciduais e disfunção de órgãos (Bone, Balk *et al.*, 1992).

Dentre as manifestações sistêmicas observadas na sepse, são de grande importância as alterações no metabolismo da glicose, pois estas são responsáveis pela incapacidade de adaptação orgânica do indivíduo (Abraham e Singer, 2007).

A ocorrência de resistência à insulina é um achado frequente no contexto de sepse. Tanto pacientes sépticos quanto modelos animais de sepse ou endotoxemia exibem capacidade de resposta à insulina atenuada (Westfall e Sayeed, 1988; Virkamaki, Puhakainen *et al.*, 1992; Virkamaki e Yki-Jarvinen, 1995). Inúmeras evidências destacam a resistência à insulina como um importante fator de risco para a sepse, além de estar diretamente associada a prognósticos ruins e ao aumento nos índices de mortalidade (Cochran, Scaife *et al.*, 2003; Hall, Peters *et al.*, 2004; Ali, O'brien *et al.*, 2008).

As alterações no metabolismo da glicose ocorrem na sepse devido ao aumento da produção hepática de glicose, redução da utilização da glicose nos tecidos periféricos que ocorre principalmente por estado de menor resposta metabólica aos níveis circulantes de insulina. Hiperglicemia e resistência à insulina são consequências dos efeitos metabólicos provocados pela excessiva produção mediadores de pró-inflamatórios e da ação de hormônios como glucagon e catecolaminas (Mccowen, Malhotra *et al.*, 2001; Marik e Raghavan, 2004; Pinsky, Brochard *et al.*, 2006).

Apesar da resistência à insulina ser conhecida como uma característica inerente ao *diabetes mellitus* tipo 2 (DM2), sabe-se que diferentes condições clínicas, como a obesidade e a sepse, estão associadas a resistência à insulina. Evidências crescentes, tanto em populações humanas como em animais, apontam que a intersecção entre estes eventos se estabelece por um processo inflamatório sistêmico (Grimble, 2002; Xu, Barnes *et al.*, 2003; Dandona, Aljada *et al.*, 2004; Pickup, 2004). Todavia, os exatos mecanismos envolvidos ainda não estão completamente elucidados.

Para que haja a compreensão dos mecanismos moleculares que induzem a resistência à insulina, faz-se necessário a caracterização dos efeitos fisiológicos da insulina e de sua via de sinalização molecular em células normais.

A insulina é um hormônio polipeptídico anabólico secretado no sangue pelas células β das Ilhotas de Langherans do pâncreas. A síntese da insulina é ativada em resposta ao aumento dos níveis circulantes de glicose e aminoácidos após as refeições.

Os efeitos metabólicos imediatos da insulina incluem: aumento da captação da glicose, especialmente nos tecidos muscular e adiposo, síntese protéica, lipogênese e glicogênese. Já no tecido hepático o hormônio inibe a produção hepática da glicose (por inibir a neoglicogênese e glicogenólise), da proteólise e da lipólise (Czech e Corvera, 1999; Bryant, Govers *et al.*, 2002). Além disso, a insulina tem efeitos na diferenciação e proliferação celular bem como na prevenção da apoptose e na promoção da sobrevida celular. Esse hormônio atua por meio de receptor próprio expresso no tecido muscular, hepático e adiposo, principais tecidos alvos da ação deste hormônio (Saltiel e Kahn, 2001).

A sinalização intracelular da insulina em tecidos insulino-sensíveis inicia-se com a ligação do hormônio a um receptor específico de membrana, o receptor de insulina (IR) (figura 1). O IR é uma proteína heterotetramérica com atividade quinase intrínseca, composta por duas subunidades alfa e duas subunidades beta. Uma vez ativado, o IR

fosforila em vários resíduos de tirosina o substrato do receptor de insulina (Rittirsch, Huber-Lang *et al.*, 2009), iniciando uma cascata sinalizatória (White, 1997; Saltiel e Pessin, 2002). A fosforilação em tirosina de IRS cria sítios de reconhecimento para moléculas contendo domínios de homolgia a Src2 (SH2), como a PI3K (fosfatidilinositol 3 quinase). A ativação da PI3K é essencial para o transporte de glicose estimulada pela insulina, além disso, a ativação da PI3K é importante na regulação da mitogênese e na diferenciação celular (Czech e Corvera, 1999).

Uma vez ativada, a PI3K aumenta a fosforilação em serina da proteína serina/treonina quinase B (Akt), proteína indispensável na ativação da glicogênio-sintase e no armazenamento do glicogênio. A ativação da via PI3K/Akt está relacionada à promoção tanto dos efeitos metabólicos quanto de crescimento desencadeados pela insulina (Saltiel e Pessin, 2002; Brozinick, Roberts *et al.*, 2003).

Em um estado de estresse agudo como a sepse, a hiperglicemia pode ser considerada como uma resposta adaptativa. No entanto, a manutenção do estado hiperglicêmico tem sido associada ao maior risco de complicações como infecções, polineuropatia e disfunção de múltiplos órgãos (Hirasawa, Oda *et al.*, 2009). A manutenção do estado hiperglicêmico influencia diretamente a resposta ao estresse por aumentar o nível de citocinas pro-inflamatórias (TNF-α, IL-1β e IL-6) e por comprometer a quimiotaxia e a fagocitose de neutrófilos (Marik e Raghavan, 2004; Van Den Berghe, 2004).

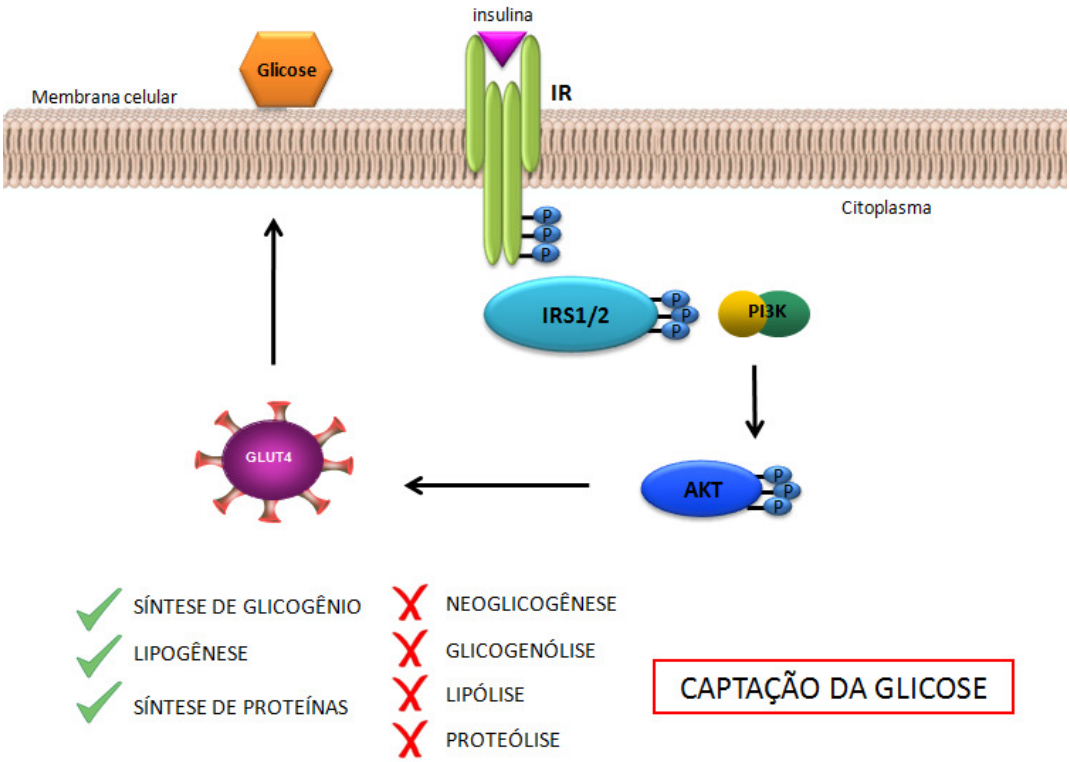

Figura 1. Via de sinalização da insulina na captação de glicose. A insulina, ao se ligar ao seu receptor de membrana, promove a autofosforilação da subunidade beta em resíduos de tirosina e desencadeia a ativação da via PI3K/Akt que irá promover os efeitos metabólicos.

A via de sinalização da insulina, que é reduzida na sepse, pode ser ativada pela insulina para mediar seus efeitos benéficos. Um estudo preliminar feito por Van Den Berghe e colaboradores demonstrou que a restauração da sinalização da insulina através da insulinoterapia intensiva pode ser uma alternativa profilática promissora para sepse. Esse estudo mostra que a insulinoterapia intensiva reduziu significativamente a morbidade e a mortalidade de pacientes sépticos, além disso, reduziu pela metade os índices de infecções na corrente sanguínea e inflamação prolongada, mostrando desta forma a ação benéfica da insulina na sepse (Van Den Berghe, Wouters *et al.*, 2001).

Cerca de oito anos após a publicação deste estudo a insulinoterapia intensiva passou a ser utilizado no manejo de pacientes em UTI para redução dos índices glicêmicos e citado como recomendação importante em diretrizes como o Surviving Sepsis Campaign (Campanha Sobrevivendo à Sepse) (Dellinger, Levy *et al.*, 2008).

Contudo, meta-análise e revisões sistemáticas demonstraram resultados divergentes com a relação à insulinoterapia intensiva (Van Den Berghe, Wouters *et al.*, 2003; Dellinger, Levy *et al.*, 2008; Wiener, Wiener *et al.*, 2008). Ao que parece, a alto número de casos de hipoglicemia acabam por sobrepujar os efeitos benéficos da insulinoterapia e agravar ainda mais os índices de mortalidade por sepse (Brunkhorst, Engel *et al.*, 2008). Por outro lado, existem fortes indícios de que a insulina tenha ação anti-inflamatória independente de seu efeito sobre o controle glicêmico (Brix-Christensen, Andersen *et al.*, 2004; Jeschke, Klein *et al.*, 2004; Jeschke, Rensing *et al.*, 2005). Todavia, o exato mecanismo pelo qual insulina reduz a inflamação na ausência de hiperglicemia é desconhecido.

Um número crescente de pesquisas revelam que a insulina reduz a inflamação através da ativação de vias de sinalização anti-inflamatórias, tais como a via de PI3K/Akt (Guha e Mackman, 2002; Schabbauer, Tencati *et al.*, 2004; Williams, Li *et al.*, 2004). Segundo Zhang et. al, a via de PI3K/Akt desempenha um papel importante na resposta imune inata, atuando como regulador negativo pela produção excessiva de mediadores pró-inflamatórios (Zhang, Wei *et al.*, 2007). Diferentes estudos apontam que via PI3K/Akt modula negativamente a ativação de vias pró-inflamatórias induzida por LPS e a produção de citocinas (Guha e Mackman, 2002; Liew, Xu *et al.*, 2005; Martin, Rehani *et al.*, 2005). Estudos conduzidos em roedores relataram ainda que a ativação da via PI3K/Akt aumenta a sobrevivência, enquanto sua inibição reduz sobrevida de camundongos endotoxêmicos (Schabbauer, Tencati *et al.*, 2004; Williams, Li *et al.*, 2004).

Uma vez que a resistência à insulina pode agravar ainda mais o quadro da sepse (Maitra, Wojnar *et al.*, 2000; Van Den Berghe, 2004; Kyle, Coss Bu *et al.*, 2010), a manutenção da normoglicemia com a insulinoterapia reduz os índices de morbidade e mortalidade da sepse (Van Den Berghe, Wouters *et al.*, 2003). Diante dessas constatações, é possível aventar a hipótese de que a droga ideal para aumentar a sobrevivência à sepse deve atenuar a resposta inflamatória, e em paralelo, deve melhorar a sinalização na via de PI3K/Akt, sem induzir a hipoglicemia.

Mecanismos moleculares associados a redução da fosforilação em tirosina de proteínas envolvidas na sinalização da insulina são o principal foco da investigação da fisiopatologia envolvida na gênese da resistência à insulina (Saad, Araki *et al.*, 1992; Aguirre, Werner *et al.*, 2002).

A fosforilação inibitória de IRS-1 em serina reduz sua fosforilação em tirosina e sua capacidade de se associar ao IR, bloqueando a sinalização e ação da insulina (Paz, Hemi *et al.*, 1997; Aguirre, Werner *et al.*, 2002). A inibição da sinalização após a ativação do receptor de insulina é o principal mecanismo pelo qual a inflamação causa resistência à insulina.

Durante os últimos 15 anos, inúmeras evidências mostraram uma clara interação molecular entre as vias de sinalização imune e metabólica em diferentes situações de resistência à insulina (Hotamisligil, Budavari *et al.*, 1994; Hotamisligil, Peraldi *et al.*, 1996; Park, Lee *et al.*, 1997; Williams, Li *et al.*, 2004).

Ácidos graxos livres e citocinas pró-inflamatórias podem induzir a fosforilação inibitória de IRS1 em serina, através da ativação de serinas quinases como JNK e IKK (Hotamisligil, Shargill *et al.*, 1993; Aguirre, Uchida *et al.*, 2000; Gao, Hwang *et al.*, 2002; Gao, Zhang *et al.*, 2004). A descoberta do envolvimento das vias IKK e JNK na indução da resistência à insulina ressalta sobreposição das vias metabólicas e inflamatórias, pois

essas são as mesmas ativadas na resposta imune inata mediada pelo TLR4 em resposta ao LPS.

Os receptores transmembrana chamados TLR4 (Toll-like receptor 4) são expressos principalmente em células apresentadoras de antígenos, como monócitos, macrófagos e células dendríticas. Os TLR4 mostram uma capacidade de reconhecer padrões moleculares associados à patógenos, como o LPS. A ativação do TLR4 ativa cascatas de sinalização pró-inflamatórias que induzem a expressão de citocinas como TNF-α, IL-1β, IL-6, IL-8, quimiocinas e outros efetores da resposta imune inata (Medzhitov, Preston-Hurlburt *et al.*, 1997).

Vias de sinalização envolvendo TLR4 desempenham uma conexão importante entre o sistema imune inato e o sistema metabólico (Kim, 2006). Uma importante pesquisa em nosso laboratório demonstrou que camundongos com a mutação inativadora do TLR4 utilizam melhor a glicose e não desenvolvem resistência à insulina mesmo quando submetidos à dieta rica em gordura (Tsukumo, Carvalho-Filho *et al.*, 2007). A ativação do TLR4 leva a ativação de vias pró-inflamatórias como JNK e IKK, que têm papel relevante na resistência à insulina (Figura 2).

Sabe-se que a JNK (c-jun N-terminal kinase) pode ser ativada por citocinas pró-inflamatórios e ácidos graxos (Ip e Davis, 1998; Hirosumi, Tuncman *et al.*, 2002). A ativação da JNK bloqueia a ação da insulina, tanto pela fosforilação em serina do receptor de insulina quanto pela ativação de fatores de transcrição como c-jun e ATF-2, que estimulam a síntese de citocinas pro-inflamatórias (Ip e Davis, 1998; Davis, 1999).

Outra via pró-inflamatória que pode levar à fosforilação em serina de substratos do receptor de insulina é a via IKK/IκB/NF-κB. Quando o complexo IKK é ativado, induz a fosforilação dos inibidores IκB. A fosforilação do IκB leva a ubiquitinação e degradação dos IκBs pelo proteassoma celular e a liberação translocação do NF-κB ao núcleo, onde

irá ativar a transcrição de diversos genes e a síntese de citocinas como TNF-α e IL-1β (Baldwin, 2001).

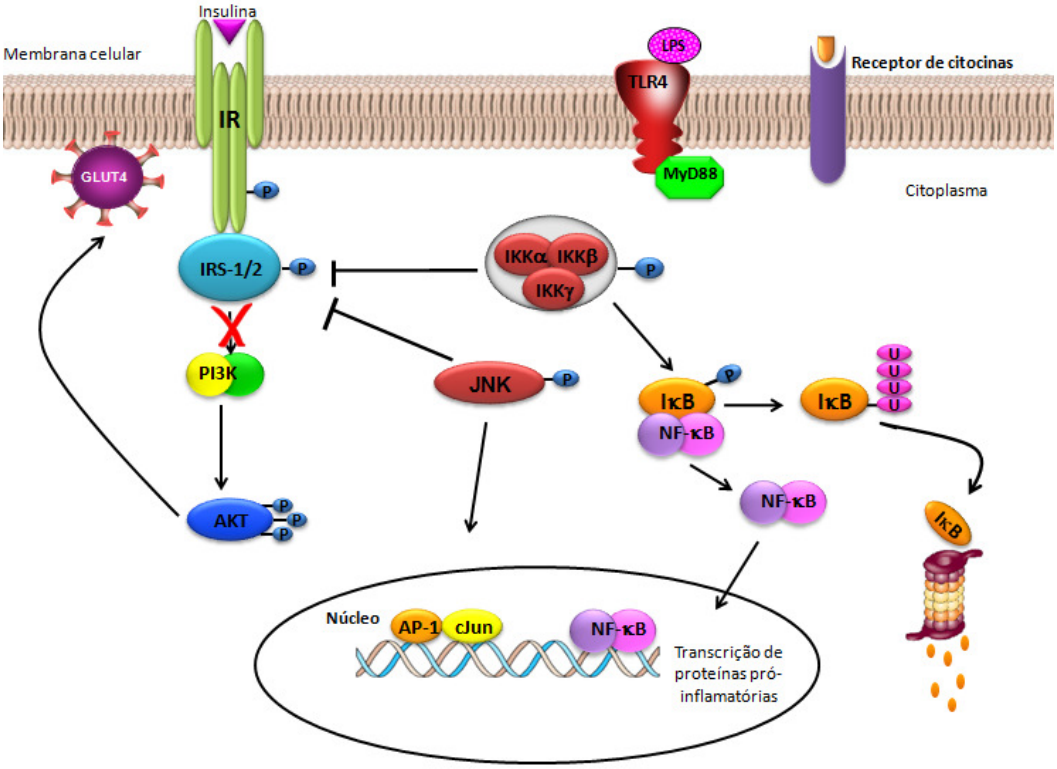

Figura 2. Sepse e resistência à insulina. A ativação de quinases na sepse, especialmente IKK e JNK, ressalta a sobreposição das vias metabólicas e inflamatórias: essas são as mesmas quinases ativadas na resposta imune inata mediada pelo TLR4 (toll-like receptor 4) em resposta ao LPS.

Face ao exposto, podemos considerar que tanto componentes bacterianos, como LPS, quanto citocinas pró-inflamatórias podem ativar mecanismos intracelulares associados à resistência à insulina na sepse.

1.3 Estresse do Retículo e Resistência à Insulina

Um importante mecanismo que parece estar envolvido na gênese da resistência à insulina é resultado de comprometimento da função do retículo endoplasmático (Ron e Walter, 2007; Hotamisligil, 2008).

O retículo endoplasmático (RE) é a organela responsável pela síntese e enovelamento de proteínas secretoras. O enovelamento de proteínas é o processo responsável pela conversão de cadeias lineares de polipeptídios em estruturas tridimensionais, que permite as proteínas tornarem-se funcionais.

Condições patológicas graves como a sepse, perturbam a homeostase do RE e podem prejudicar o processo de enovelamento, resultando na formação de proteínas imaturas no lúmen do RE. O acúmulo de proteínas imaturas no RE aciona uma resposta adaptativa conhecida como a UPR (unfolded protein response) (Ron e Walter, 2007).

A UPR aumenta a expressão de chaperonas e quinases dobradoras de proteínas que promovem a conformação tridimensional adequada, reduzem a carga de proteínas imaturas e restabelecem a homeostase do RE. O controle da função reticular e sinalização da UPR em células eucarióticas ocorrem através três proteínas de membrana do RE: PERK (quinase do retículo endoplasmático semelhante à proteína quinase do pâncreas), IRE1 (enzima ativada por inositol 1) e ATF6 (Fator 6 ativador de transcrição) (Figura 3).

A UPR auxilia a recuperação celular após o estresse reparando proteínas mal formadas e protegendo as células contra a apoptose através da redução da síntese de proteínas e do aumento da produção de chaperonas que ativam o dobramento de proteínas na organela (Ron e Walter, 2007).

Apesar de ser uma resposta adaptativa necessária para manutenção da homeostase do RE e para a sobrevivência do organismo, a ativação persistente de UPR é

prejudicial. Evidências crescentes demonstram que proteínas de UPR interagem com diferentes vias de sinalização pró-inflamatórias e de estresse (Hotamisligil, 2010). Recentemente, demonstrou-se que a ativação de UPR é capaz de ativar diretamente as vias pro-inflamatórias JNK e IKK (Urano, Wang *et al.*, 2000; Hu, Han *et al.*, 2006).

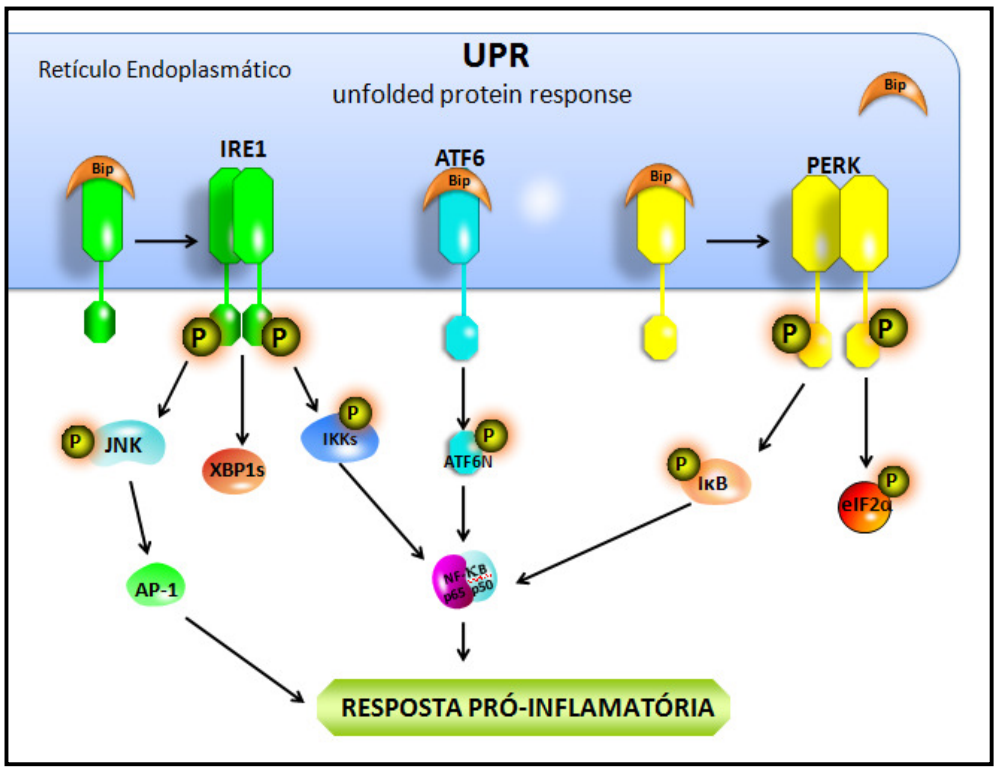

Figura 3 - Sinalização molecular da UPR. Em situação de estresse, o acúmulo de proteínas imaturas no RE, leva ao recrutamento das chaperonas BiP (Binding Protein) que se desligam das três proteínas de membrana, permitindo a homodimerização e auto-fosforilação das quinases PERK e IRE1 e clivagem do ATF6.

Diante das constatações, podemos considerar que a redução do estresse do ER seja um alvo terapêutico potencial para atenuar os danos à sinalização da insulina induzida pela a resposta inflamatória. Neste contexto, acreditamos que droga ideal para

aumentar a sobrevivência à sepse deve atenuar a resposta inflamatória, e em paralelo, melhorar a sinalização da insulina, sem induzir a hipoglicemia.

1.4 Atorvastatina

A atorvastatina é um membro da classe de drogas conhecidas como estatinas e é extensamente utilizada no tratamento de dislipidemias. Seu principal mecanismo de ação é através da inibição da enzima HMG-CoA redutase, enzima limitadora da biosíntese hepática de colesterol, que converte o composto HMG-CoA a mevalonato (Endo, 2008).

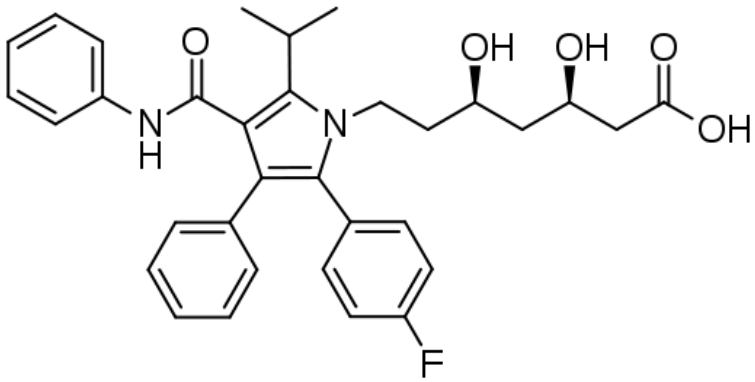

Figura 4 - Fórmula estrutural da Atorvastatina

A inibição da síntese do colesterol é considerada o principal mecanismo de ação das estatinas, no entanto, tem-se comprovado que estes fármacos reduzem a inflamação, aumentam a estabilidade da placa e melhoram a função endotelial em pacientes com aterosclerose, reduzindo desta forma o risco de acidente vascular (Amarenco e Labreuche, 2009). Outrossim, Pleiner e cols. consideram que estes fármacos podem ser úteis no tratamento de pacientes com inflamação sistêmica aguda por serem potentes antioxidantes e vasoprotetores (Pleiner, Schaller *et al.*, 2004).

Em 2001, um estudo retrospectivo evidenciou que o índice de mortalidade por bacteremia era significativamente menor entre os pacientes que utilizavam estatinas, em relação aos que não usavam (Liappis, Kan *et al.*, 2001). A partir deste estudo pioneiro, progressivas evidências epidemiológicas fortaleceram a hipótese do possível efeito benéfico das estatinas sobre a redução da mortalidade por bacteremia (Almog, Shefer *et al.*, 2004; Kruger, Fitzsimmons *et al.*, 2006; Thomsen, Hundborg *et al.*, 2006) e sobre a redução do risco de infecções (Schmidt, Hennen *et al.*, 2006; Gupta, Plantinga *et al.*, 2007; Schlienger, Fedson *et al.*, 2007).

Um importante estudo que avaliou a relação entre o uso de estatina e risco de sepse em pacientes com aterosclerose, verificou que a incidência de sepse e a progressão para sepse grave eram menores em pacientes que usavam estatinas. Adicionalmente, verificou-se que o efeito benéfico das estatinas sobre a sepse foi substancial, mesmo em pacientes em grupos de risco, como pacientes com DM2, falência renal crônica ou com histórico de infecção (Hackam, Mamdani *et al.*, 2006).

De fato, inúmeros estudos demonstraram que, além do serem um potentes hipolipemiantes, as estatinas têm ação anti-inflamatória, antioxidante e imunomoduladora (Almog, 2003; Spitzer e Harris, 2006; Terblanche, Almog *et al.*, 2006). No entanto, os mecanismos moleculares subjacentes a estes efeitos pleiotrópicos não são completamente elucidados. Recentemente, tem sido destacado que as estatinas podem trazer benefícios a eventos de resistência à insulina induzida por processos inflamatórios.

Uma pesquisa prospectiva constatou que as estatinas reduzem a incidência de diabetes tipo 2 (Freeman, Norrie *et al.*, 2001). Corroborando tais estudos, outros dados demonstraram que as estatinas melhoram a sensibilidade à insulina em pacientes com síndrome metabólica (Paniagua, Lopez-Miranda *et al.*, 2002; Costa, Casamitjana *et al.*, 2003; Sonmez, Baykal *et al.*, 2003; Guclu, Ozmen *et al.*, 2004; Huptas, Geiss *et al.*, 2006).

No entanto, os exatos mecanismos pelos quais as estatinas exercem esse efeito positivo sobre a resistência à insulina permanecem desconhecidos.

Têm-se apontado, que ação anti-inflamatória das estatinas é mediada pelo estímulo de PPAR-α e γ, bloqueio de NFκB e inibição da adesão leucocitária (Munford, 2001; Mcfarlane, Muniyappa *et al*., 2002; Weitz-Schmidt, 2002). Mais recentemente, demonstrou-se que as estatinas reduzem o estresse do RE em macrófagos ativados presentes nas paredes arteriais em modelo animal de aterosclerose (Breder, Coope *et al*., 2010), fato que também pode estar relacionado ao seu efeito protetor sobre a mortalidade na sepse.

A partir das evidências sobre a ação anti-inflamatória das estatinas e dos dados positivos sobre a mortalidade na sepse, consegue-se perceber que a compreensão dos mecanismos moleculares subjacentes aos efeitos pleiotrópicos deste fármaco sobre a sepse são de extrema importância.

1.5 Diacereína

A diacereína (1,8-diacetoxy-9,10-dioxo-dihydroanthracene-3-carboxylic acid) (Figura 5) é um fármaco utilizado no tratamento da osteoartrite, com propriedades anti-inflamatórias, além de moderada ação analgésica e antipirética (Spencer e Wilde, 1997). Pesquisas clínicas têm comprovado que diacereína tem efeitos benéficos sobre os sintomas da osteoartrite, incluindo efeito antiartrítico e condroprotetor (Smith, Myers *et al.*, 1999).

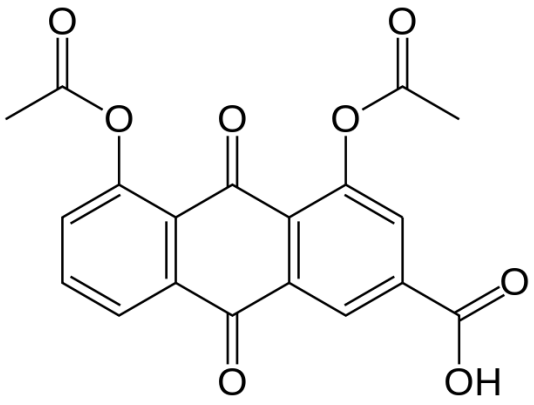

Figura 5 - Fórmula estrutural da diacereína

A reína, o principal metabólito ativo da diacereína, tem demonstrado inibir a síntese e a atividade de citocinas pró-inflamatórias, como IL-1β, IL-6 e TNF-α (Moore, Greenslade *et al.*, 1998; Nicolas, Tod *et al.*, 1998; Pelletier, Mineau *et al.*, 1998; Pelletier, Lajeunesse *et al.*, 2001). O composto também age diretamente sobre as células inflamatórias inibindo a liberação de enzimas lisossomais, a quimiotaxia e atividade fagocitária de neutrófilos e macrófagos (Mian, Brunelleschi *et al.*, 1987; Del Rosso, Fibbi *et al.*, 1990; Boittin, Redini *et al.*, 1993; Martel-Pelletier, Mineau *et al.*, 1998; Pelletier, Mineau *et al.*, 1998). Estudos mostraram ainda que a diacereína e a reína inibem a

ativação de NF-κB e a expressão de genes dependente NF-κB (Mendes, Caramona *et al.*, 2002).

Além de seus efeitos inibitórios sobre genes pró-inflamatórios, tem sido demonstrado que a reína, têm atividade anti-tumoral em várias linhagens celulares de câncer (Delpino, Paggi *et al.*, 1992; Lin e Zhen, 2009).

Pesquisas revelaram que diacereína é capaz de reduzir a incidência do diabetes tipo I em animais NOD (*Non Obese Diabetic*) pela redução da expressão das citocinas pró-inflamatórias IL-1β, TNF-α, IFN-γ e IL-12 (Malaguti, Vilella *et al.*, 2008). Um estudo recente realizado em nosso laboratório constatou que o tratamento com a diacereína melhorou a tolerância à glicose e a sensibilidade à insulina em modelo de obesidade e inibiu a ativação de vias de sinalização pró-inflamatórias como IKKβ e JNK (Tobar, Oliveira *et al.*, 2011).

Frente ao exposto, podemos sugerir que a diacereína tem potencial para atenuar a resposta inflamatória na sepse e melhorar a sensibilidade à insulina, sem induzir a hipoglicemia.

OBJETIVOS

2.1 Objetivo Geral

- Avaliar os efeitos da administração da Atorvastatina e Diacereína sobre a sobrevivência, sinalização inflamatória, estresse do reticulo e resistência à insulina em modelo de sepse induzida por CLP.

2.2 Objetivos Específicos

- Avaliar os efeitos da administração da Atorvastatina e Diacereína sobre a sobrevivência de ratos sépticos.

-Estudar o efeito dessas drogas sobre a cascata de sinalização da insulina através da expressão e/ou fosforilação das proteínas envolvidas nesta via (IR, IRS-1 e Akt)na sepse.

- Estudar o efeito dessas drogas sobre a cascata de sinalização pró-inflamatória envolvida na modulação negativa da via da insulina (JNK e IKKβ).

- Avaliar a ação destas drogas sobre as proteínas sinalizadoras de estresse do retículo endoplasmático na sepse.

- Avaliar a ação destas drogas sobre a expressão de mediadores inflamatórios (IL-6, IL-1β e TNF-α) diante do quadro de inflamatório induzidos por sepse.

CAPÍTULO 1

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Atorvastatin Improves Survival in Septic Rats: Effect on Tissue Inflammatory Pathway and on Insulin Signaling

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Abstract

The aim of the present study was to investigate whether the survival-improving effect of atorvastatin in sepsis is accompanied by a reduction in tissue activation of inflammatory pathways and, in parallel, an improvement in tissue insulin signaling in rats. Diffuse sepsis was induced by cecal ligation and puncture surgery (CLP) in male Wistar rats. Serum glucose and inflammatory cytokines levels were assessed 24 h after CLP. The effect of atorvastatin on survival of septic animals was investigated in parallel with insulin signaling and its modulators in liver, muscle and adipose tissue. Atorvastatin improves survival in septic rats and this improvement is accompanied by a marked improvement in insulin sensitivity, characterized by an increase in glucose disappearance rate during the insulin tolerance test. Sepsis induced an increase in the expression/activation of TLR4 and its downstream signaling JNK and IKK/NF-κB activation, and blunted insulin-induced insulin signaling in liver, muscle and adipose tissue; atorvastatin reversed all these alterations in parallel with a decrease in circulating levels of TNF-α and IL-6. In summary, this study demonstrates that atorvastatin treatment increased survival, with a significant effect upon insulin sensitivity, improving insulin signaling in peripheral tissues of rats during peritoneal-induced sepsis. The effect of atorvastatin on the suppression of the TLR-dependent inflammatory pathway may play a central role in regulation of insulin signaling and survival in sepsis insult.

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Introduction

Sepsis is one of the most prevalent diseases and one of the main causes of death among hospitalized patients [1]. During the onset of sepsis, the inflammatory system becomes hyperactive, leading to an over-production of pro-inflammatory mediators [2], which contribute to septic shock, multiple organ failure, and death. Hyperglycemia and insulin resistance occur during sepsis, as a consequence of the metabolic effects of stress hormone and cytokine production [3,4,5,6]. Although in the past years there has been considerable progress in our understanding of the pathological pathways that contribute to sepsis and septic shock, pharmacological interventions are currently limited to insulin and activated protein C [7]. Insulin is infused in septic patients with hyperglycemia to normalize glucose levels [7,8], and it is hypothesized that this reduction in glycaemia is associated with decreased inflammation and endothelial cell damage [4,5,6,9,10].

Conversely, results from animal studies indicate that insulin may have direct anti-inflammatory effects, independent of its effect on hyperglycemia [11,12,13]. However, the mechanism by which insulin reduces inflammation in the absence of hyperglycemia is unknown. Recent data demonstrate that insulin reduces inflammation by activating anti-inflammatory signaling pathways, such as the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway. Moreover, it is now well established that this pathway negatively regulates LPS-induced signaling and pro-inflammatory cytokine production [14,15,16,17] and activation of PI3K/Akt pathway enhanced survival, whereas inhibition of PI3K reduced survival of endotoxemic mice [15,18]. In animal models of sepsis, there is a down regulation of the PI3K/Akt pathway that may be the consequence of TLR4 activation and downstream in activation of well established inducers of insulin resistance as JNK, IKK-β and iNOS. Taken together, these data indicate that this insulin signaling pathway, which is reduced in sepsis, may be activated by insulin to mediate the protective effects of insulin in endotoxemia. However, insulin-induced hypoglycemia may counteract the beneficial effects of aggressive insulin therapy in patients with severe sepsis [19].

These data suggest that the ideal drug to improve survival in sepsis should reduce the over-reaction of the inflammatory response and, in parallel, should improve insulin signaling in the PI3K/Akt pathway, without inducing hypoglycemia. Recently, knowledge about statins, a class of powerful hypolipemic drugs with pleiotropic effects, such as anti-inflammatory, antioxidative, immunomodulatory properties, suggest that these agents may offer a novel therapeutic or prophylactic strategy to sepsis [1,2,20]. Relevant epidemiological evidence suggests that use of statins may decrease progression to severe sepsis [21], reduce mortality rates [22,23,24,25,26,27,28,29] and reduce the risk of infections and infection-related complications [30,31,32,33,34]. However, the

mechanisms by which statins induce this protective effect is not well established. The aim of the present study was to investigate whether the ability of atorvastatin to improve survival in sepsis is accompanied by a reduction in tissue activation of inflammatory pathways and, in parallel, an improvement in tissue insulin signaling.

Results

Atorvastatin Improves Survival and Protects Against Insulin Resistance in Septic Rats

In the first part of the study, we examined the effect of atorvastatin on the survival curve in animals in which sepsis was induced via CLP. Atorvastatin (10 mg/kg) or placebo was administered by gavage 3 h after surgery or the procedure in sham-operated animals. No deaths occurred in the sham-operated animals, whether or not they had been treated with atorvastatin. The survival curves for rats, in which CLP was performed, are shown in Figure 1A and clearly delineate the benefit sustained from atorvastatin treatment due to improved survival ($P<0.0001$). As shown in Fig. 1B and 1C, septic animals were more insulin resistant than sham rats; fasting plasma glucose and insulin levels were higher in septic rats than in the control group, and atorvastatin treatment reduced these levels. As depicted by Fig. 1D the plasma glucose disappearance rates measured during the insulin tolerance test (Kitt) were lower in septic animals and atorvastatin treatment reversed these alterations. This improvement is also suggested by the HOMA-IR index, calculated from fasting glycaemia and insulinemia, which is increased in septic animals and significantly decreased in those treated with atorvastatin (Fig. 1E). Atorvastatin treatment had no effect on insulin tolerance in the sham group. Taken together, these data suggest that atorvastatin reversed the sepsis-induced insulin resistance.

Effect of Atorvastatin on Serum Levels of IL-6 and TNF-α

The serum levels of IL-6 and TNF-α were also higher in septic animals compared with sham-operated rats. After atorvastatin treatment there was a significant decrease in IL-6 (Fig. 1F) and TNF-α (Fig. 1G) circulating levels.

Atorvastatin Improves Insulin Signaling in Liver, Muscle and Adipose Tissue of Septic Animals

In the sepsis group, insulin-induced IR and IRS-1 tyrosine phosphorylation were decreased in liver, muscle and adipose tissue when compared with sham rats and these alterations were reversed by atorvastatin (Fig. 2A–F). In parallel, there was a decrease in insulin-induced Akt serine phosphorylation in the liver, muscle and adipose tissue of septic animals when compared with sham rats and atorvastatin was able to reverse these reductions in Akt phosphorylation (Fig. 2G–I). The modulation in IR, IRS-1 and Akt phosphorylation, induced by sepsis and reversed by atorvastatin, was independent of changes in tissue protein expression (Fig. 2A–I). The protein concentration of IR, IRS-1, and Akt did not change between the groups. Equal protein loading in the gels was confirmed by reblotting the membranes with an anti-β-actin antibody (lower panels).

Atorvastatin Attenuates Sepsis-Induced Inflammatory Changes

Toll-like receptor 4 (TLR4) is a transmembrane receptor that participates in pathogen recognition during the inflammatory response, and leads to cytokine and other immune-related gene expression [35,36]. During sepsis, the activation of TLR4 signaling induces upregulation of intracellular inflammatory pathways, such as the IκB kinase β (IKK-β)/nuclear factor kappa B (NFκ-B) pathway.

Next, we examined the immunomodulatory effects of atorvastatin on TLR4 activation in liver, muscle and adipose tissue of septic animals. Fig. 3 shows that sepsis induced TLR4 protein levels and activation, as demonstrated by an increase in TLR4/MyD88 interaction in the three tissues investigated, and atorvastatin reduced this early step of TLR4 activation and also TLR4 expression (Fig. 3A–C).

Downstream of TLR4 activation, we examined the IKK–NF-κB pathway, an important regulator of inflammation and insulin resistance. The main function of the IKK complex is the activation of NFκB through phosphorylation and degradation of IκBα [37,38]. NFκB activity was monitored using IKKβ and IκBα phosphorylation, as described previously [39]. As expected, IKKβ and IκBα phosphorylation were increased in liver, muscle and adipose tissue of septic animals and atorvastatin decreased these phosphorylations in the tissues investigated (Fig. 3D–I).

JNK activation was determined by monitoring phosphorylation of JNK (Thr183 and Tyr185) and c-Jun (Ser63), which is a substrate of JNK. JNK phosphorylation in liver, muscle and adipose tissue were increased in septic animals and atorvastatin induced a downmodulation in the phosphorylation of this serine kinase (Fig. 4A–C). Consistent with JNK activation, c-Jun phosphorylation was induced by sepsis and reversed by atorvastatin in the tissues investigated (Fig. 4D–F).

We also investigated Ser307 phosphorylation of IRS-1 in liver, muscle, and adipose tissue in the four groups of rats. Ser307 phosphorylation was induced by sepsis in the three tissues and the treatment with atorvastatin reversed this alteration (Fig. 4G–I). NFκB nuclear subunit p50 expression was determined in nuclear extracts and we found an increase in this subunit in nuclear extracts of septic animals, but there was a clear decrease in the three tissues after atorvastatin treatment (Fig. 5A–C). The tissue expressions of iNOS (Fig. 5D–F) and IL-6 (Fig. 5G–I) were higher in liver, muscle and adipose tissue of septic rats that received saline, and were significantly reduced by atorvastatin treatment.

Previous studies have shown that sepsis is also characterized by endoplasmic reticulum stress. It is clear that ER stress can also induce activation of JNK and IKKβ. We then investigated the effect of sepsis (treated or not with atorvastatin) on proteins that reflect ER stress. Our data showed that sepsis induced ER-stress, as determined by the increased phosphorylation of the ER membrane kinase, PERK (PKR-like endoplasmic reticulum kinase) and its substrate (Fig. 6A–C), eIF2α (eukaryotic translation initiation factor 2α) (Fig. 6D–F) and increased the expression of ATF6α (Fig. 6G–I). Treatment with atorvastatin significantly reduced the expression of all markers of ER-stress. (Fig. 6A and D)

Discussion

In sepsis, the acute immune response is organized and executed by innate immunity. This response starts with sensing of danger by pattern-recognition receptors on the immune competent cells and endothelium. The pattern-recognition receptors, mainly toll-like receptor (TLR), are also activated in other tissues such as liver, muscle and adipose tissue [40]. The sensed danger signals, through specific signaling pathways, activate transcription factors and gene regulatory systems, which up-regulate the expression of pro-inflammatory mediators. However, the over-reaction of this pro-inflammatory response has an important role in the development of multiple organ failure and death. The combinations of the

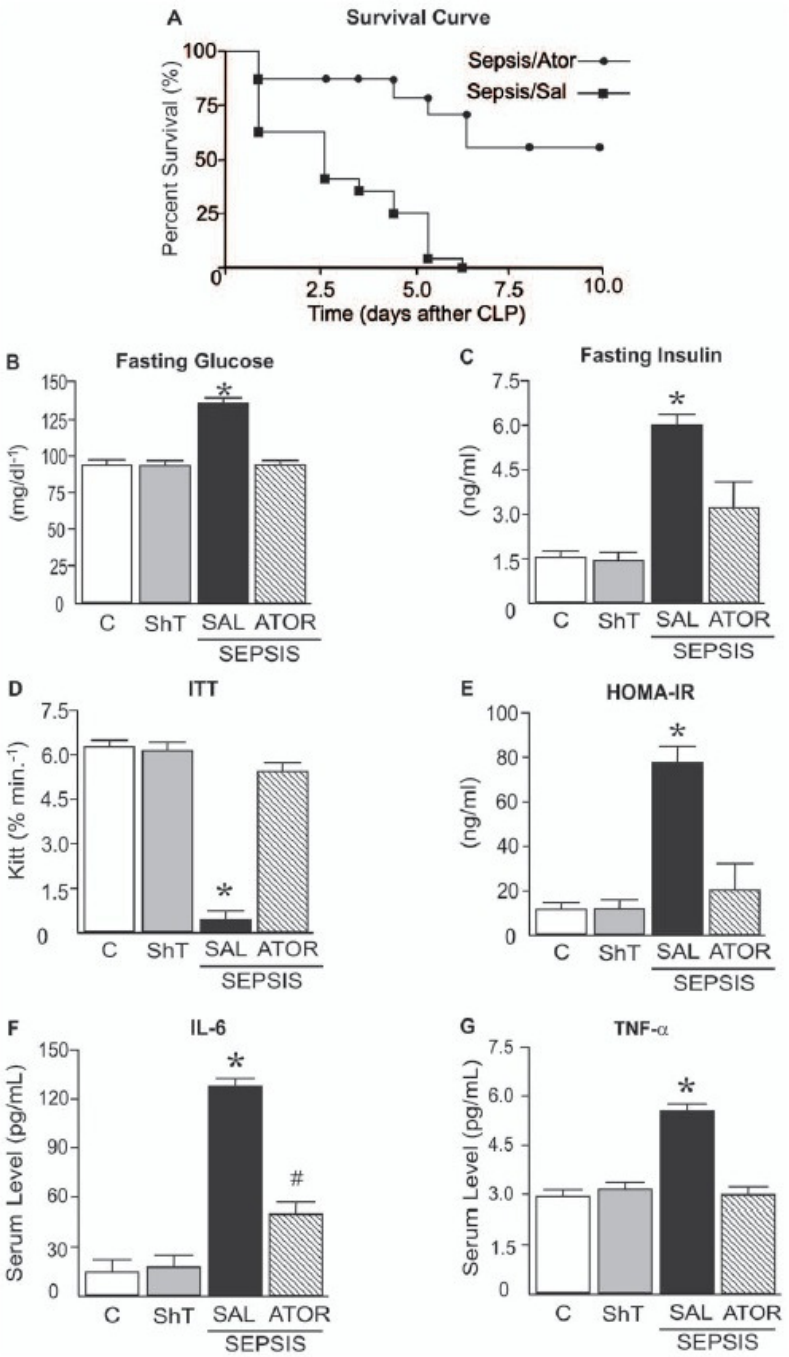

Figure 1. Effect of atorvastatin on survival in CLP sepsis model. Male Wistars rats, 8 weeks old, were given saline (Sepsis/Sal, n = 20) or atorvastatin 10 mg/kg (Sepsis/Ator, n = 20), 3 h and once a day after CLP. Survival of the rats was monitored at intervals of 12 h for 15 days. The overall difference in survival rate between the groups with and without atorvastatin was significant ($P<0.0001$) (A). Fasting blood glucose (B). Fasting insulin levels (C). Glucose disappearance rate (D). HOMA-IR index (E). Serum levels of TNF-α (F) and IL-6 (G). Data are presented as means and S.E. of six to eight rats per group. *$P<0.05$ (Sepsis saline vs. all others groups).
doi:10.1371/journal.pone.0014232.g001

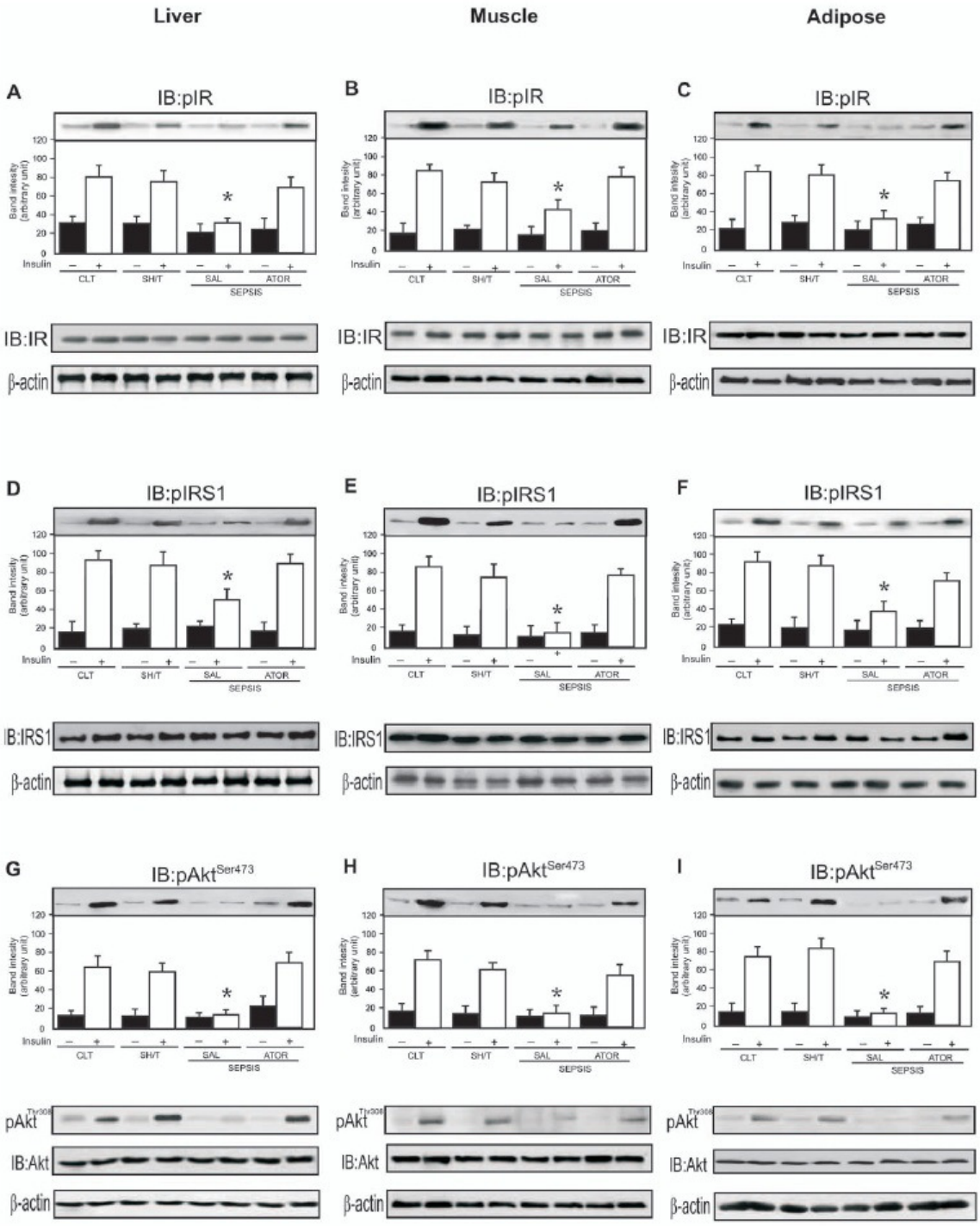

Figure 2. Effects of atorvastatin treatment on insulin signaling in the CLP rat. Representative blots show insulin-induced tyrosine phosphorylation of Insulin Receptor β (IRβ) in liver (A), muscle (B) and adipose (C) of sham and septic rats. Total protein expression of IRβ (A–C, lower panels). Insulin-induced tyrosine phosphorylation of Insulin Receptor Substrate 1 (IRS1) in liver (D), muscle (E) and adipose tissue (F) of sham and septic rats. Total protein expression of IRS1 (D–F, lower panels). Insulin-induced serine phosphorylation of Akt in liver (G), muscle (H) and adipose (I) of

sham and septic rats. Insulin-induced threonine phosphorylation and total protein expression of Akt (G–I, lower panels). In this case, blots were stripped and reprobed with β-actin (A–I, lower panels) to confirm equal loading of proteins. Data are presented as means +/− S.E.M from 6–8 rats per group. *P<0.05 (Sepsis/Sal vs. all others groups). IB, immunoblot; CLT: Sham/Saline; ShT: Sham/Atorvastatin; SAL: saline; ATOR: atorvastatin.
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activation of pattern recognition receptors, specifically TLR4, and the cytokine storm of sepsis have an important role in insulin resistance. In this regard, tissue insulin resistance may be used as an important indicator of the resultant actions of pattern recognition receptors and the induction of the pro-inflammatory cytokines, being a tissue marker of severity of sepsis, before organ failure. In addition, this insulin resistance may also aggravate sepsis, as previously described [3,4,41]. In this regard, we believe that the evaluation of insulin signaling pathway through PI3K/Akt in liver, muscle and adipose tissue may be important indicators of this over-reaction at the tissue level, and the improvement in this signaling pathway, induced by some treatments, in parallel with a decrease in tissue inflammation, may predict the effectiveness of this treatment.

In the present study, we demonstrated that atorvastatin improves survival in septic rats, decreases circulating inflammatory cytokines and improves insulin resistance, in parallel with a decrease in TLR4 signaling and an improvement in insulin signaling in liver, muscle and adipose tissue. The improvement in survival of septic animals when using statins has been previously described, and was related to a complete preservation of cardiac function and hemodynamic status and also a reversion of increased monocyte adhesion to the endothelium, all of which were altered in septic animals without treatment [42,43].

Our data show that atorvastatin, administered 3 hours after the induction of sepsis, is able to improve the survival curve, attenuating higher levels of IL-6 and TNF-α and reduce insulin resistance, as demonstrated during the insulin tolerance test. In septic animals, insulin resistance was accompanied by a reduction in insulin-induced IR, IRS-1 tyrosine phosphorylation and in insulin-induced Akt phosphorylation, in liver, muscle and adipose tissue. Since Akt has a critical role in protection from apoptosis, it is possible that the reduced insulin signaling through this IRSs/PI3K/Akt pathway, in sepsis, may contribute to multi-organ failure by preventing or delaying apoptosis [14,15,44]. In this study, we demonstrate that pretreatment with atorvastatin inhibits sepsis-induced insulin resistance by improving insulin signaling via the IR–IRS-1–Akt pathway in target tissues. This restoration of insulin signaling in these tissues would allow the animal to have an appropriate control of hepatic glucose output and of the peripheral glucose uptake and storage. In addition, skeletal muscle and adipose tissue contribute to IL-6 expression during sepsis [45,46,47], and since the anti-inflammatory effect of insulin is mediated through the PI3K pathway [14,15,18], we can speculate that the restoration of this pathway in the insulin-dependent tissues, induced by atorvastatin in septic animals, may have also contributed to the anti-inflammatory effect of this drug.

During the past ten years, accumulating evidence shows a clear molecular interaction between metabolic and immune signaling systems in different situations of insulin resistance [40,48,49]. We and others have previously demonstrated that TLR4 signaling is activated in diet induced obesity (DIO), and that this activation culminates in an increase in the activation of downstream effectors such as IKKβ, JNK and iNOS, which have critical roles in insulin resistance [40]. In septic animals, there was an increase in TLR4 activation and also in downstream effectors that may have an important role in the insulin resistance of these animals [50]. Our data show that atorvastatin decreases TLR4 expression/activation, a modulation that might have a role in the attenuated expression of inflammatory mediators in response to a septic insult. The reduction in TLR4 expression/activation, observed in our study, is in accordance with a previous study that also observed this effect on TLR4 expression/activation with different statins and cell types [51].

The immune modulator activity of atorvastatin was evident downstream from TLR4, at the level of IKK/IκB/NF-κB pathway activation. Atorvastatin induced a significant decrease in IKK phosphorylation and, as expected, an increase in IκB phosphorylation, suggesting a deactivation of this pathway. NF-κB has been documented to play a major role in sepsis induced inflammatory cytokine expression [52,53,54]. Our findings suggest that NF-κB, which is normally translocated from the cytoplasm to the nucleus after sepsis insult, was strongly inhibited by atorvastatin in the three target tissues studied. This result suggests that the atorvastatin-mediated inhibition of cytokine production may be the consequence of the modulation of the IKK/IκB/NF-κB pathway by this drug.

The activation of NF-κB with its translocation to the nucleus is able to induce the increase in TNF-α, IL-6 and in iNOS in septic animals [37,38,55]. TNF-α is one of the crucial pro-inflammatory cytokines; however, when over produced by deregulation or persistent infection, TNF-α may induce septic shock and contributes to insulin resistance, and its levels are drastically elevated in a number of forms of human sepsis, in turn correlating with increased mortality [56]. Specifically, iNOS and IL-6 have been shown to be produced early in the response and have been suggested to play critical roles in driving physiological/pathological responses that lead to septic shock [57]. We further investigated the effects of atorvastatin on the production of these inflammatory proteins in tissues and serum of septic rats. In accordance with previous data, atorvastatin treatment inhibited iNOS, TNF-α and IL-6 expression in the muscle, liver and adipose tissues of septic rats, and also the circulating levels of TNF-α and IL-6. [58]. Previous studies have demonstrated that skeletal muscle and adipose tissue contribute to IL-6 production during endotoxemia [45,46,47], and the anti-inflammatory effects of atorvastatin on IL-6 production in sepsis may be the result of the direct activation of PI3K pathway in these tissues. The atorvastatin-mediated reduction of these negative modulators of insulin signaling may have an important role in the improvement of insulin signaling observed in the three tissues of septic animals.

Another TLR4 downstream pathway by which atorvastatin could attenuate the inflammatory response induced by sepsis is through JNK, a serine kinase that is responsible for activation of the inflammatory pathway by phosphorylation of the c-Jun and ATF2 transcription factors [59,60]. Several studies suggest that JNK contributes to insulin resistance by phosphorylating IRS-1 at serine 307, and this phosphorylation leads to inhibition of IRS-1 function [26,27,30,33,61,62,63,64], although this has very recently been questioned [65]. Here, we observed that sepsis led to serine phosphorylation of IRS-1 and that atorvastatin reversed this phenomenon in three target tissues, in parallel with a reduction in JNK activity. Our data showing that atorvastatin inhibits JNK phosphorylation/activation in septic rats indicate that the beneficial effect of this drug in improving survival and reducing insulin resistance is mediated by different pathways.

Besides being activated by TLR4, JNK activity is induced in different pathophysiological states including infection, inflamma-

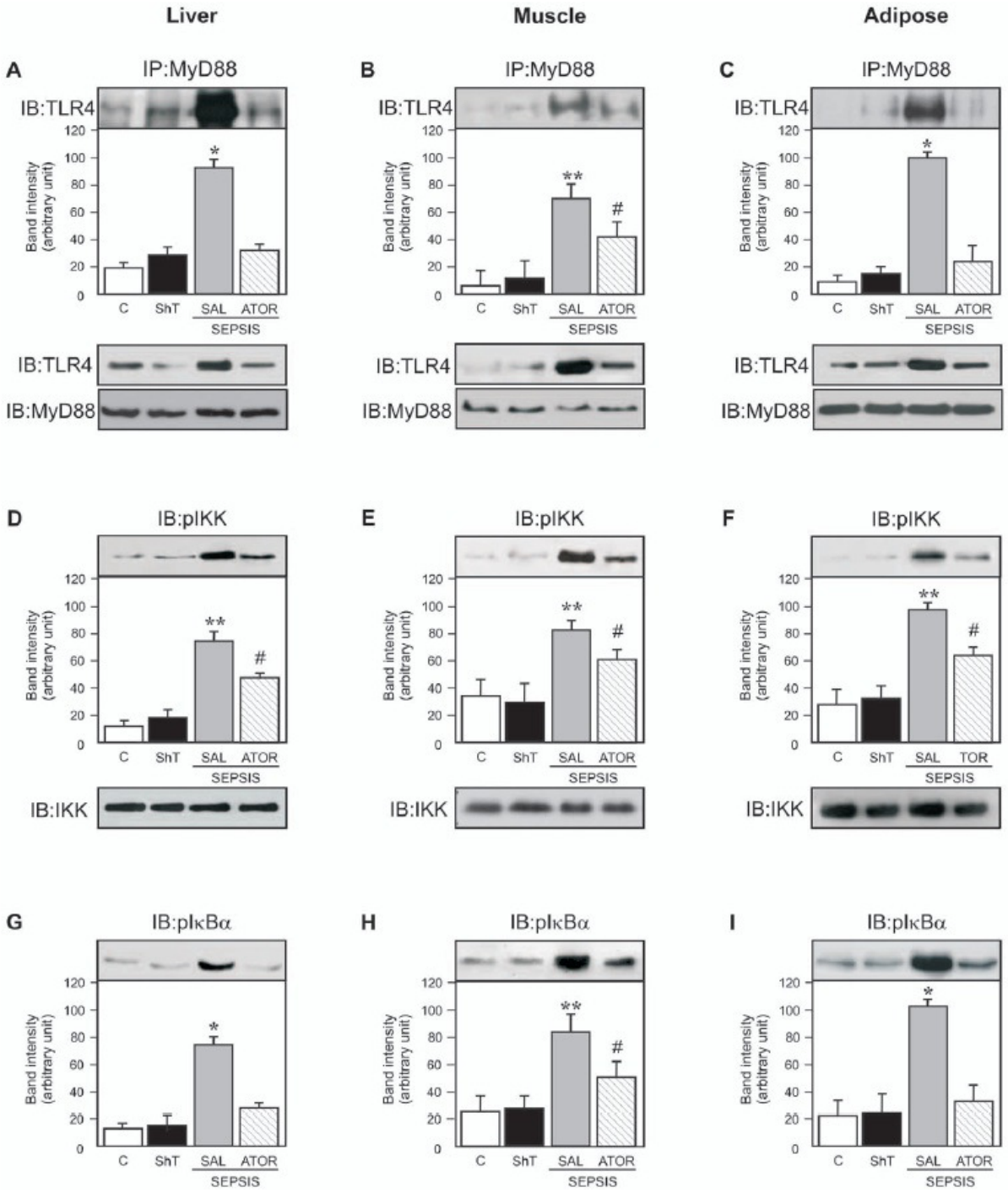

Figure 3. To evaluate the association of TLR4 with MyD88, immunoprecipitations were performed with MyD88 antibody followed by immunoblotting with TLR4 specific antibody. Representative blots show TLR4 activation (upper panels) and expression (lower panels) in liver (A), muscle (B) and adipose tissue (C) of sham and septic rats. IKKβ phosphorylation in liver (D), muscle (E) and adipose (F) of sham and septic rats. Total protein expression of IKKβ (D–F, lower panels). Phosphorylation of IκBα in liver (G), muscle (H) and adipose (I) of sham and septic rats. Data are presented as means ±S.E.M from 6–8 rats per group. *P<0.05 (Sepsis/Sal vs. all other groups); **P<0.001 (Sepsis/Sal vs. control); #P<0.05 (Sepsis/Sal vs. Sepsis/Ator). IB, immunoblot; CLT: Sham/Saline; ShT: Sham/Atorvastatin; SAL: saline; ATOR: atorvastatin.
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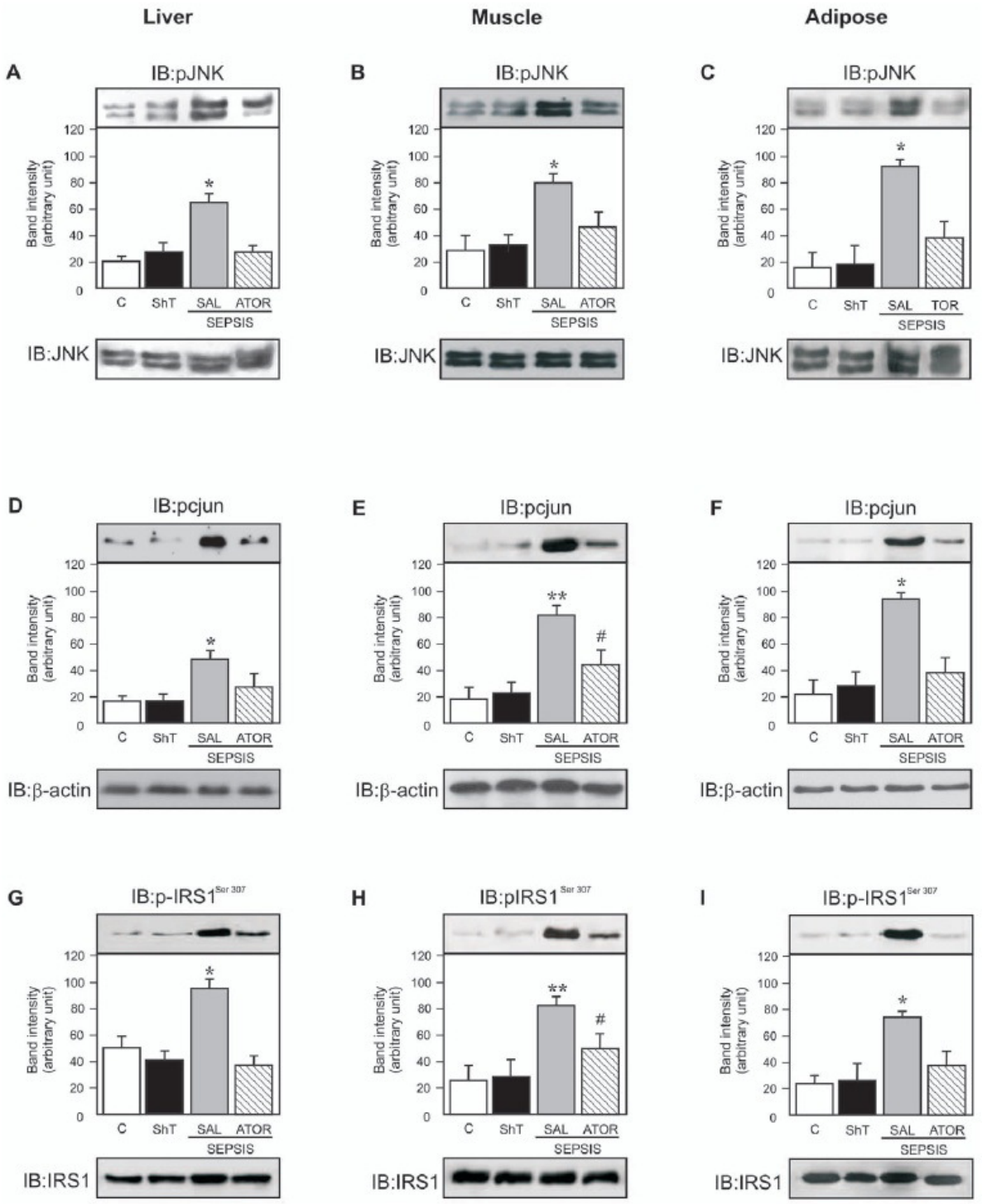

Figure 4. Representative blots show the JNK phosphorylation in liver (A), muscle (B) and adipose tissue (C) of sham and septic rats (upper panels). Total protein expression of JNK (A–C, lower panels). Phosphorylation of c-jun in liver (D), muscle (E) and adipose tissue (F) of sham and septic rats. Serine 307 Phosphorylation of IRS1 in liver (G), muscle (H) and adipose tissue (I) of sham and septic rats (upper panels). Total protein expression of IRS-1 (G–I, lower panels). Data are presented as means ± S.E.M from 6–8 rats per group. *P<0.05 (Sepsis/Sal vs. all others groups); **P<0.001 (Sepsis/Sal vs. control); #P<0.05 (Sepsis/Sal vs. Sepsis/Ator). IB, immunoblot; CLT: Sham/Saline; ShT: Sham/Atorvastatin; SAL: saline; ATOR: atorvastatin.

doi:10.1371/journal.pone.0014232.g004

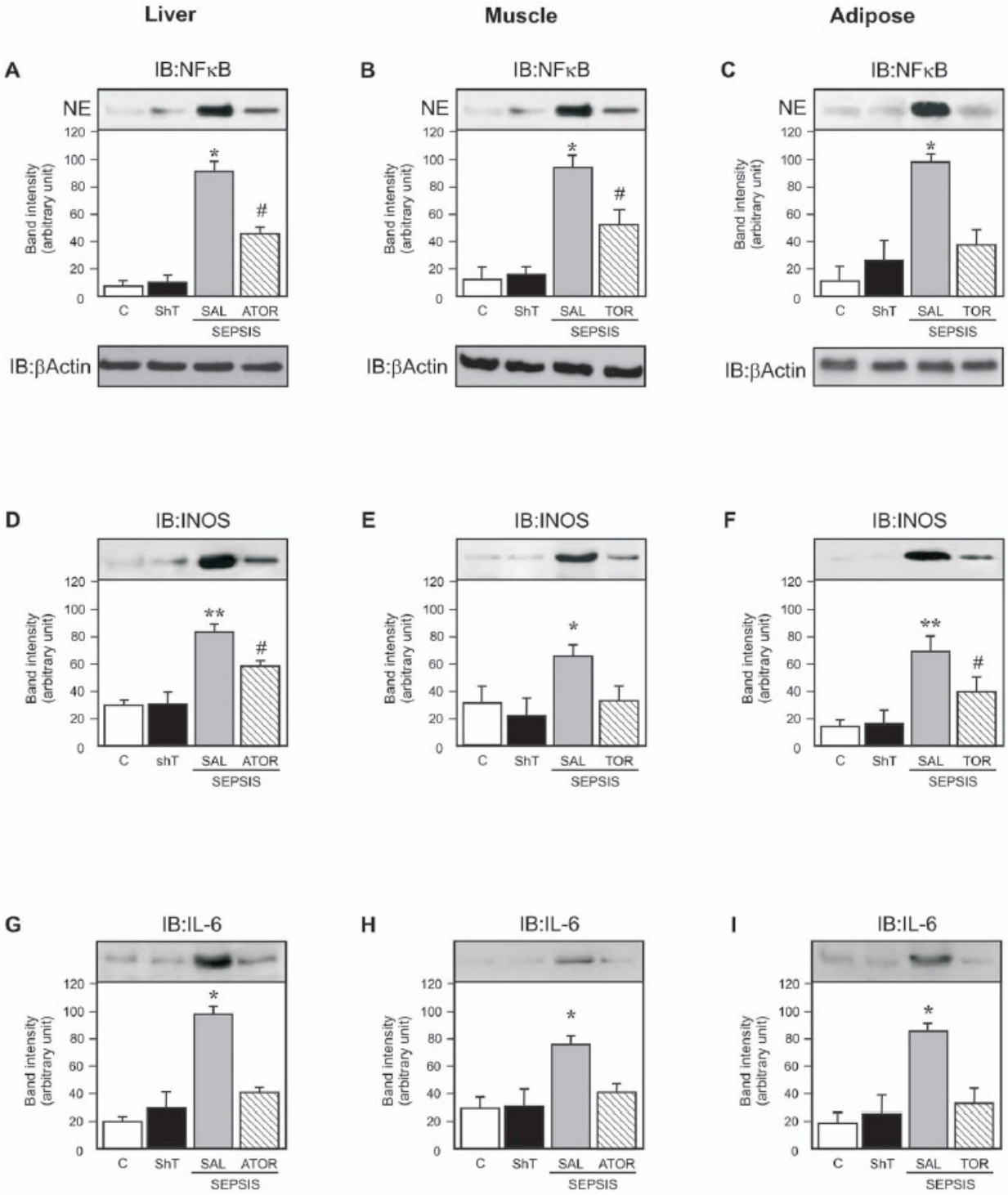

Figure 5. Representative blots show the NFkB activation in nuclear fractions of liver (A), muscle (B) and adipose tissue (C) of sham and septic rats. In this case blots were stripped and reprobed with actin (A–C, lower panels) to confirm equal loading of proteins. Tissue levels of iNOS (D–F) and IL-6 (G–I) expression in liver, muscle and adipose tissue of sham and septic rats. Data are presented as means ± S.E.M from 6–8 rats per group. *P<0.05 (Sepsis/Sal vs. all others groups); **P<0.001 (Sepsis/Sal vs. control); #P<0.05 (Sepsis/Sal vs. Sepsis/Ator). IB, immunoblot; CLT: Sham/Saline; ShT: Sham/Atorvastatin; SAL: saline; ATOR: atorvastatin.
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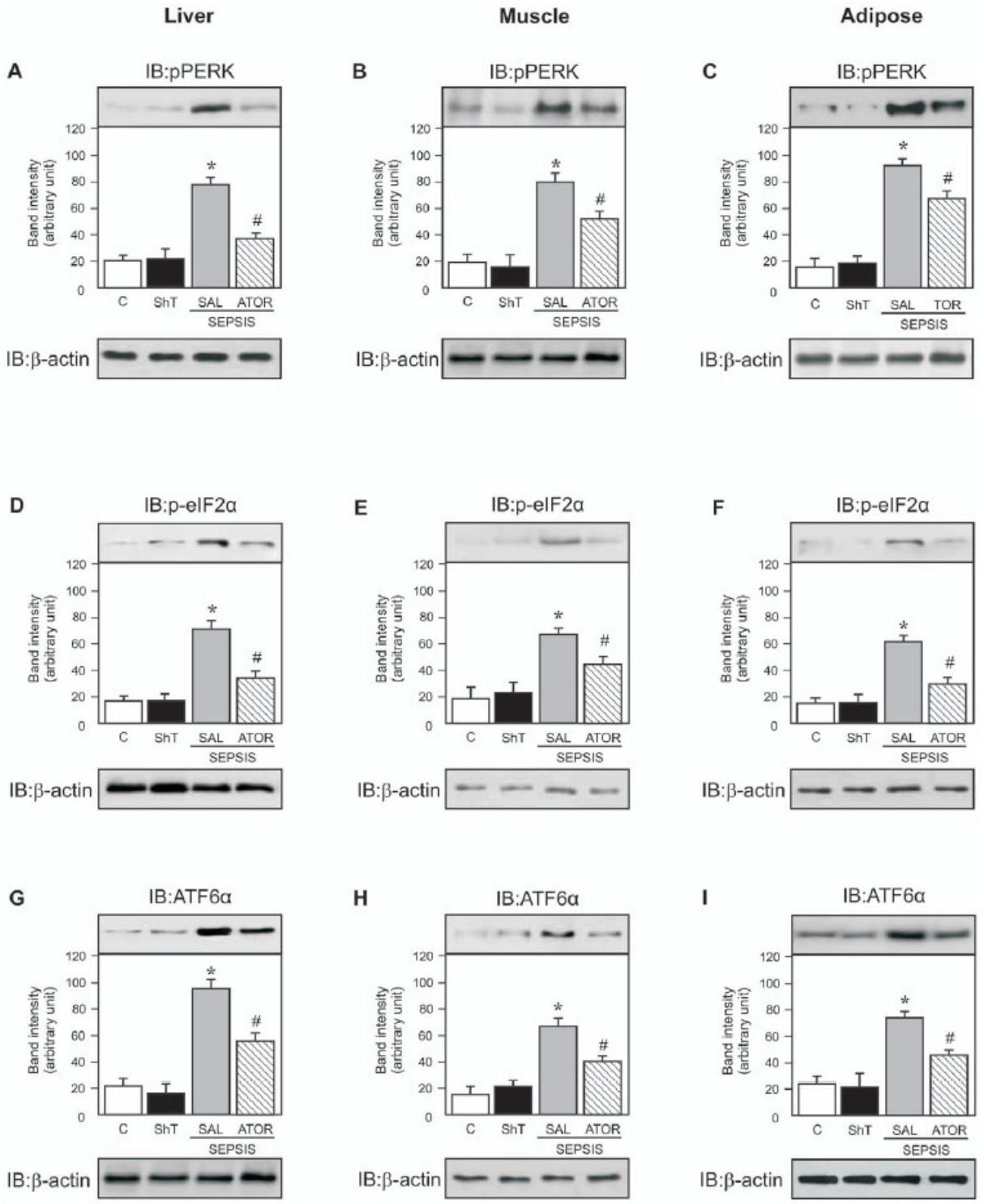

Figure 6. Representative blots show the PERK phosphorylation in liver (A), muscle (B) and adipose tissue (C) of sham and septic rats. eIF2α phosphorylation (D–F) and ATF6 (G–I) expression in liver, muscle and adipose tissue of sham and septic rats. In this case, blots were stripped and reprobed with actin (A–I, lower panels) to confirm equal loading of proteins. Data are presented as means ± S.E.M from 6–8 rats per group. *P<0.05 (Sepsis/Sal vs. all others groups); #P<0.05 (Sepsis/Sal vs. Sepsis/Ator). IB, immunoblot; CLT: Sham/Saline; ShT: Sham/Atorvastatin; SAL: saline; ATOR: atorvastatin.
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tion, obesity and hyperlipidemia, also as a consequence of ER stress [66,67,68]. The cellular response to ER stress, referred to as UPR, results in the activation of three linked signal transduction pathways emanating from three principle ER stress sensors: IRE1α, double-stranded RNA–dependent protein kinase–like kinase (PERK) and ATF6α [49,69]. Mechanistically, activation of the UPR contributes to the decrease in insulin sensitivity through IRE1α-dependent activation of c-Jun N-terminal kinase (JNK) [70,71]. Recently, it was demonstrated that statins are able to prevent ER stress [72]. In the present study, we confirm this finding by showing that atorvastatin strongly inhibited phosphorylation of IRE1α and PERK and ATF6α expression, suggesting that this drug can attenuate the ER-stress induced by sepsis.

Overall, our data demonstrate that atorvastatin exerts direct regulatory effects on TLR4 expression/activation in an animal model of sepsis that influences TLR4 signaling. Atorvastatin reduces TLR4 surface expression on liver, muscle and adipode tissues, causing downregulation of proinflammatory pathways such as IKK and JNK, leading to a decrease in NF-κB activation and cytokine expression. In addition, atorvastatin also decreases ER stress and, consequently, the activation of JNK and IKK. Thus, we suggest that the effects of atorvastatin on TLR4 expression/activation and on ER stress are mechanistically relevant to improve the sepsis-induced insulin resistance. There are several potential limitations to the present study.

To date, pharmacological interventions in sepsis have usually been limited to insulin and Protein C [7]. The beneficial effects of activated protein C are partially independent from its anticoagulant activity and may be related to anti-inflammatory and anti-apoptotic effects, but its effect on insulin signaling is unknown. Insulin has anti-inflammatory effects [11,12,13,19], which are dependent on PI3K signaling, and the infusion of this hormone induces the PI3K/Akt pathway. However, the beneficial effect of insulin may be overcome by hypoglycemia. It is important to mention that, in sepsis, many inflammatory pathways are activated in parallel with a reduction in the PI3K/Akt pathway, thus, merely blocking a single component of the inflammatory pathways or inducing the activation of PI3K may be insufficient to arrest the process. In this regard, our data show that atorvastatin is able to modulate entire families of inflammatory mediators, associated with a clear improvement in tissue insulin signaling and in insulin sensitivity, suggesting mechanisms for its efficiency in sepsis. Additionally, our data may suggest that the investigation of drugs in sepsis should take into account tissue measurements of inflammatory pathways and insulin signaling, or other early tissue markers that indicate the severity of sepsis.

In summary, this study demonstrated that treatment with atorvastatin increased survival with a significant effect upon insulin sensitivity, improving insulin signaling in peripheral tissues of the rat during peritoneal-induced sepsis. This drug reduces TLR4 activation, in association with downstream JNK and IKK/NF-κB activation and downregulated the serum levels of cytokine release. The effect of atorvastatin on TLR-dependent inflammatory pathway suppression may play a central role in the regulation of insulin signaling and survival following the sepsis insult.

Materials and Methods

Materials

Anti-IR-β (α-IR), anti-IRS-1, anti-Akt, anti-p-JNK, anti-iNOS, anti-NFκB, anti-IL6, anti-TLR4, anti-IKKβ, anti-pIKKβ, anti-pIκBα, anti-MyD88, anti-p-cjun, anti-pJNK, anti-pPERK anti-PERK, anti-ATF6α and anti-IRE1α antibodies were from Santa Cruz Technology (Santa Cruz, CA, USA). Anti-pAkt was from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-IRS-1^{scr307} was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Anti-peIF2α was from Abcam (Cambridge, MA, USA). Atorvastatin was obtained from Pfizer (Loughbeg, County Cork, Ireland). Human recombinant insulin was from Eli Lilly and Co. (Indianapolis, Indiana, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless specified elsewhere.

Animal Care and Experimental Procedures

All experiments were approved by the Ethics Committee at the University of Campinas, CEEA/Unicamp 1267-1. Male Wistar-Hannover (8 wks old) were maintained in a room with 12-hour day/night cycles and room temperature of 21°C, with food and water *ad libitum*. Wistar rats were randomly divided into four groups; atorvastatin-treated sepsis (Sepsis/Ator), saline-treated sepsis (Sepsis/Sal), atorvastatin-treated sham (Sham), and saline-treated sham (Control).

Cecal ligation and puncture (CLP) was performed, as previously described [73], and is a commonly-used surgical technique in rodents and thought to be a clinically relevant animal model of sepsis. Anesthesia was induced by IP administration of ketamine (80 mg/kg BW) and xylazine (15 mg/kg). Through a 1-cm abdominal midline incision, the cecum was ligated below the ileocecal valve with careful attention to avoid obstruction of the ileum or colon. The cecum was subjected to a four "through-and-through" perforations (20-gauge needle). The abdominal incision was closed in layers. Sham-operated rat underwent the same procedure, except for ligation and perforation of the cecum. All procedures were performed under sterile conditions. Animals were allowed to recover and were observed twice a day. Three hours after the induction of sepsis and every 24 hours rats received atorvastatin (10 mg/kg) or an equivalent volume of saline by oral gavage. The dose of atorvastatin was chosen on the basis of previous findings [74] and was consistent with the maximum human dose (1.1 mg/kg per day), and the higher metabolic rate of the drug in rodents [75].

Homeostasis Model Assessment

Insulin resistance was assessed from fasting insulin and glucose levels, using the previously validated homeostasis model of assessment (HOMA-IR), as previously described [76,77]. HOMA-IR was calculated by the formula: fasting plasma glucose (mmol/l) x fasting plasma insulin (mU/l)/22.5. Fasting blood glucose was measured by the glucose oxidase method. Plasma insulin was assayed using commercial rat-specific radioimmunoassay kits (Linco Research Inc, St. Louis, MO, USA).

Insulin tolerance Test (ITT)

The insulin tolerance test (ITT) was performed on these rats at 24 hours after sepsis, as previously described [78]. Insulin (1.5 U/kg) was administered by i.p. injection and blood samples were collected at 0, 5, 10, 15, 20, 25, and 30 min to determine serum glucose. The constant rate for glucose disappearance (Kitt) was calculated using the formula 0.693/t1/2. Glucose t1/2 was calculated from the slope of the least-squares analysis of plasma glucose concentrations during the linear decay phase [76].

Cytokines Assays

IL-6 and TNF-α were determined using commercially available ELISA kits (Pierce Biotechnology Inc., Rockford, IL, USA), following the instructions of the manufacturer.

Tissue Extraction, Immunoprecipitation and Immunoblotting

Rats were anaesthetized by intraperitoneal injection of sodium thiopental and were used 10–15 min later, *i.e.* as soon as anesthesia was assured by the loss of pedal and corneal reflexes. Five minutes after saline (0.2 ml) or insulin injection (3.8 U/Kg ip), liver, muscle and adipose tissue were removed, minced coarsely and homogenized immediately in extraction buffer, as described elsewhere. Extracts were used for immunoprecipitation with MyD88 and Protein A-Sepharose 6MB (Pharmacia, Uppsala, Sweden). NFκB p50 activation was determined in nuclear extracts from liver, muscle and adipose tissue, as previously described [79]. The precipitated proteins and/or whole tissue extracts were subjected to SDS-PAGE and immunoblotting, as previously described [40,78].

Statistical Analysis

The overall difference in survival rate was determined by the Kaplan–Meier test followed by log–rank test. Specific protein bands present in the blots were quantified by digital densitometry (ScionCorp Inc., Frederick, MD, USA). Means ± S.E.M. obtained from densitometric scans, area measurements, and the values for blood IL-6, TNF-α and glucose were compared by ANOVA with post hoc test (Bonferroni). A P value of $p<0.05$ was accepted as statistically significant.

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Author Contributions

Conceived and designed the experiments: KLC MJAS. Performed the experiments: KLC BdMC FCM ACAC DG MJAS. Analyzed the data: KLC BdMC ERR JBCC MJAS. Contributed reagents/materials/analysis tools: JBCC MJAS. Wrote the paper: KLC ERR MJAS.

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CAPÍTULO 2

Diacerhein attenuates the inflammatory response and improves survival in a model of severe sepsis

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Keywords

Sepsis; Insulin resistance; Diacerhein

Abstract

Introduction: Hyperglycemia and insulin resistance has been associated with a worse outcome in sepsis. Despite tight glycemic control through insulin therapy shows to reduce morbidity and mortality rates, the effect of intensive insulin therapy in patients with severe sepsis is controversial because of the increased risk of serious adverse events related to hypoglycemia. Recently, knowledge about diacerhein, an anthraquinone drug with powerful anti-inflammatory properties, revealed that this drug improves insulin sensitivity, mediated by the reversal of chronic subclinical inflammation. The aim of the present study was to evaluate whether the anti-inflammatory effects of diacerhein after onset of sepsis-induced glycemic alterations is beneficial and whether the survival rate is prolonged in this situation.

Methods: Diffuse sepsis was induced by cecal ligation and puncture surgery (CLP) in male Wistar rats. Serum glucose and inflammatory cytokines levels were assessed 24 h after CLP. The effect of diacerhein on survival of septic animals was investigated in parallel with insulin signaling and its modulators in liver, muscle and adipose tissue. Results: Here we demonstrated that diacerhein treatment improves survival during peritoneal-induced sepsis and inhibits sepsis-induced insulin resistance by improving insulin signaling via increased IRS-1-associated PI3-kinase activity and Akt phosphorylation. Diacerhein also decreases the activation of endoplasmic reticulum stress signaling that involves up-regulation of pro-inflammatory pathways, such as the IKK and JNK, which blunts insulin-induced insulin signaling in liver, muscle and adipose tissue. Additionally, our data shows that this drug promoted down-regulation of pro-inflammatory signaling cascades which culminate in transcription of immunomodulatory factors such IL-1β, IL-6 and TNF-α.

Conclusions: This study demonstrated that diacerhein treatment increased survival, attenuates the inflammatory response with a significant effect upon insulin sensitivity. On the basis of efficacy and safety profile, diacerhein represents a novel anti-inflammatory therapy for management of insulin resistance in sepsis and a potential approach for future clinical trials.

Introduction

Sepsis is defined as a systemic inflammatory response syndrome caused by the body's response to an infection [2]. During the onset of sepsis, the inflammatory system becomes hyperactive, leading to an production of pro-inflammatory molecules and cytokine release [3], which contribute to septic shock, multiple organ failure and death. Hyperglycemia and insulin resistance occur as a consequence of the metabolic effects of stress hormones and the over-production of pro-inflammatory mediators in sepsis [4-5]. In this regard, tissue insulin resistance may be used as an important indicator of the resultant actions of the pro-inflammatory cytokines, being a tissue marker of the severity of sepsis prior to organ failure. In addition, this insulin resistance may also aggravate sepsis, as previously described [5-7].

Both bacterial components such as LPS, and pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, resulting from the immune response to sepsis, may activate intracellular mechanisms associated with insulin resistance such as the IKK/NF-κB and JNK pathways. JNK has been shown to promote insulin resistance through serine phosphorylation of IRS-1, preventing signaling from the insulin receptor. Furthermore, IKK induces insulin resistance through activation of NF-κB, which in turn induces the transcription of several genes related to pro-inflammatory cytokine release (IL-1β, TNF-α, IL-6 and IL-8) [8]. IKK activation leads to phosphorylation, ubiquitination and degradation of IκB, which releases NF-κB, allowing it to translocate to the nucleus and activate transcription of target genes [9].

In this regard, we believe that the evaluation of insulin signaling pathways through PI3K/Akt in liver, muscle and adipose tissue may be important indicators of this over-reaction at the tissue level, and the improvement in this signaling pathway, induced by

some treatments, in parallel with a decrease in tissue inflammation, may predict the effectiveness of this treatment. Substantial resources have been invested in developing and evaluating potential therapies to sepsis and in increasing knowledge of systemic inflammation and multiple-system organ failure [10-11], although pharmacologic interventions available at the present time are not effective in decreasing the high mortality rates.

Diacerhein (4,5-diacetoxy-9,10-dihydro-9,10-dioco-2-anthracenecarboxylic acid is an anthraquinone that shows anti-inflammatory properties, in addition to moderate analgesic and antipyretic characteristics [11], and is used in the treatment of osteoarthritis. Studies have suggested that diacerhein can exert beneficial effects on the symptoms of osteoarthritis including antiarthritic and chondroprotective effects [12]. Rhein, the active metabolic of diacerhein, has been demonstrated to inhibit the synthesis and activity of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β [13-16]. The compound also acts directly on inflammatory cells, inhibiting superoxide anion production by human neutrophils, release of lysosomal enzymes, chemotaxis and phagocytic activity of neutrophils and macrophages [17-21].

Further studies have suggested that diacerhein inhibits nitric oxide production induced by the reduction of IL-1β [22-23]. Besides its inhibitory effects on pro-inflammatory genes, rhein has been shown to have anti-tumor activity on several cancer cell lines [24-25]. Moreover, studies suggest that diacerhein and rhein inhibit NF-κB activation and expression of NF-κB-dependent genes [26]. In this regard, diacerhein has the potential to improve insulin resistance in sepsis and to reduce the over-reaction of the inflammatory response, without inducing hypoglycemia.

The aim of the present study was to investigate whether diacerhein, by reducing tissue activation of inflammatory pathways, can improve insulin signaling and survival in sepsis.

Materials and Methods

Anti-IR-β (α-IR), anti-p-IRS-1, anti-p-JNK, anti-JNK1, anti-pIKKβ, anti-pIkBa, anti-NF-kB, anti-pPERK and anti-IRE1 antibodies were from Santa Cruz Technology (Santa Cruz, CA, USA). Anti-pAkt was from Cell Signaling Technology (Beverly, MA, USA). Anti-p-IRS-1ser307 was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Anti-p-eIF2a was from Abcam (Cambridge, MA, USA). Diacerhein was kindly ceded by TRB-Pharma (Campinas, Brazil). Human recombinant insulin was from Eli Lilly and Co. (Indianapolis, Indiana, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless specified elsewhere.

Animal Care and Experimental Procedures

All experiments were approved by the Ethics Committee at the University of Campinas, CEEA/Unicamp 1267-1. Male Wistar- Hannover (8 wks old) were maintained in a room with 12-hour day/night cycles and room temperature of 21°C, with food and water ad libitum. Wistar rats were randomly divided into four groups; diacerhein -treated sepsis (Sepsis/Dia), vehicle-treated sepsis (Sepsis/Veh), diacerhein-treated sham (Sham), and vehicle treated sham (Control).

Polymicrobial sepsis was induced by subjecting rats to CLP, as previously described [27], and is a commonly-used surgical technique in rodents and thought to be a clinically relevant animal model of sepsis. Anesthesia was induced by IP administration of ketamine (80 mg/kg BW) and xylazine (15 mg/kg). Through a 1-cm abdominal midline incision, the cecum was ligated below the ileocecal valve with careful attention to avoid obstruction of the ileum or colon. The cecum was subjected to a four ‘‘through-and through’’ perforations (20-gauge needle). The abdominal incision was closed in layers.

Sham-operated rat underwent the same procedure, except for ligation and perforation of the cecum. All procedures were performed under sterile conditions. Animals were allowed to recover and were observed twice a day. Dried diacerhein was diluted in 0.01M PBS 3% DMSO, to a final concentration of 18 mg/ml.

Three hours after the induction of sepsis and every 24 hours rats received diacerhein (100 mg/kg per day) or an equivalent volume of vehicle by oral gavage. The dose of diacerhein was chosen on the basis of previous findings [28] and was consistent with the higher metabolic rate of the drug in rodents.

Homeostasis Model Assessment

The Insulin tolerance test (ITT) was performed on these rats at 24 hours after sepsis, as previously described [31]. Insulin (1.5 U/kg) was administered by i.p. injection and blood samples were collected at 0, 5, 10, 15, 20, 25, and 30 min to determine serum glucose by the glucose oxidase method.. The constant rate for glucose disappearance (Kitt) was calculated using the formula 0.693/t1/2. Glucose t1/2 was calculated from the slope of the least-squares analysis of plasma glucose concentrations during the linear decay phase [29].

Cytokines Assays

IL-1β, IL-6 and TNF-α were determined using commercially available ELISA kits (Pierce Biotechnology Inc., Rockford, IL, USA), following the instructions of the manufacturer.

Tissue Extraction and Immunoblotting

Rats were anaesthetized by intraperitoneal injection of sodium thiopental and were used 10–15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and

corneal reflexes. Five minutes after saline (0.2 ml) or insulin injection (3.8 U/Kg ip), liver, muscle and adipose tissue were removed, minced coarsely and homogenized immediately in extraction buffer, as described elsewhere. NF-kB p50 activation was determined in nuclear extracts from liver, muscle and adipose tissue. The precipitated proteins and/or whole tissue extracts were subjected to SDS-PAGE and immunoblotting, as previously described [32].

Statistical Analysis

The overall difference in survival rate was determined by the Kaplan–Meier test followed by log–rank test. Specific protein bands present in the blots were quantified by digital densitometry (ScionCorp Inc., Frederick, MD, USA). Means ± S.E.M. obtained from densitometric scans, area measurements, and the values for blood cytokines and glucose were compared by ANOVA with post hoc test (Bonferroni). A P value of $p<0.05$ was accepted as statistically significant.

Results

Diacerhein Improves Survival

To test the hypothesis that diacerhein decreases sepsis mortality we monitored the survival in animals in which sepsis was induced via CLP. Diacerhein (100mg/kg/day) or placebo was administered by gavage 3 hours post surgery, or procedure, in sham-operated animals. No deaths occurred in the sham-operated animals, whether or not they had been treated with diacerhein. The survival curves showed a significantly improved survival ($P<0.0001$) after diacerhein treatment (Figure 1A).

As shown in Fig. 1B, septic animals were more insulin resistant than sham-operated rats, fasting plasma glucose was higher in septic rats than in the control group and diacerhein treatment reduced both of these levels. As depicted in Fig. 1C, the plasma glucose disappearance rates measured during the insulin tolerance test (Kitt), were lower in septic animals, and diacerhein treatment attenuate this alteration. Diacerhein treatment had no effect on glucose tolerance in the sham group. Taken together, these data suggest that diacerhein improves sepsis-induced insulin resistance.

Effect of Diacerhein on Serum Levels of IL-1, IL-6 and TNF-α

IL-1β, IL-6 and TNF-α serum levels were examined in the four groups studied. As expected, cytokine expressions in the septic rats were higher than in the sham-operated rats. After diacerhein treatment there was a significant decrease in IL-1β (Fig. 1D), IL-6(Fig. 1E) and TNF-α (Fig. 1F) circulating levels.

Diacerhein Improves Insulin Signaling in Septic Animals

We then examined the effects of diacerhein administration on the insulin- signaling pathway in its main target tissues. In the sepsis group, insulin-induced IR and IRS-1 tyrosine phosphorylation were decreased in liver, muscle and adipose tissue when compared with sham rats, and these alterations were reversed by diacerhein (Fig. 2A–C). Also, there was a decrease in insulin-induced Akt serine phosphorylation in liver, muscle and adipose tissue of septic animals when compared with sham rats, and diacerhein was able to reverse this reduction in Akt phosphorylation (Fig. 2A–C). Equal protein loading in the gels was confirmed by re-probing the membranes with an anti-β-actin antibody (lower panels).

Diacerhein Attenuates Sepsis-Induced Inflammatory Changes

During sepsis, the activation of pro-inflammatory signaling involves up-regulation of intracellular inflammatory pathways, such as the IKKβ and JNK pathway. We examined the anti-inflammatory effects of diacerhein on the IKK/NF-κB pathway by monitoring the main function of IKK phosphorylation and degradation of the NF-κB inhibitor (IκBa) [33]. NF-κB was monitored through analysis of NF-κB p65 nuclear expression. As expected, IKKβ and IκB phosphorylation were increased in liver, muscle and adipose tissue of septic animals. When treated with diacerhein, septic rats showed a reduction in IKKβ and IκB phosphorylation in all tissues studied (Fig. 3A–C). Likewise, we assessed the nuclear translocation of NF-κB p65 and, as expected, in nuclear tissue extracts from treated septic rats, we detected reduced expression of NF-κB p65 compared with the non-treated group (Fig. 3A–C).

JNK activation was determined by monitoring phosphorylation of JNK1 and total expression of this protein. JNK phosphorylation in liver, muscle and adipose tissue was increased in septic animals, and diacerhein induced a down modulation in the phosphorylation of this serine kinase in liver and adipose tissue (Fig. 4A–C). We also investigated Ser307 phosphorylation of IRS-1 in the three tissues from the four groups of rats. Ser307 phosphorylation was induced by sepsis and the treatment with diacerhein reduce this alteration (Fig. 4A–C).

Previous studies have shown that sepsis is also characterized by endoplasmic reticulum (ER) stress. It is clear that ER stress can also induce activation of JNK and IKKβ. We therefore investigated the effect of sepsis (treated or untreated with diacerhein) on proteins that reflect ER stress. Our data showed that sepsis induced ER stress, with activation of the membrane kinase PERK (PKR-like endoplasmic reticulum kinase) and its substrate eIF2α (eukaryotic translation initiation factor 2α), and increased the expression

of IRE1 (Fig. 5A–C). Treatment with diacerhein significantly reduced the activation of IRE1, PERK and its substrate eIF2α (Fig. 5A-C).

Discussion

In the present study, we demonstrated that administration of the anti-inflammatory diacerhein improves survival during peritoneal-induced sepsis, with a significant effect upon insulin sensitivity. In addition, this drug promoted down-regulation of pro-inflammatory signaling cascades which culminate in the transcription of immunomodulatory factors such interleukins and TNF-α. The improvement in survival of septic animals when treated with diacerhein has not been previously described. However, multiple studies have found that the anti-inflammatory effects of diacerhein include anti-catabolic and anti-proliferative properties, and this may explain its value in the treatment of cancer [34] and joint diseases [35].

Our data show that diacerhein, administered 3 hours after the induction of sepsis, is able to improve the survival curve, attenuating increased levels of IL1- β, IL-6 and TNF-α, and reduce insulin resistance, as demonstrated by the insulin tolerance test. The improvement in insulin sensitivity was probably due to the increased IRS-1-associated PI3-kinase activity and Akt phosphorylation. It is possible that reduced insulin signaling through the IRSs/PI3K/Akt pathways in sepsis, may contribute to multi-organ failure by activation of apoptosis [36]. Additionally, studies have shown that prevention of apoptosis may be a potential treatment of sepsis in humans [37-39]. Diacerhein, by improving insulin-induced PI3K and Akt, may play a critical role in protection from apoptosis in sepsis.

Growing evidence suggests that the PI3K/Akt pathway plays an important role as a negative regulator of the innate response by excessive production of pro-inflammatory mediators. The PI3K-Akt pathway has been shown to negatively regulate NF-κB and the

expression of inflammatory genes [40-42]. Inhibition of the PI3K-Akt pathway enhances LPS-induced TNF-α and TF gene expression [43] and activates the mitogen-activated protein kinase pathways (ERK1/2, p38, and JNK) and the downstream target AP-1 [44]. Relevant in this regard is a report showing that PI3K knockout mice fail to respond to LPS [45]. In addiction, studies have demonstrated that activation of the PI3K/AKT pathway by α-lipoic acid has a beneficial effect upon inflammatory response and mortality improvement in endotoxemic mice [46].

In this study, we demonstrated that treatment with diacerhein inhibits sepsis-induced insulin resistance by improving insulin signaling via the IR–IRS-1–Akt pathway in target tissues. Studies show that tight glycemic control through insulin therapy reduces morbidity and mortality rates [47]. Concomitantly, studies indicate that insulin may have direct anti-inflammatory effects, and this effect appears to be mediated by the PI3K pathway [43-44, 48]. In this context, is possible that the restoration of this pathway, induced by diacerhein, may contribute to the anti-inflammatory effect of this drug.

During the past years, studies have shown a direct link between metabolic and immune signaling systems in different conditions of insulin resistance [7, 49-51]. We and others have previously demonstrated that triggering inflammatory signaling through IKKβ and JNK is activated in metabolic disorders and that this activation culminates in an increase in pro-inflammatory gene expression, which may have critical roles in insulin resistance [32, 51-52].

Our data show that diacerhein decreases IKKβ/IκB/NF-κB pathway activation, a modulation that may play a role in the attenuated expression of inflammatory mediators in response to a septic insult. IKK is a serine kinase and its activation phosphorylates IκB, a cytoplasmic protein that inhibits nuclear translocation of NF-κB. After its phosphorylation, IκB is ubiquitinated and consequently degraded in the proteasome, releasing NF-κB for translocation to the nucleus and activation of gene expression and the transcription of pro-

inflammatory and immunomodulatory factors such TNF-α and IL-1 [53-54]. Diacerhein induced a significant decrease in IKK phosphorylation, and an increase in IκB total expression, suggesting a deactivation of this pathway. IKK pathway activation causes an increase in serine residue phosphorylation of IR and IRS-1, inducing insulin resistance [55-56]. It has been proposed that increased IKKβ activity can inhibit insulin-stimulated PI3-kinase activity [57]. In this respect, the capacity of insulin to stimulate PI3-kinase activity and Akt phosphorylation was completely restored in septic animal treated with diacerhein.

Evidence supporting a pivotal role for NF-κB activation in inflammatory response is undeniable. Enhanced NF-κB activation is associated with a worse outcome in sepsis [58-60]. Increased NF-κB activity is observed in every form of inflammation and inhibiting NF-κB activation prevents the development of those pathological conditions [61]. NF-κB is critical for maximal expression of many cytokines involved in the pathogenesis of inflammation [62-63]. NF-κB nuclear translocation induces the release of IL-1β, IL-6 and TNF-α [64]. TNF-α levels are drastically elevated in human sepsis, in turn correlating with increased mortality [49]. Cytokines plays a crucial role in pro-inflammatory events and contribute to insulin resistance. In this regard, the inhibition of NF-κB activation explains the reduced levels of TNF-α, IL-1β and IL-6 detected in the serum of diacerhein-treated animals, and consequently, the improvement in the sepsis-induced insulin resistance process. The picture of hyperglycemia and insulin resistance observed in sepsis, often referred to as “stress diabetes”, reflects the activation of signaling pathways and expression of inflammatory mediators which inhibit insulin action, such as IL-6 and TNF-α.

An initial investigation by Van den Berghe and colleagues [66] suggested that controlling blood glucose levels by intensive insulin therapy decreased mortality and morbidity following surgery on critically ill patients. Moreover, intensive insulin therapy halved the prevalence of bloodstream infections and prolonged inflammation, showing the anti-inflammatory action of insulin. These findings are supported by further studies

demonstrating that insulin has anti-inflammatory effects via activation of the PI3K-Akt pathway and has been shown to negatively regulate NF-κB [43-44]. On the other hand, the role of intensive insulin therapy in patients with severe sepsis is uncertain because the beneficial effect of insulin may be overcome by the increased risk of serious adverse events related to hypoglycemia [67]. We propose that drugs capable of reversing sepsis-induced insulin resistance, and maintenance of adequate glycemic control, may be a potential therapy for sepsis. Since many inflammatory pathways are triggered, merely blocking a single component is likely to be insufficient to halt the process [68-69]. Indeed, therapies modulating entire families of mediators seem to be more efficacious [68-70]. In this line, diacerhein may be a potential therapeutic strategy for sepsis, with a significant effect upon insulin sensitivity and insulin signaling in peripheral tissues.

Another mechanism involved in host response to sepsis is activation of the pro-inflammatory JNK pathway. JNK acts by phosphorylation of the c-Jun and ATF2 transcription factors [71-72]. Several studies suggest that JNK contributes to insulin resistance. JNK is capable of blocking insulin signaling through two mechanisms. Firstly, JNK directly phosphorylates IRS-1 on serine residues [52,73-74], although this has recently been questioned [75]. Secondly, JNK activates several negative modulators of insulin signaling, as well as TNF-α [76]. Our data show that diacerhein inhibits JNK phosphorylation in septic rats and indicates that the beneficial effect of this drug in improving survival and reducing insulin resistance is mediated by different pathways.

Moreover, we observed that sepsis led to serine phosphorylation of IRS-1 and that diacerhein reduced this phenomenon in three target tissues, in parallel with a reduction in JNK activity. Thus, negative modulators of the insulin intracellular cascade such as JNK and IKK are partly responsible for the establishment of insulin resistance and represent potential therapeutic targets for sepsis induced insulin resistance. This hypothesis is supported by evidence that JNK inhibitors improve insulin sensitivity in insulin resistant

mice [77]. Numerous lines of evidence show that JNK can function as a pro-apoptotic kinase [78-79]. Despite being activated by inflammation and infection, JNK activity is induced in many different pathophysiological states, including obesity and hyperlipidemia, also as a consequence of ER stress.

One mechanism that, based on newly emerging data, appears to have a central role in the activation of inflammatory pathways is ER stress [80]. Under stress conditions, unfolded proteins accumulate in the ER and initiate an adaptive response known as the unfolded protein response (UPR). UPR is initiated through three ER trans-membrane sensors, namely IRE1α, double-stranded RNA–dependent protein kinase–like kinase (PERK) and activating transcription actor-6 (ATF-6), which activate an adaptive response that results in cessation of protein translation and a transcriptional increase in protein-folding chaperones and ER-associated degradation genes [81]. If ER stress continues for a certain period then programmed cell death is triggered. This response has a close relation to sepsis, once sepsis generates conditions that increase the demand on the ER. In both in vitro and in vivo studies, ER stress leads to activation of JNK and thus contributes to insulin resistance [82-84]. Interestingly, ER stress also activates IKK and thus may represent a common mechanism for the activation of these two important signaling pathways [85].

In the present study, we showed that diacerhein strongly inhibited phosphorylation of PERK and its substrate eIF2α, as well as IRE1expression, suggesting that this drug can attenuate ER stress induced by sepsis. Over the last decade, accumulating evidence shows that inflammatory signaling pathways can also become activated by metabolic stresses originating from inside the cell, as well as by extracellular signaling molecules. ER stress plays a central role in the activation of inflammatory signaling. Acute inflammation, oxidative stress and ER stress seem to contribute to the association of sepsis with insulin resistance. The ER is an attractive potential therapeutic target in sepsis because proper

ER function may prevent apoptosis [86]. The pharmacological attenuation of all aforementioned stresses leads to improved insulin sensitivity and consequently favorable sepsis outcomes.

In summary, our data show that the administration of the anti-inflammatory diacerhein in septic animals increased survival, with a significant effect upon insulin sensitivity and insulin signaling in peripheral tissues. The treatment also reduced NF-κB activation, in association with upstream JNK and IKK activation, down-regulated serum levels of cytokines and improved ER stress. These results indicate that diacerhein treatment attenuates insulin resistance in sepsis through modulation of inflammatory pathways, and that this drug may be an alternative therapy for management of insulin resistance in sepsis.

Conclusion

This is the first report demonstrating that diacerhein reverses insulin resistance in sepsis through attenuation of the inflammatory response and improves survival in CLP model of sepsis in rats. On the basis of efficacy, safety profile and rare side effects, this drug may be an alternative therapy for management of insulin resistance in sepsis.

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Figure 1

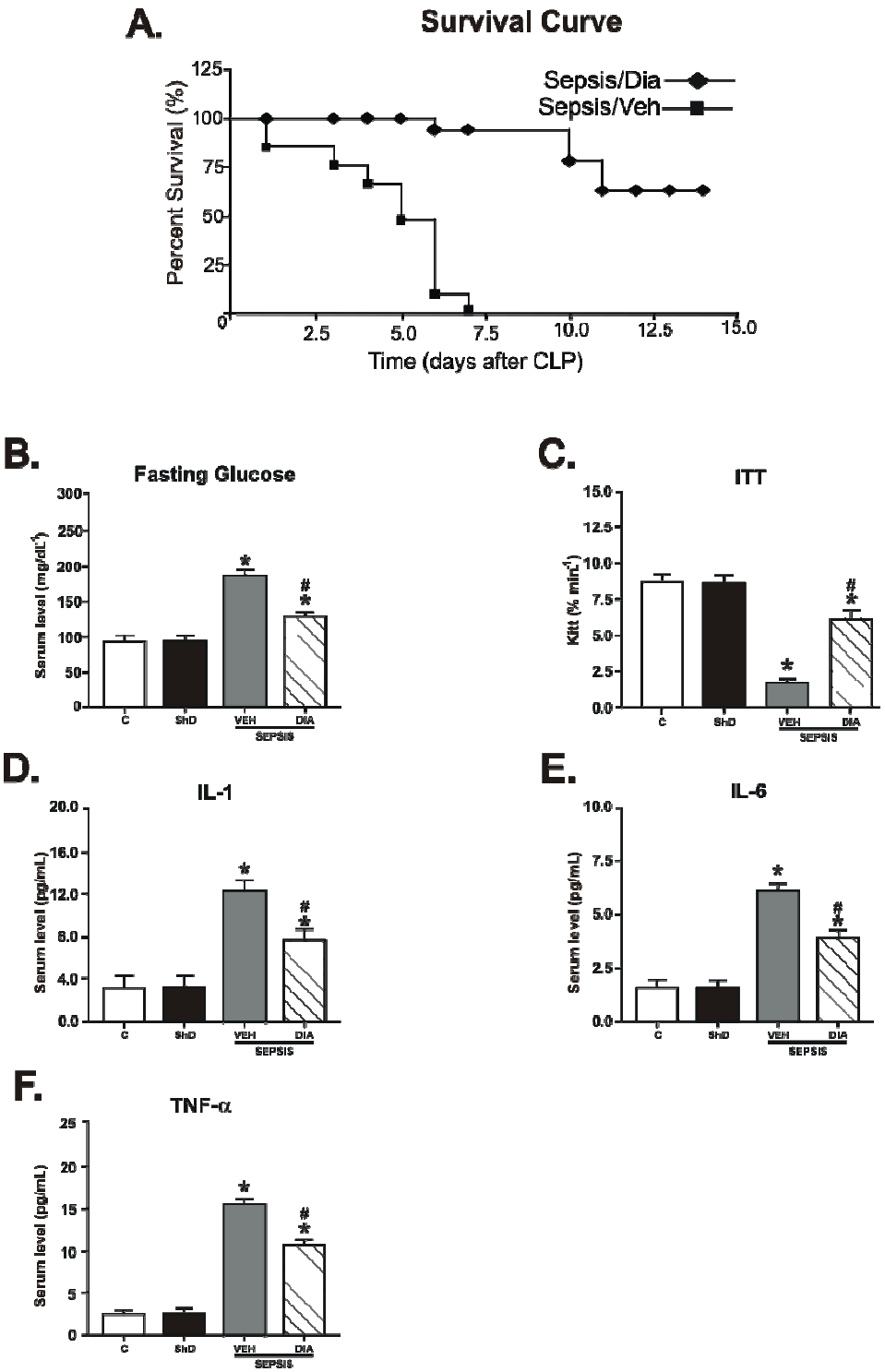
A.
Survival Curve
Sepsis/Dia
Sepsis/Veh
Percent Survival (%)
Time (days after CLP)
B.
Fasting Glucose
Serum level (mg/dL)
C.
ITT
Kitt (% min-1)
D.
IL-1
Serum level (pg/mL)
E.
IL-6
Serum level (pg/mL)
F.
TNF-α
Serum level (pg/mL)
C
ShD
VEH
DIA
SEPSIS

Figure 2

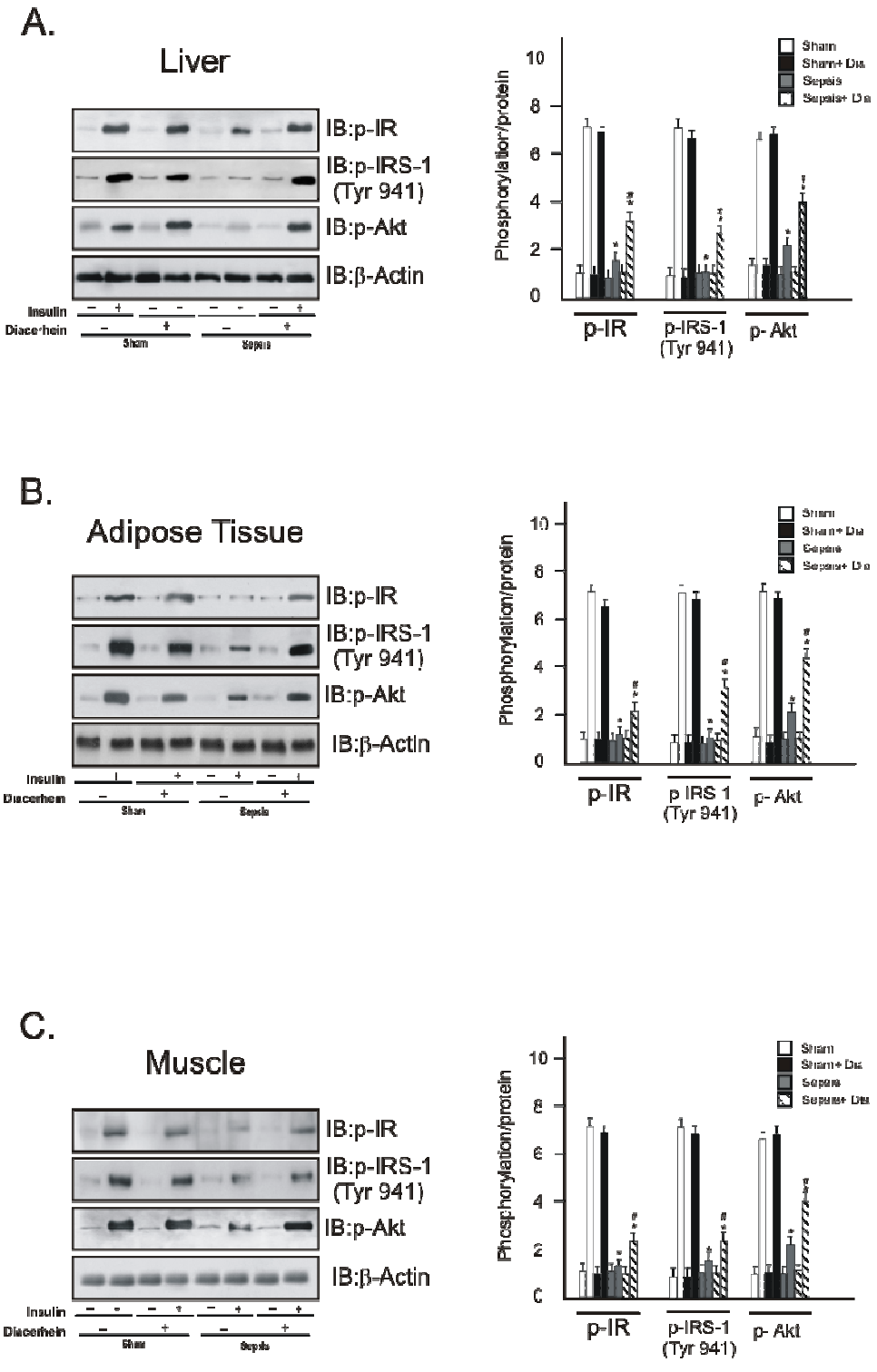
A.
Liver
IB:p-IR
IB:p-IRS-1 (Tyr 941)
IB:p-Akt
IB:β-Actin
Insulin
Diacerhein
Sham
Sepsis
Phosphorylation/protein
Sham
Sham+Dia
Sepsis
Sepsis+Dia
p-IR
p-IRS-1 (Tyr 941)
p-Akt
B.
Adipose Tissue
IB:p-IR
IB:p-IRS-1 (Tyr 941)
IB:p-Akt
IB:β-Actin
Insulin
Diacerhein
Sham
Sepsis
Phosphorylation/protein
Sham
Sham+Dia
Sepsis
Sepsis+Dia
p-IR
p IRS 1 (Tyr 941)
p- Akt
C.
Muscle
IB:p-IR
IB:p-IRS-1 (Tyr 941)
IB:p-Akt
IB:β-Actin
Insulin
Diacerhein
Sham
Sepsis
Phosphorylation/protein
Sham
Sham+Dia
Sepsis
Sepsis+Dia
p-IR
p-IRS-1 (Tyr 941)
p- Akt

Figure 3

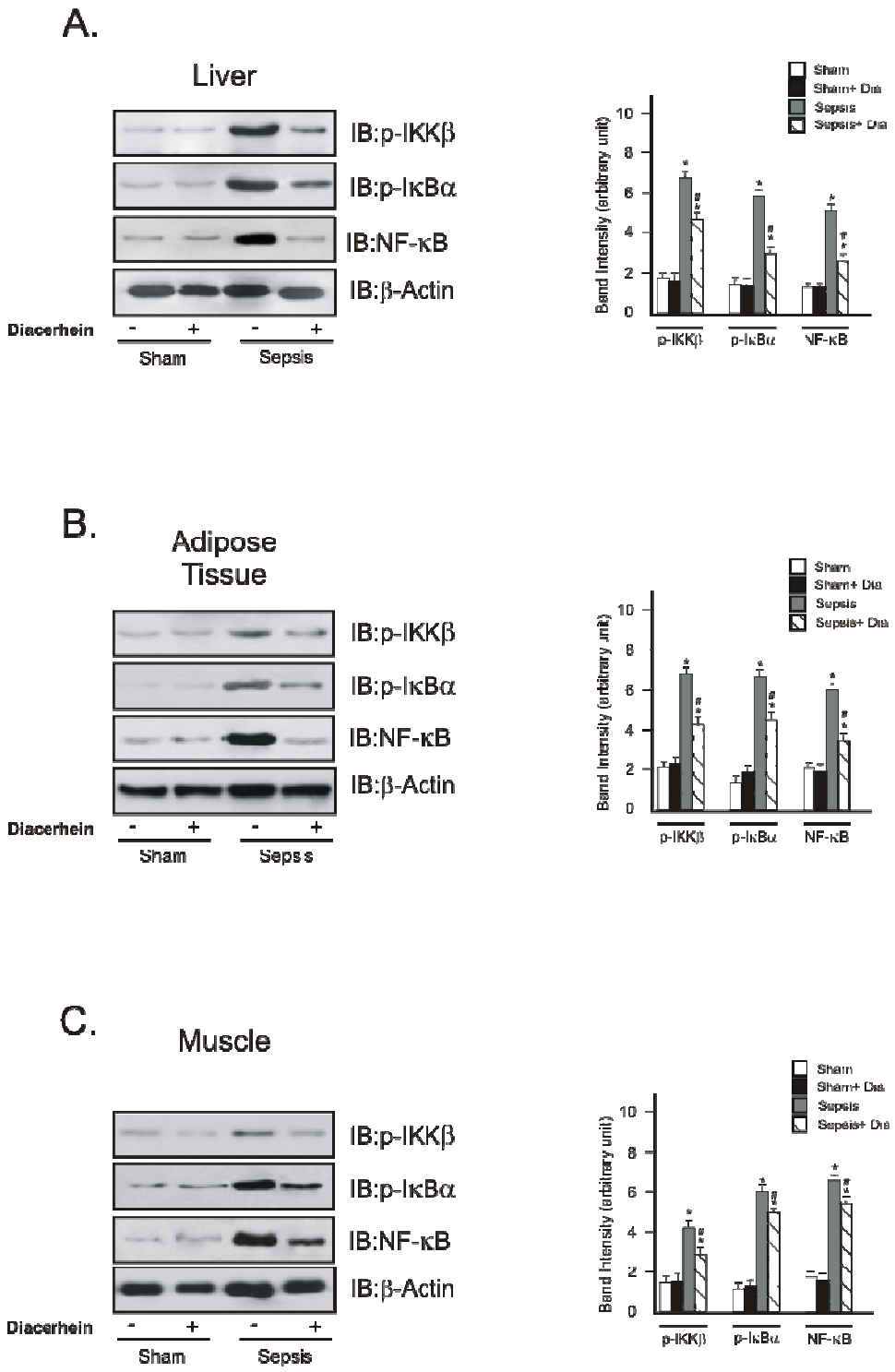
A.
Liver
IB:p-IKKβ
IB:p-IκBα
IB:NF-κB
IB:β-Actin
Diacerhein
- + - +
Sham
Sepsis
Sham
Sham+ Dia
Sepsis
Sepsis+ Dia
Band Intensity (arbitrary unit)
p-IKKβ
p-IκBα
NF-κB
B.
Adipose Tissue
IB:p-IKKβ
IB:p-IκBα
IB:NF-κB
IB:β-Actin
Diacerhein
- + - +
Sham
Sepsis
Sham
Sham+ Dia
Sepsis
Sepsis+ Dia
Band Intensity (arbitrary unit)
p-IKKβ
p-IκBα
NF-κB
C.
Muscle
IB:p-IKKβ
IB:p-IκBα
IB:NF-κB
IB:β-Actin
Diacerhein
- + - +
Sham
Sepsis
Sham
Sham+ Dia
Sepsis
Sepsis+ Dia
Band Intensity (arbitrary unit)
p-IKKβ
p-IκBα
NF-κB

Figure 4

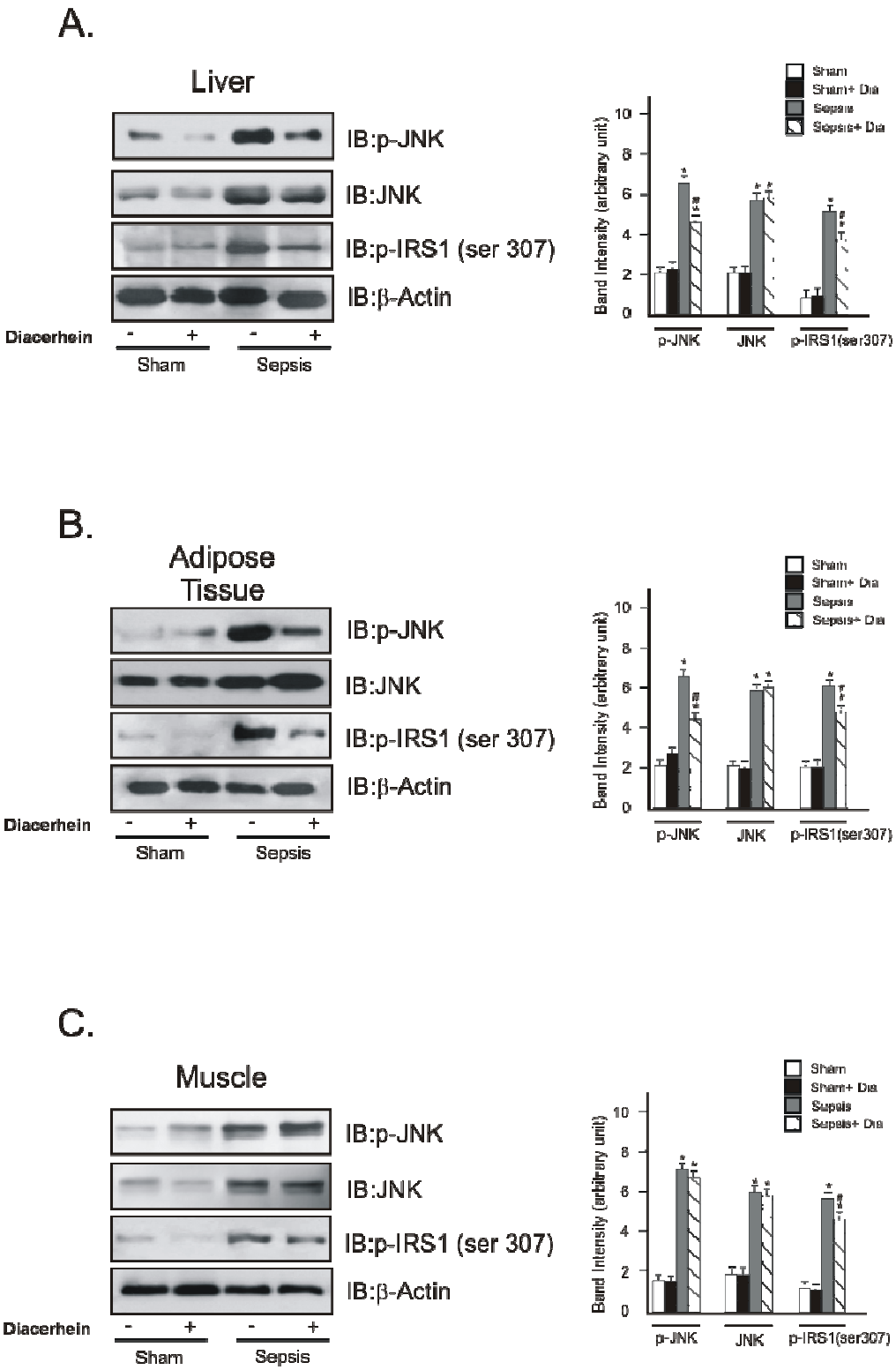
A.
Liver
IB:p-JNK
IB:JNK
IB:p-IRS1 (ser 307)
IB:β-Actin
Diacerhein
- + - +
Sham
Sepsis
Sham
Sham+ Dia
Sepsis
Sepsis+ Dia
Band Intensity (arbitrary unit)
p-JNK
JNK
p-IRS1(ser307)
B.
Adipose Tissue
IB:p-JNK
IB:JNK
IB:p-IRS1 (ser 307)
IB:β-Actin
Diacerhein
- + - +
Sham
Sepsis
Sham
Sham+ Dia
Sepsis
Sepsis+ Dia
Band Intensity (arbitrary unit)
p-JNK
JNK
p-IRS1(ser307)
C.
Muscle
IB:p-JNK
IB:JNK
IB:p-IRS1 (ser 307)
IB:β-Actin
Diacerhein
- + - +
Sham
Sepsis
Sham
Sham+ Dia
Sepsis
Sepsis+ Dia
Band Intensity (arbitrary unit)
p-JNK
JNK
p-IRS1(ser307)

Figure 5

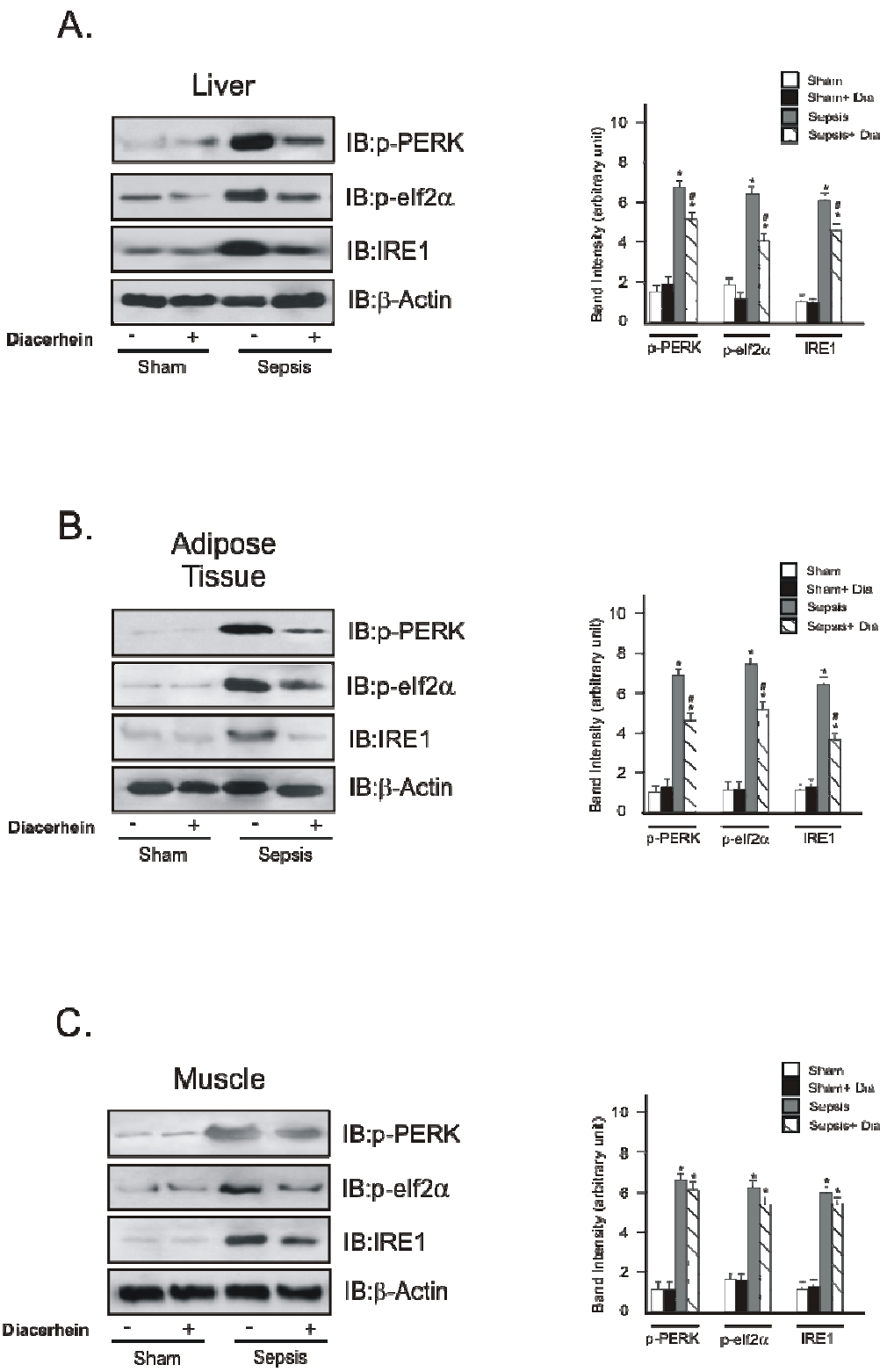
A.
Liver
IB:p-PERK
IB:p-elf2α
IB:IRE1
IB:β-Actin
Diacerhein
- + - +
Sham
Sepsis
Sham
Sham+ Dia
Sepsis
Sepsis+ Dia
Band Intensity (arbitrary unit)
p-PERK
p-elf2α
IRE1
B.
Adipose Tissue
IB:p-PERK
IB:p-elf2α
IB:IRE1
IB:β-Actin
Diacerhein
- + - +
Sham
Sepsis
Sham
Sham+ Dia
Sepsis
Sepsis+ Dia
Band Intensity (arbitrary unit)
p-PERK
p-elf2α
IRE1
C.
Muscle
IB:p-PERK
IB:p-elf2α
IB:IRE1
IB:β-Actin
Diacerhein
- + - +
Sham
Sepsis
Sham
Sham+ Dia
Sepsis
Sepsis+ Dia
Band Intensity (arbitrary unit)
p-PERK
p-elf2α
IRE1

Figure Legends

Figure 1 - Effect of diacerhein on survival in CLP sepsis model.

Male Wistars rats, 8 weeks old, were given vehicle (Sepsis/Veh, n = 15) or diacerhein 100 mg/kg (Sepsis/Dia, n = 15), 3 h and once a day after CLP. Survival of the rats was monitored at intervals of 12 h for 15 days. The overall difference in survival rate between the groups with and without diacerhein was significant ($p < 0.05$) (A). Fasting blood glucose (B). Glucose disappearance rate (C). Serum levels of IL-6 (D), IL-6 (E) and TNF-α (F). Data are presented as means and S.D. of six to eight rats per group. * $p < 0.05$ vs. Sham/Vehicle; # $p < 0.05$ (Sepsis/Veh vs. Sepsis/Dia). C: Sham/Vehicle; ShD: Sham/Diacerhein; SAL: Sepsis/ Vehicle; DIA: Sepsis/Diacerhein.

Figure 2 - Effects of diacerhein treatment on insulin signaling in the CLP rat.

Representative blots show insulin-induced tyrosine phosphorylation of IRβ, IRS1 and serine phosphorylation of Akt in liver (A), adipose tissue (B) and muscle (C) along with phosphorylation ratio analysis. Blots were stripped and reprobed with β-actin (lower panels) to confirm equal loading of proteins. Data are presented as means ±SEM from 6–8 rats per group. * $p < 0.05$ vs. Sham/ Vehicle; # $p < 0.05$ (Sepsis/Veh vs. Sepsis/Dia). IB, immunoblot; Sham: Sham/Vehicle; Sham+Dia: Sham/Diacerhein; Sepsis: Sepsis/ Vehicle; Sepsis+Dia: Sepsis/Diacerhein.

Figure 3 - Representative blots show the IKKβ, IκBα phosphorylation and protein expression of NF-κB in liver (A), adipose tissue (B) and muscle (C) along with phosphorylation ratio analysis. Blots were stripped and reprobed with β-actin (lower panels) to confirm equal loading of proteins. Data are presented as means ±SEM from 6–8 rats per group. * $p < 0.05$ vs. Sham/ Vehicle; # $p < 0.05$ (Sepsis/Veh vs. Sepsis/Dia). IB, immunoblot; Sham: Sham/Vehicle; Sham+Dia: Sham/Diacerhein; Sepsis: Sepsis/ Vehicle; Sepsis+Dia: Sepsis/Diacerhein.

Figure 4 - Representative blots show the JNK phosphorylation, total protein expression of JNK and serine 307 phosphorylation of IRS1 in liver (A), adipose tissue (B) and muscle (C) along with phosphorylation ratio analysis. Blots were stripped and reprobed with β-actin (lower panels) to confirm equal loading of proteins. Data are presented as means ±SEM from 6–8 rats per group. * $p < 0.05$ vs. Sham/ Vehicle; # $p < 0.05$ (Sepsis/Veh vs. Sepsis/Dia). IB, immunoblot; Sham: Sham/Vehicle; Sham+Dia: Sham/Diacerhein; Sepsis: Sepsis/ Vehicle; Sepsis+Dia: Sepsis/Diacerhein.

Figure 5 - Representative blots show the PERK, eIF2α phosphorylation and IRE-1 expression in liver (A), adipose tissue (B) and muscle (C) along with phosphorylation ratio analysis. Blots were stripped and reprobed with β-actin (lower panels) to confirm equal loading of proteins. Data are presented as means ±SEM from 6–8 rats per group. * $p < 0.05$ vs. Sham/ Vehicle; # $p < 0.05$ (Sepsis/Veh vs. Sepsis/Dia). IB, immunoblot; Sham: Sham/Vehicle; Sham+Dia: Sham/Diacerhein; Sepsis: Sepsis/ Vehicle; Sepsis+Dia: Sepsis/Diacerhein.

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DISCUSSÃO

No presente estudo, demonstramos que a atorvastatina aumenta a sobrevivência, diminui os níveis de citocinas pró-inflamatórias circulantes, reduz a sinalização pró-inflamatória mediada por TLR4 e em paralelo melhora a sinalização da insulina no fígado, músculo e tecido adiposo de ratos sépticos. A melhora na sobrevivência de animais sépticos tratados com estatina foi previamente descrita, e foi relacionada a preservação da função cardíaca, estado hemodinâmico e também a redução na adesão de monócitos ao endotélio (Merx, Liehn *et al.*, 2004; Merx, Liehn *et al.*, 2005).

A atorvastatina, administrada 3 horas após a indução da sepse, é capaz reduzir os altos níveis de IL-6 e TNF-α, além de reduzir a resistência à insulina, como demonstrado durante o teste de tolerância à insulina.

Em animais sépticos, a resistência à insulina é acompanhada por uma redução na fosforilação em tirosina de IR, IRS-1, bem como na fosforilação em serina da Akt induzida pela insulina no fígado, músculo e tecido adiposo. Neste estudo, demonstramos que o tratamento com atorvastatina reduz a resistência à insulina na sepse, melhorando a sinalização da via IR-IRSs-Akt nos tecidos alvos. Uma vez que o efeito anti-inflamatório da insulina é mediado pela via da PI3K/Akt (Guha e Mackman, 2002; Schabbauer, Tencati *et al.*, 2004; Williams, Li *et al.*, 2004) podemos especular que a restauração desta via nos tecidos insulinodependentes, pode ter contribuído para o efeito anti-inflamatório desta droga.

Durante os últimos 10 anos, inúmeras evidências mostram uma clara interação molecular entre vias de sinalização metabólica e imune em diferentes situações de resistência à insulina (Hotamisligil, 2006; Shoelson, Lee *et al.*, 2006; Tsukumo, Carvalho-Filho *et al.*, 2007). Atualmente sabe-se que o TLR4 é uma conexão importante entre esses sistemas.

TLRs são receptores transmembrana expressos principalmente em células apresentadoras de antígenos, como monócitos, macrófagos e células dendríticas (Medzhitov, Preston-Hurlburt *et al.*, 1997). O reconhecimento de produtos microbianos por TLRs leva a ativação de uma variedade de vias de transdução de sinal que regulam a natureza, magnitude e duração da resposta inflamatória (Dinarello, 2000). O TLR4 é um subtipo de TLRs capaz de reconhecer e responder ao estímulo por LPS (Medzhitov, Preston-Hurlburt *et al.*, 1997). Apesar do TLR4 ser importante para ativação de vias de sinalizações envolvidas na resposta imune à bactérias Gram-negativas, a ativação extrema da via do TLR4 está relacionada à indução de resistência à insulina (Tsukumo, Carvalho-Filho *et al.*, 2007).

Nosso estudo mostra que a atorvastatina diminui expressão e a ativação do TLR4 e essa modulação pode ter um papel central na modulação negativa da resposta pró-inflamatória ativada por LPS. Nossos dados corroboram um estudo anterior onde o pré-tratamento com simvastatina inibiu a expressão pós-transcricional do TLR4 em monócitos, de humanos com endotoxemia (Niessner, 2006).

A sinalização do TLR4 ativa vias pró-inflamatórias como a via da JNK e da IKK (Brunkhorst, Engel *et al.*, 2008). A atorvastatina reduziu significativamente a fosforilação de IKK e, como esperado, reduziu a fosforilação IκB. Ao mesmo tempo, translocação nuclear de NF-kB foi fortemente inibida pelo tratamento nos três tecidos estudados, demonstrando a desativação desta via. A ativação de NF-kB é observado em todas as formas de inflamação (Liu e Malik, 2006) e está associada à piores prognósticos na sepse (Arnalich, Garcia-Palomero *et al.*, 2000; Yang, Arcaroli *et al.*, 2003; Arcaroli, Silva *et al.*, 2006).

A translocação de NF-κB para o núcleo aumenta a expressão de TNF-α, IL-6 e iNOS (Baeuerle e Baltimore, 1996; Baldwin, 1996; Li, Browder *et al.*, 1999). iNOS e IL-6, especificamente, são produzidos logo no início da resposta imune e desempenhem um

papel central na evolução para o choque séptico (Mcgown e Brookes, 2007). Em acordo com dados anteriores, nosso estudo demonstrou que os níveis circulantes de TNF-α e IL-6, bem como a expressão iNOS, TNF-α e IL-6 foram reduzidos nos três tecidos dos animais tratados com a atorvastatina (Ando, Takamura *et al.*, 2000).

Estudos anteriores demonstraram que o músculo esquelético e tecido adiposo contribuem para a produção de IL-6 durante a endotoxemia (Chang, 2004; Brix-Christensen, 2005; Bultinck, 2006). O efeito inibitório da atorvastatina sobre a síntese de IL-6 pode ser resultado da ativação direta da via PI3K nestes tecidos. Além disso, a redução desses moduladores negativos da sinalização da insulina foi fundamental na melhora da sinalização observada nos animais sépticos.

Outra via inflamatória ativada pelo LPS é a via da JNK. Essa via tem sido implicada tanto em mecanismos de ativação da apoptose quanto em mecanismos de sobrevivência celular (Liu e Lin, 2005). Quando ativada, a JNK induz a fosforilação de IRS-1 em resíduos de serina, impedindo a sinalização da insulina.

Aqui, observamos que a fosforilação de IRS-1 em serina está aumentada nos animais sépticos e o tratamento com a atorvastatina reverteu este fenômeno, e em paralelo reduziu na atividade JNK. A atorvastatina inibe a fosforilação e a ativação da JNK, indicando que o efeito benéfico desta droga sobre o processo inflamatório e sinalização da insulina é mediado por diferentes vias de sinalização.

Além da ativação induzida pela resposta inflamatória, a JNK também pode ser ativada como conseqüência do estresse do RE (Ozcan, Cao *et al.*, 2004; He, 2006; Boden, Duan *et al.*, 2008).

O estresse do RE leva a redução da sensibilidade à insulina pela ativação de JNK e IKK, proteínas quinases envolvidas na fosforilação inibitória de IRS-1 em resíduos de serina (Gao, Zhang *et al.*, 2004; Herschkovitz, Liu *et al.*, 2007). Recentemente, foi

demonstrado *in vitro* e *in vivo* que as estatinas são capazes de reduzir o estresse de RE (Breder, Coope *et al.*, 2010). No presente estudo, confirmamos esse achado, mostrando que a atorvastatina inibe a fosforilação de IRE1α e PERK, e a expressão de ATF6α, sugerindo que esta droga pode atenuar o estresse do RE induzido pela sepse.

De uma maneira sumária, os resultados mostraram que a atorvastatina exerce ação reguladora direta sob a expressão e ativação do TLR4 no fígado, músculo e tecido adiposo, o que reduziu a ativação da JNK e IKK, e a translocação nuclear de NF-kB. A inibição de NF-kB justifica, ao menos em parte, a redução nos níveis séricos e na expressão de citocinas pró-inflamatórias. A atorvastatina reduz o estresse do RE e, o que também pode ter colaborado para a forte inibição das vias de JNK e IKK induzida pelo tratamento com este fármaco. Assim, sugerimos que os efeitos da atorvastatina sobre expressão/ativação TLR4, ativação da via PI3K e sobre o estresse do RE foram cruciais para melhorar a resistência à insulina induzida pela sepse.

No presente estudo, demonstramos também que a administração do anti-inflamatório diacereína, aumenta a sobrevida na sepse induzida por peritonite, com um efeito significativo sobre a sensibilidade à insulina. Este fármaco demonstrou-se capaz de reduzir a resposta inflamatória através da inibição da transcrição de mediadores pró-inflamatórios como interleucinas e TNF-α.

A melhora na sobrevida de animais sépticos tratados com diacereína nunca havia sido descrita antes. Apesar disso, múltiplos estudos têm demonstrado que este fármaco possui ação anti-catabólica e anti-proliferativa, demonstrando desta forma seu valor no tratamento de artropatias (Legendre, Bogdanowicz *et al.*, 2007) e até mesmo do câncer (Huang, Lu *et al.*, 2007).

Nossos resultados mostram que diacereína, administrada 3 horas após a indução da sepse, é capaz de melhorar a sensibilidade à insulina que é reduzida na sepse, melhorando a sinalização de insulina via IR-IRS-1-Akt em tecidos alvos.

Como descrito previamente, a ativação da via da IKK causa a resistência à insulina pela ativação transcricional do NF-kB que induz a liberação de interleucinas e TNF-α. O tratamento com a diacereína reduziu a fosforilação de IKK, aumentou a expressão de IκB, e a reduziu a translocação nuclear de NF-kB. Além disso, os animais tratados com este fármaco tiveram uma redução nos níveis séricos de IL-1β, IL-6 e TNF-α em relação aos não tratados, sugerindo uma forte atenuação de IKK.

Tem sido proposto que a ativação de IKK pode inibir estímulo da PI3K pela insulina (Kim, Kim *et al*., 2001). A este respeito, a capacidade da insulina de ativar a PI3K e a Akt foi completamente restaurada nos animais sépticos tratados com diacereína. Ao mesmo tempo, o tratamento com a diacereína inibiu a fosforilação da JNK,e em paralelo, reduziu a fosforilação em serina de IRS-1. Moduladores negativos da via de sinalização da insulina, tais como JNK e IKK são parcialmente responsáveis pelo estabelecimento da resistência à insulina, como já descrito.

Paralelamente, a diacereína inibiu fortemente fosforilação de PERK e eIF2α, e a expressão de IRE1, sugerindo que esta droga pode atenuar o estresse do RE induzido por sepse.

Em resumo, nossos dados mostram que o tratamento de animais sépticos com o anti-inflamatório diacereína, aumentou sobrevivência desses animais com um efeito significativo sobre a sensibilidade e sinalização da insulina nos tecidos alvos da ação desse hormônio. O tratamento também atenuou a resposta inflamatória como demonstrado pela redução nos níveis séricos de citocinas. Nossos resultados revelam que o tratamento com a diacereína é capaz de melhorar à resistência à insulina, mesmo em condições agudas como a sepse. Tendo em vista a supressão de mediadores pró-inflamatórios promovida por este fármaco pode ser uma terapia potencial para o tratamento da resistência à insulina induzida por processos inflamatórios.

A diacereína é bem tolerada e amplamente utilizada no tratamento de artropatias

Com base no seu perfil de segurança e nos seus efeitos sobre vias inflamatórias, a diacereína demonstra-se uma alternativa terapêutica nova para atenuar a resposta inflamatória e melhorar o quadro de resistência à insulina na sepse.

A sepse é uma condição letal e extremamente custosa sem opções de terapêuticas específicas disponíveis. Diversas estratégias são estudadas, no intuito de controlar essa hiperatividade inflamatória.

A crença antiga de que tratamento com antibióticos específicos seria suficiente para o controle da doença não é de todo verdadeiro. Atualmente é sabido que, tão importante quanto à terapia com antibiótico adequado, é o controle do processo inflamatório. Contudo, está suficientemente claro que a abolição dos fenômenos pró-inflamatórios não é eficaz no tratamento e resulta em resposta analogamente deletéria. Estudos demonstraram que o bloqueio de TNF-α em um modelo de sepse reduziu a sobrevida dos animais (Eskandari, Bolgos *et al.*, 1992). Em estudos clínicos, a administração de um antagonista de TNF-α aumentou a taxa de mortalidade (Fisher, Agosti *et al.*, 1996) e em razão dos resultados negativos, esses conceitos começaram a ser repensados.

O manejo atual da sepse envolve o tratamento da infecção subjacente com o antibiótico adequado além de medidas de suporte orgânico e hemodinâmico (Rivers, Nguyen *et al.*, 2001; Kumar, Roberts *et al.*, 2006). Intervenções farmacológicas estão limitadas à insulina (Dellinger, Levy *et al.*, 2008), corticóides (Bone, Fisher *et al.*, 1987) e proteína C ativada (Shapiro, Howell *et al.*, 2006), contudo, as alternativas terapêuticas disponíveis atualmente não se mostram eficazes em reduzir os altos índices de mortalidade.

Estudos preliminares feitos por Van Den Berghe e colaboradores [66] demonstraram que o controle dos níveis de glicêmicos com insulinoterapia intensiva reduziu a mortalidade e morbidade em pacientes criticamente enfermos. Por outro lado, o

papel da insulinoterapia intensiva em pacientes com sepse grave é incerto, porque o efeito benéfico da insulina pode ser superado pelo aumento do risco de eventos adversos graves relacionados com a hipoglicemia (Brunkhorst, Engel *et al.*, 2008).

Atualmente, a insulina é infundida em pacientes sépticos com hiperglicemia para normalizar os níveis de glicêmicos (Russell, 2006). Estudos demonstram que essa redução na glicemia está associada com redução na inflamação e danos nas células endoteliais (Van Den Berghe, Wouters *et al.*, 2001; Marik e Raghavan, 2004; Van Den Berghe, 2004; Langouche, Vanhorebeek *et al.*, 2005; Van Den Berghe, Wilmer *et al.*, 2006). Além disso, a insulina parece ter ação anti-inflamatória, independente de seu efeito sobre glicemia (Brix-Christensen, Andersen *et al.*, 2004; Jeschke, Klein *et al.*, 2004; Jeschke, Rensing *et al.*, 2005).

A ocorrência de resistência à insulina durante a sepse é acompanhada pela redução na tolerância glicose e hiperinsulinemia (White, Frayn *et al.*, 1987). Além disso, a toxicidade aguda provocada por altos níveis de glicemia pode resultar em estresse oxidativo, com grave dano à função mitocondrial, em células onde a utilização da glicose é dependente de insulina (Abraham e Singer, 2007). Nossos dados mostram que ambos os tratamentos reduziram à resistência à insulina e aumentaram a ativação de proteínas da via de sinalização da insulina, IRS1/PI3K/Akt, nos tecidos-alvos.

Interessantes pesquisas mostram que a prevenção da apoptose de células do sistema imune pode ser uma terapia eficaz para sepse (Hotchkiss, Coopersmith *et al.*, 2005; Hotchkiss e Nicholson, 2006; Parrino, Hotchkiss *et al.*, 2007). Uma vez que a ativação da Akt tem um papel importante na redução da apoptose, é possível que a redução da ativação da via IRS/PI3K/Akt, pode ativar apoptose e contribuir para a falência de múltiplos órgãos na sepse (Kidd, Schabbauer *et al.*, 2008). Em nosso estudo, os animais sépticos tratados mostraram um aumento na ativação da via IRS/PI3K/Akt.

Paralelamente, estudos recentes indicam que a ação anti-inflamatória da insulina ocorre pela ativação da via da PI3K (Kidd, Schabbauer *et al.*, 2008). É agora bem estabelecido que a via de PI3K/Akt regula negativamente sinalização pró-inflamatórias e produção de citocinas induzidas por LPS (Guha e Mackman, 2002; Kim, Oh *et al.*, 2002; Schabbauer, Tencati *et al.*, 2004). Evidências sugerem que a via PI3K exerça um papel importante como regulador negativo de resposta imune pelo controle da produção excessiva mediadores pró-inflamatórios (Park, Lee *et al.*, 1997; Zhao, Lee *et al.*, 2008). Além disso, a inibição da via de PI3K/Akt é associada à ativação de quinases como ERK1/2 e JNK (Guha e Mackman, 2002) e aumento da expressão de TNF-α (Schabbauer, Tencati *et al.*, 2004). É importante ressaltar que a ativação da via PI3K aumenta a sobrevivência, enquanto que a inibição da PI3K reduz a sobrevida de camundongos endotoxêmicos (Fruman, Snapper *et al.*, 1999; Williams, Li *et al.*, 2004; Zhang, Wei *et al.*, 2007).

Uma vez que o efeito anti-inflamatório da insulina é mediada através da via PI3K/Akt (Guha e Mackman, 2002; Schabbauer, Tencati *et al.*, 2004; Williams, Li *et al.*, 2004), podemos especular que a restauração desta via nos tecidos insulinodependentes, também podem ter contribuído para o efeito anti-inflamatório observado em ambos os tratamentos.

Neste contexto, acreditamos que a avaliação da via de sinalização da insulina através da via de PI3K/Akt no tecido muscular, adiposo e fígado podem ser importantes indicadores da lesão a nível tecidual. E a melhora da sinalização nesta via, em paralelo com a atenuação da inflamação nos tecidos, pode ajudar a predizer a eficácia deste tratamento.

É importante mencionar que, na sepse, muitas vias inflamatórias são ativadas em paralelo com uma redução da ativação via PI3K/Akt, assim, o bloqueio de apenas um único componente das vias inflamatórias ou a ativação da via PI3K pode ser insuficiente

para deter o processo (Bohrer, Qiu *et al*., 1997; Marshall, 2004). Na verdade, terapias que modulam famílias inteiras de mediadores têm se mostrado mais eficazes (Marshall, 2004; Terblanche, Almog *et al*., 2006).

Propomos que drogas capazes de reverter resistência à insulina induzida pela sepse, manter o controle glicêmico adequado e em paralelo modular a inflamação, podem ser uma potencial estratégia profilática para sepse. Nesta linha, tanto a diacereína quanto a atorvastatina se mostraram eficazes em atenuar o processo inflamatório, através da redução na sinalização das vias pró-inflamatória ativadas por LPS e melhora na sensibilidade e sinalização da insulina.

Nossos dados e de outras pesquisas (Schabbauer, Tencati *et al*., 2004; Williams, Li *et al*., 2004) indicam que as drogas que primem a restauração da via de sinalização da insulina, que está prejudicada na sepse, podem limitar os efeitos deletérios induzidos por LPS e reduzir a mortalidade associada à sepse (Guha e Mackman, 2002), sugerindo, desta forma, que esses agentes possam oferecer uma nova estratégia terapêutica ou profilática a sepse, uma vez que melhoraram a sinalização da insulina, sem induzir hipoglicemia.

Conclusão

- Os tratamentos com a Atorvastatina e a Diacereína foram capazes de aumentar a sobrevivência de ratos à sepse induzida por peritonite.

- O tratamento de ratos sépticos com a Atorvastatina e a Diacereína reduziu expressivamente a ativação de vias de sinalização pró-inflamatórias (JNK/IKKβ)no fígado, músculo e tecido adiposo desses animais.

- A inibição da cascata de sinalização pró-inflamatória permitiu a significativa redução nos níveis séricos de citocinas pró-inflamatórias.

- Os tratamentos promoveram uma expressiva melhora na sinalização intracelular da insulina no fígado, músculo e tecido adiposo desses animais. A melhora decorre, sobretudo, da inibição dos moduladores negativos desta via (JNK/IKKβ).
- A atenuação da inflamação nos tecidos, mediada por diferentes vias de sinalização pró-inflamatórias, pode ajudar predizer a eficácia deste tratamento.

Conclusão Geral

A sepse é uma condição letal e extremamente custosa sem opções de terapêuticas específicas disponíveis. Com base no perfil de eficácia e segurança, a Atorvastatina e a Diacereína revelam-se como uma nova abordagem terapêutica para reverter ou ao menos melhorar o quadro de resistência à insulina associados à sepse.

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