MATHEUS FERRACINI

PAPEL DOS LEUCOTRIENOS NA FAGOCITOSE VIA FcγR POR MACRÓFAGOS ALVEOLARES DE RATOS SADIOS E DIABÉTICOS

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Área de concentração: Imunologia

Orientadora: Profª Drª Sonia Jancar Negro

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Examinador(a): Assinatura: .....
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UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS

Cidade Universitária "Armando de Salles Oliveira"
Av. Prof. Lineu Prestes, 2415 – CEP. 05508-000 São Paulo, SP – Brasil
Telefone :(55) (011) 3091.7733 – telefax : (55) (011) 3091.7438
e-mail:

CERTIFICADO

Certificamos que o protocolo registrado sob nº **142** nas fls. **39** do livro **2** para uso de animais em experimentação, sob a responsabilidade de Sonia Jancar, Coordenador(a) da Linha de pesquisa "***Papel da insulina na potencialização por leucotrienos da atividade fagocítica e microbicida de macrófagos alveolares e mecanismos moleculares envolvidos***" do qual participou(aram) o(s) alunos **Matheus Ferracini e o pesquisador Joilson de Oliveira Martins,** está de acordo com os Princípios Éticos de Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela *COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL* (CEEA) em **21.11.2006.**

São Paulo, 22 de novembro de 2006.

Prof. Dr. UBIRATAN FABRES MACHADO
Coordenador da CEEA - ICB/USP

Profa. Dra. PATRÍCIA CASTELUCCI
Secretária da CEEA – ICB/USP

Dedico este trabalho ao
meu pai, Eber Ferracini.

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Posso estar sendo injusto por não querer citar nomes neste espaço, mas também estou sendo justo por não me esquecer de ninguém!

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"... O que já não tenho é sorte. Quem sabe?
Talvez a tenha hoje. Cada dia é um novo dia.
É preferível ter sorte. Mas eu prefiro ser exacto.
Assim, quando a sorte vem, está-se pronto para ela."

Trecho de "O velho e o mar"
de Ernest Hemingway

RESUMO

FERRACINI, M. **Papel dos leucotrienos na fagocitose via FcγR por macrófagos alveolares de ratos sadios e diabéticos.** 2009. 103 f. Dissertação (Mestrado em Imunologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2009.

Indivíduos diabéticos são mais susceptíveis a infecções e isto parece estar relacionado a defeitos na função dos fagócitos. Macrófagos alveolares (MAs) são a primeira barreira contra infecções respiratórias, e está bem estabelecido que leucotrienos (LTs) potencializam a fagocitose e a atividade microbicida destas células. Neste estudo, nós comparamos MAs de ratos saudáveis e diabéticos quanto a fagocitose via FcγR, as vias de sinalização acionadas e o papel dos LTs. Além disso, avaliamos o efeito da adição de insulina aos MAs na fagocitose. O diabetes foi induzido em ratos Wistar machos pela injeção de aloxana (42 mg/kg, iv). Somente animais com glicemia > 200 mg/dL foram considerados diabéticos. Células obtidas por lavagem broncoalveolar foram deixadas para adesão por 90min e, após remoção das não-aderidas, foram colocadas em cultura a 37 ºC/5% CO_2. No dia seguinte, foram adicionadas hemácias de carneiro opsonizadas com IgG (IgG-SRBC) e 1h depois foi feita a determinação do índice fagocítico em células coradas por hematoxilina/eosina (produto do nº de MAs que fagocitaram pelo nº de IgG-SRBC fagocitadas num total de 100 MAs observados). A determinação de LTs foi feita nos sobrenadantes das culturas por ELISA. Os MAs foram tratados com LTs (100 nM) ou com inibidor de sua síntese (zileuton, 10 μM) ou antagonistas de seus receptores (CP105.696 e MK571, ambos 10 μM) antes da adição da suspensão de IgG-SRBC na presença ou não de insulina (10 mU/mL). A fosforilação de Akt e PKC-δ foi determinada por *immunoblotting* em lisados de MAs após 15min da adição de IgG-SRBC. Neste trabalho, comparamos a capacidade fagocítica de MAs de ratos sadios e diabéticos para IgG-SRBC e os resultados obtidos mostram que: a) MAs de ratos diabéticos fagocitam menos que os de ratos sadios; b) o tratamento de MAs com inibidor da síntese ou antagonistas de LTs reduziu a fagocitose nos sadios mas não teve efeito nos diabéticos; c) a adição de LTB_4 ou LTD_4 aos MAs em cultura aumentou a fagocitose em sadios e diabéticos; d) MAs de sadios e diabéticos liberam quantidades equivalentes de LTB_4 e LTC_4; e) a adição de insulina aos MAs aumentou a capacidade fagocítica em sadios e diabéticos. Ainda, em ratos sadios, a fagocitose de IgG-SRBC induziu fosforilação de Akt e PKC-δ, de modo dependente

de LTs endógenos, enquanto que em ratos diabéticos ocorreu fosforilação da PKC-δ, mas não da Akt. Estes resultados mostram que a fagocitose de IgG-SRBC por MAs é dependente de LTs produzidos pelo engajamento do FcγR os quais amplificam a fosforilação da PKC e da Akt. Isto parece ser, de alguma forma, dependente da ação da insulina, pois MAs provenientes de ratos diabéticos fagocitam menos, a fagocitose não é dependente de LTs endógenos e o engajamento do FcγR não é capaz de ativar a Akt.

Palavras-chave: Leucotrienos. Diabetes mellitus. Insulina. Fagocitose. Macrófago.

ABSTRACT

FERRACINI, M. **Role of leukotrienes in phagocytosis via FcγR by alveolar macrophages from healthy and diabetic rats.** 2009. 103 p. Master thesis (Immunology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2009.

Diabetic individuals are more susceptible to infections and this seems to be related to impaired phagocytes functions. Alveolar macrophages (AMs) are the first barrier to prevent respiratory infections, and it is well stablished that leukotrienes (LTs) are able to potentiate phagocytosis via Fcγ receptor (FcγR) by these cells. Here, we compared the phagocytosis via FcγR by AMs from healthy and diabetic rats, the role of LTs and the signaling pathways activated by these stimuli. Besides, we evaluated the effect of insulin addition on AMs culture during phagocytosis. Diabetes was induced in male Wistar rats by alloxan injection (42 mg/kg, iv). Only animals presenting glycemia > 200 mg/dL were considered diabetics. Cells obtained by bronchoalveolar lavage were allowed to adhere for 90min and, after removal of non-adherent cells, AMs were incubated at 37 ºC/5% CO_2. In the next day, IgG-opsonized red blood cells (IgG-SRBC) were added and, after 1h, the phagocytic index was assessed in cells stained with hematoxylin/eosin (product of the percentage of AMs that ingested IgG-SRBC by the number of ingested cells). LTs in culture supernatants were quantified by ELISA. AMs were treated with LTs (100 nM) or LTs synthesis inhibitor (zileuton, 10 μM) or receptor antagonists (CP105.696 and MK571, both 10 μM) before addition of IgG-SRBC suspension in the presence/absence of insulin (10 mU/mL). The Akt and PKC-δ phosphorylation was assessed in lysates of AMs 15min after addition of IgG-SRBC by immunoblotting. In this work, we compared the phagocytic capacity of AMs from healthy and diabetic rats and the results obtained showed that: a) AMs from diabetic rats showed lower phagocytic capacity than AMs from healthy rats; b) the phagocytosis was dependent of endogenous LTs in AMs from healthy but not diabetic rats, since treatment with LTs synthesis inhibitor or receptors antagonists reduced the phagocytosis in healthy but not diabetic AMs; c) addition of LTB_4 or LTD_4 to cultures enhanced the phagocytosis by AMs from healthy and diabetic rats; d) AMs from healthy and diabetic rats produced similar levels of LTB_4 and LTC_4; e) addition of insulin to AMs enhanced the phagocytic capacity in healthy and diabetics cells. Moreover, whereas in healthy rats the phagocytosis via FcγR induced phosphorylation of Akt and PKC-δ, which was amplyfied by endogenous LTs, in

diabetic rats only PKC-δ was phosphorylated. These results show that phagocytosis of IgG-opsonized targets by AMs is dependent of LTs produced under FcγR engagement which amplify the PKC and Akt phosphorylation. This seems to be, in a way, dependent of insulin action, because AMs from diabetic rats have lower phagocytic capacity, the phagocytosis is not dependent of endogenous LTs and the FcγR engagement is not capable to activate Akt.

Keywords: Leukotrienes. Diabetes mellitus. Insulin. Phagocytosis. Macrophage.

LISTA DE ABREVIATURAS E SIGLAS

5-LO – 5 lipoxigenase

5-HPETE – ácido 5-hidroperoxieicosatetraenóico

Akt – proteína quinase B

BLT1 e BLT2 – receptores de LTB_4

cisteinil-LT – leucotrienos peptídicos (LTC_4, LTD_4 e LTE_4)

CMV – citomegalovírus

cys-LT1 e cysLT2 – receptores de cisteinil-leucotrienos

DM – diabetes mellitus

ERK1/2 – "extracellular signal-regulated kinases 1/2"

FcγR – receptor da porção Fc de imunoglobulina G ligada

HIV – vírus da imunodeficiência humana adquirida

ICAM-1 – "intercellular adhesion molecule-1"

IL – interleucina

IgG – imunoglobulina G

IgG-SRBC – hemácia de carneiro opsonizada por imunoglobulina G

IF – índice fagocítico

LPS – lipopolissacarídeo

LT – leucotrieno

LTA_4 – leucotrieno A_4

LTB_4 – leucotrieno B_4

LTC_4 – leucotrieno C_4

LTD_4 – leucotrieno D_4

LTE_4 – leucotrieno E_4

MAs – macrófagos alveolares

NADPH – fosfato de dinucleotídeo adenina-nicotinamida

p38MAPK – "p38 mitogen-activated protein kinases"

PAMPs – padrões moleculares associados a patógenos

PBS – salina tamponada com fosfato

PI3K – fosfoinositídeo-3 quinase

PKC – proteína kinase C

PMA – "phorbol-12-myristate-13-acetate"

SDS – dodecilsulfato de sódio

SRBC – hemácias de carneiro

Syk – “spleen tyrosine kinases”

TNF-α - fator de necrose tumoral-α

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Introdução

1 INTRODUÇÃO

O **diabetes mellitus** (DM) é uma síndrome complexa caracterizada pela perda da homeostasia do metabolismo de carboidratos. O DM está associado à baixa qualidade e expectativa de vida devido às altas taxas mortalidade e morbidade atribuídas a essa doença. Alguns pesquisadores estimam que a mortalidade atribuída ao DM chega a 3 milhões de mortes por ano no mundo (ROGLIC *et al.*, 2005). No Brasil o DM ocupa o quinto lugar no *ranking* das causas de morte (WORLD HEALTH ORGANIZATION, 2006).

Esta alta mortalidade deve-se, em parte, a uma maior susceptibilidade dos pacientes diabéticos às infecções quando comparados com os não-diabéticos (BERTONI; SAYDAH e BRANCATI, 2001; SHAH e HUX, 2003). Alguns tipos de infecção são mais freqüentes em diabéticos, como infecções superficiais, de tecidos moles e dos tratos urinário e respiratório. As infecções superficiais e de tecidos moles, mais graves em diabéticos, são comumente causadas por cocos Gram positivos, geralmente nas extremidades dos membros. A gravidade destas infecções em diabéticos é devida à alta invasividade dos microrganismos, que pode resultar em complicações como osteomielite e amputação do membro afetado (PELEG *et al.*, 2007). Em mulheres, o DM é considerado fator de predisposição para infecção urinária. Estudos mostraram que 63% das pacientes com pielonefrite eram diabéticas e, mais ainda, que em pacientes diabéticas, a incidência de bacteriúria assintomática era cerca de três vezes maior quando comparadas com não-diabéticas. Além disso, diabéticos apresentam maior incidência de infecções bilaterais e na porção superior do trato urinário, que podem evoluir para sepse e causar a morte (JOSHI *et al.*, 1999; GREGG *et al.*, 2007).

Infecções pulmonares são freqüentes e recorrentes em indivíduos diabéticos. Pneumonias causadas por bactérias Gram negativas e *Mycobacterium tuberculosis* ocorrem com maior freqüência, porém a alta mortalidade e morbidade estão relacionadas com pneumonias por *Streptococcus pneumoniae* e influenza vírus. Em casos de pneumonia pneumocócica, o DM é considerado fator de predisposição para bacteremia (JOSHI *et al.*, 1999; PELEG *et al.*, 2007; KORNUM *et al.*, 2008).

A maior incidência de infecções em diabéticos parece estar relacionada com deficiências na migração e função dos fagócitos. Entretanto, muito mais estudos são necessários para esclarecer os mecanismos envolvidos nesta complexa doença. Desta forma, o uso de modelos experimentais de DM tornou-se fundamental. Drogas diabetogênicas, como a aloxana e a estreptozotocina, se acumulam nas ilhotas pancreáticas causando lesão irreversível das células β produtoras de insulina. A ação citotóxica de ambos agentes diabetogênicos é mediada por espécies reativas do oxigênio (SZKUDELSKI, 2001). A aloxana é uma droga hidrofílica e instável, sua meia-vida é de apenas 90 segundos, tempo, entretanto, suficiente para permitir que a droga destrua as células β quando administrada por via endovenosa (LENZEN e MUNDAY, 1991). Porém, ambas as drogas exibem efeitos tóxicos. A aloxana pode causar, particularmente em altas doses, necrose da célula tubular renal (SZKUDELSKI, 2001). Por outro lado, a estreptozotocina apresenta toxicidade para células linfóides do baço e do timo (GAULTON; SCHWARTZ e EARDLEY, 1985).

Pacientes e animais diabéticos apresentam deficiências na resposta inflamatória, particularmente na migração de fagócitos, que poderiam explicar o aumento da susceptibilidade às infecções. Anjos-Valota *et al.* (2006) mostraram que ratos com DM induzido por aloxana apresentavam baixa adesão e migração celular e baixa expressão de ICAM-1 ("intercellular adhesion molecule-1") na fáscia espermática interna após estímulo com fator de necrose tumoral (TNF)-α, e que o tratamento destes animais com insulina restaurava todos estes parâmetros. Neste mesmo modelo de DM foi encontrado que a instilação intratraqueal de lipopolissacarídeo (LPS) induziu menor migração de neutrófilos e baixos níveis de TNF-α, interleucina (IL)-1β, IL-6 e IL-10 no espaço broncoalveolar quando comparados com ratos sadios. O tratamento dos animais diabéticos com dose única de insulina foi capaz de restaurar estes parâmetros (DE OLIVEIRA MARTINS *et al.*, 2006; MARTINS *et al.*, 2009).

Além disso, os fagócitos de diabéticos apresentam baixa atividade fagocítica e microbicida, o que também poderia explicar a alta suscetibilidade de diabéticos às infecções. Usando o modelo experimental de DM induzida por aloxana, foi observado que os neutrófilos de ratos diabéticos possuem atividade fagocítica para *Candida albicans* diminuída (PANNEERSELVAM e GOVINDASAMY, 2003). A

fagocitose de partículas de zymosan e a produção de peróxido de hidrogênio sob estímulo de PMA (*phorbol myristate acetate*) por neutrófilos e macrófagos de ratos diabéticos também estão diminuídos em comparação com células de ratos sadios (COSTA ROSA *et al.*, 1996; ALBA-LOUREIRO *et al.*, 2006).

A **insulina** ativa seus receptores presentes na membrana plasmática de diversas células, inclusive macrófagos (BAR *et al.*, 1976), com conseqüente fosforilação intracelular do substrato do receptor de insulina (IRS) que é responsável pelo início da ativação de diversas vias de sinalização. Uma quinase-chave na cascata de sinalização acionada pela insulina é a Akt, um regulador *downstream* da PI3K, que pode atuar como amplificadora do sinal desencadeado pela insulina (MANNING e CANTLEY, 2007). A importância desta quinase para as funções metabólicas do organismo foi demonstrada por Cho *et al.* (2001), que demonstraram que camundongos deficientes de Akt desenvolvem resistência a insulina.

A insulina, além de restaurar os parâmetros fisiológicos alterados em diabéticos, também exerce importante papel modulador na função dos fagócitos, independente da normalização da glicemia. A adição de insulina a fagócitos de animais não-diabéticos *in vitro* foi capaz de aumentar a fagocitose de zymosan por macrófagos peritoneais (COSTA ROSA *et al.*, 1996). Mais ainda, neutrófilos e monócitos de indivíduos não-diabéticos, mas com hiperinsulinemia, apresentam aumento na quimiotaxia, fagocitose e produção de espécies reativas de oxigênio após estímulo com PMA (WALRAND *et al.*, 2006). Em trabalho do nosso grupo, mostramos que a insulina adicionada a cultura de macrófagos alveolares (MAs) de ratos inibe a ativação de várias vias de sinalização além da expressão de óxido nítrico sintase induzível (iNOS) e cicloxigenase (COX)-2 induzidas por LPS (MARTINS *et al.*, 2008b; a).

Nos últimos anos, tem-se acumulado evidências de que um grupo de mediadores lipídicos derivados do metabolismo do ácido araquidônico, os **leucotrienos** (LTs), tem importante papel na defesa contra infecções. Estes mediadores são produzidos por fagócitos e potencializam a fagocitose e a atividade microbicida destas células. Os LTs são mediadores lipídicos gerados a partir da ação da enzima 5-lipoxigenase (5-LO) sobre o ácido araquidônico. Este ácido graxo insaturado faz parte dos fosfolipídeos de membranas celulares de onde é clivado por ação de fosfolipase A2 citoplasmática. A 5-LO gera um precursor hidroperóxido (5-

HPETE) o qual é transformado em LTA_4 com a formação de um grupamento epóxido (oxigênio ligando os carbonos 5 e 6). Este é instável e pode sofrer hidrólise enzimática e, assim, gerar o LTB_4, ou então receber um resíduo de glutationa e gerar o LTC_4. A retirada enzimática do ácido glutâmico do LTC_4 dá origem ao LTD_4 e, com a retirada da glicina do LTD_4, forma-se o LTE_4. Estes leucotrienos ligados a glutationa ou resíduos desta são conhecidos coletivamente como cisteinil-leucotrienos (cisteinil-LTs). O LTB_4 atua em receptores celulares BLT1 e BLT2 de alta e baixa afinidade, respectivamente, e os cisteinil-LTs nos receptores cysLT1 e cysLT2 com afinidades distintas para os LTC_4, LTD_4 e LTE_4 (PETERS-GOLDEN e HENDERSON, 2007).

Trabalhos do grupo do Dr Peters-Golden foram pioneiros no estudo do papel dos **LTs na função dos fagócitos**. Mancuso *et al.* (1998) mostraram que a capacidade fagocítica de MAs de ratos é aumentada por LTs produzidos durante a fagocitose de alvos opsonizados com imunoglobulina IgG, portanto via receptor Fcγ (FcγR), e que a inibição da síntese dos LTs endógenos ou o antagonismo de seus receptores diminuiu significantemente a fagocitose. Canetti *et al.* (2003) demonstraram que, sob o estímulo do FcγR de MAs, a ativação da *Syk* (uma proteína tirosina-quinase envolvida na fagocitose via FcγR) é amplificada por LTB_4 mas não por cisteinil-LTs, apesar de estes também aumentarem a fagocitose via FcγR. Além disso, trabalho do mesmo grupo mostrou que os receptores do LTB_4 e cisteinil-LTs possuem distintas proteínas G acopladas, sendo o aumento da fagocitose causado pelo LTB_4 dependente da proteína $G\alpha_{q11}$ e $G\alpha_{i3}$ e, pelo LTD_4, dependente de $G\alpha_q$ (PERES *et al.*, 2007). Em outro artigo, Serezani *et al.* (2005) mostraram que os LTs (principalmente LTB_4) aumentam a capacidade dos MAs de matar *Klebsiella pneumoniae* opsonizada com IgG. Os autores mostraram que este efeito dos LTs é devido a alta produção de peróxido de hidrogênio pela nicotinamida adenina dinucleotídeo (NADPH) oxidase ativada pela translocação da subunidade p47phox que, por sua vez, foi promovida pela ação do LTB_4 de maneira dependente de proteína quinase (PKC)-δ. Mais recentemente, um estudo realizado em nosso laboratório e em colaboração com o grupo do Dr Peters-Golden analisou as vias de sinalização acionadas por LTB_4 e LTD_4 durante a fagocitose via FcγR por MAs. Foi relatado que, apesar de ambos os LTB_4 e D_4 exógenos (adicionados a cultura) aumentarem a fosforilação de PKC-α e -δ, ERK1/2 e p38, a ação do LTB_4 exógeno

sobre a fagocitose é independente da ativação de p38, enquanto que a do LTD_4 é dependente somente de PKC-δ e p38. Já a ação dos LTs endógenos (produzidos pelos próprios MAs sob o estímulo do FcγR) sobre a fagocitose se dá de modo diferente. Enquanto que o LTB_4 atua via ativação de PKC-α, ERK1/2 e PI3K, os cisteinil-LTs atuam via PKC-δ, p38 e também PI3K (CAMPOS *et al.*, 2009). Este estudo mostra que, além de haver diferenças entre as vias de sinalização desencadeadas por LTB_4 e cisteinil-LTs, existem também diferenças entre as vias acionadas por LTs endógenos (produzidos pelas próprias células sob o estímulo do FcγR) e exógenos (adicionados na cultura).

As deficiências nas funções de fagócitos de animais diabéticos, já descritas na literatura, como baixa produção de mediadores e baixa atividade fagocítica e microbicida, certamente contribuem para a alta incidência de infecções e mau prognóstico que indivíduos diabéticos apresentam. Porém, as causas específicas e os mecanismos responsáveis pela resposta deficiente destas células a estímulos de ativação não estão bem esclarecidos. Os LTs são produzidos desde os estágios iniciais da infecção e sua ação potencializadora sobre as atividades fagocítica e microbicida de fagócitos está bem caracterizada, como comentado acima. Com base nestas observações, foi objetivo deste trabalho investigar o possível envolvimento dos LTs na resposta deficiente de fagócitos de ratos diabéticos. Para testar esta hipótese, comparamos a atividade fagocítica via FcγR de macrófagos alveolares de ratos sadios e diabéticos e o papel dos LTs. Adicionalmente investigamos o efeito da adição *in vitro* de insulina sobre a fagocitose.

Objetivos

2 OBJETIVOS

Os objetivos deste trabalho foram:

- Comparar a atividade fagocítica de macrófagos alveolares de ratos sadios e diabéticos sobre hemácias opsonizadas com IgG (via receptor Fcγ);

- Estudar o papel dos leucotrienos endógenos nesta atividade fagocítica, assim como o efeito da adição de LTs aos MAs;

- Comparar vias de sinalização (Akt e PKC-δ) ativadas durante a fagocitose em macrófagos alveolares de ratos sadios e diabéticos e investigar se os LTs endógenos interferem com estas vias;

- Avaliar o efeito da adição de insulina *in vitro* sobre a fagocitose.

Material e métodos

3 MATERIAL E MÉTODOS

3.1 Animais utilizados

Ratos Wistar machos com oito semanas de vida (~ 200g) foram obtidos do Biotério Central do Instituto de Ciências Biomédicas da Universidade de São Paulo. Os animais foram mantidos a 23 ± 2 ºC em ciclo claro/escuro de 12h com acesso a comida e água *ad libitum*. Os protocolos de pesquisa e tratamento dos animais estão de acordo com os princípios e recomendações do Colégio Brasileiro de Experimentação Animal (COBEA) e aprovados pela Comissão de Ética em Experimentação Animal (CEEA) do Instituto de Ciências Biomédicas da Universidade de São Paulo.

3.2 Indução do DM

O DM foi induzido pela injeção i.v. de aloxana (42 mg/kg de animal) em solução fisiológica (NaCl 0,9%). Após dez dias, a glicemia dos animais foi determinada utilizando o aparelho Accu-Chek Advantage II (Roche Diagnóstica, São Paulo, SP-Brasil). Somente animais com glicemia maior que 200 mg/dL foram considerados diabéticos para este estudo. Os animais-controle (sadios) foram injetados pela mesma via com igual volume do veículo.

3.3 Obtenção dos macrófagos alveolares e cultura

Os MAs foram obtidos por lavagem broncoalveolar com PBS (137 mM NaCl, 2,7 mM KCl, 4,3 mM Na_2HPO_4, 1,47 mM KH_2PO_4, pH 7,4) gelado, centrifugados a 259 *g* por 10min a 4 ºC, ressuspensos em meio RPMI-1640 e a concentração de células determinada em câmara de Neubauer. Volumes da suspensão contendo 5 x 10^5 MAs foram distribuídos sobre lamínulas de vidro dentro de placas de 24 poços e incubados em estufa a 37 ºC, 5% CO_2 para adesão. Após

90 min, as lamínulas foram lavadas três vezes com PBS pré-aquecido e incubadas com meio RPMI-1640 + soro fetal bovino (SFB) a 5% em estufa 37 ºC, 5% CO_2 *overnight*.

3.4 Ensaio de fagocitose de hemácias opsonizadas com IgG

3.4.1 Opsonização de hemácias de carneiro com IgG

Hemácias de carneiro (SRBC) foram lavadas três vezes por centrifugação (239 *g* por 5 min), ressuspensas em PBS e sua concentração determinada em câmara de Neubauer. Alíquotas contendo 1 x 10^9 SRBC foram ajustadas para volume de 1 mL e, a esta suspensão, foi adicionada IgG anti-SRBC em quantidade subaglutinante. Após incubação de 30min a 37 ºC e 1h a 4 ºC, as hemácias de carneiro opsonizadas com IgG (IgG-SRBC) foram novamente lavadas por centrifugação para remoção de IgG não ligada. A concentração de células na suspensão foi novamente determinada em câmara de Neubauer para, enfim, se ajustar o volume da suspensão antes de sua adição à cultura de MAs (1,5 x 10^7 IgG-SRBC em 300 µL de RPMI 1640). As SRBC descritas como não-opsonizadas nesta dissertação foram submetidas às mesmas etapas descritas para IgG-SRBC, porém foi utilizado soro normal de coelho no lugar de IgG anti-SRBC (ARAKI; JOHNSON e SWANSON, 1996).

3.4.2 Ensaio de fagocitose e determinação do índice fagocítico

Após obtenção dos MAs (descrita acima) e adição de agentes farmacológicos ou LTs (descrita abaixo), 300 µL da suspensão de IgG-SRBC foram adicionados a cada lamínula (proporção de 30 alvos por MA). Após incubação de 1h a 37 ºC/5% CO_2 para fagocitose, as lamínulas foram lavadas três vezes com PBS pré-aquecido para remoção de alvos não fagocitados e coradas com hematoxilina/eosina. O índice fagocítico (IF), que corresponde ao produto do número de MAs que fagocitaram ao menos uma IgG-SRBC pelo número de IgG-SRBC

fagocitadas (num total de 100 MAs observados), foi determinado por contagem cega em microscópio óptico (aumento de 1000x) (CAMPOS *et al.*, 2009).

3.5 Tratamento dos MAs

Os agentes farmacológicos foram adicionados, ou não, individualmente nas culturas de MAs logo antes da adição da suspensão de IgG-SRBC. As concentrações e tempos foram previamente padronizados por nosso grupo ou descritos por outros autores (CANETTI *et al.*, 2003; CAMPOS *et al.*, 2009): zileuton (inibidor da 5-LO) a 10 μM por 30 min; CP105.696 (antagonista de receptor de LTB_4) a 10 μM por 20 min; MK571 (antagonista de receptor de LTD_4) a 10 μM por 20 min; LTB_4 ou LTD_4 por 5min a 100 nM ou como especificado nas figuras. Os respectivos veículos foram adicionados nas culturas-controle que não sofreram tratamento.

A insulina foi adicionada à cultura de MAs juntamente com as IgG-SRBC. A concentração utilizada foi de 10 mU/mL ou está especificada nas figuras.

3.6 Dosagem de LTB_4 e LTC_4

Após uma hora de fagocitose, conforme descrito anteriormente, o sobrenadante de cultura de MAs (5×10^5) de ratos sadios ou diabéticos foi coletado e centrifugado a 239 *g* por 10min a 4 ºC para remoção de IgG-SRBC não fagocitada. A dosagem de LTB_4 e LTC_4 foi feita por imunoensaio enzimático por competição utilizando-se *kit* comercial (Cayman Chemical). Os procedimentos foram realizados de acordo com o fabricante. Resumidamente, diluições dos sobrenadantes foram misturadas com LTB_4 ou LTC_4 conjugados com acetilcolinesterase e também com o antissoro de coelho específico para o respectivo LT e adicionados em placa de 96 poços pré-sensibilizadas com anticorpo anti-IgG de coelho. Após 18 horas de incubação a 4 ºC, as placas foram lavadas e o substrato da enzima (reagente de Ellman) foi adicionado por 60 a 120min a 25 ºC. A densidade ótica das amostras foi determinada a 412 nm em leitor de microplacas e a concentração dos LTs calculada a partir de curva-padrão. Um controle-positivo para dosagem de LTs foi feito com a

adição ionomicina (ionóforo de cálcio, 1 μM por 1 h) em cultura de MAs de ratos sadios e diabéticos.

3.7 *Western blotting*

Após obtenção do MAs por lavagem broncoalveolar (descrito anteriormente), 2 x 10^6 células foram plaqueadas em placas de 6 poços e incubadas a 37 ºC/5% CO_2 para adesão. Após 2 h, as células foram lavadas com PBS pré-aquecido e incubadas a 37 ºC/5% CO_2 com RPMI 1640 + SFB (5%) *overnight*. As células foram, então, pré-tratadas com zileuton (10 μM por 30 min) ou com o respectivo veículo antes da adição da suspensão de IgG-SRBC na proporção de 30:1 (IgG-SRBC:MA). Após 15min de incubação, a cultura foi lavada com PBS pré-aquecido e os MAs lisados por sonicação em tampão de lise (150 mM Tris-HCl, 100 mM NaCl, 1 mM ortovanadato de sódio, 1% Sigma protease inhibitor cocktail; pH 8,0). Depois de centrifugação para remoção de organelas e ácidos nucléicos, a concentração de proteínas do sobrenadante dos lisados foi determinada e alíquotas contendo 20 μg de proteínas foram misturadas com tampão de amostra (62,5 mM Tris-HCl, 10% glicerol, 2% SDS, 5% β-mercaptoetanol e 0,001% azul de bromofenol), aquecidas a 95 ºC por 5 min, submetidas a eletroforese em gel de poliacrilamida a 10% contendo dodecilsulfato de sódio (SDS-PAGE) e finalmente transferidas para membranas de PVDF. Em seguida, estas tiveram seus sítios inespecíficos bloqueados pela adição de solução de albumina de soro bovino (BSA) a 5% em tampão Tris-tween-20 por 2h e foram sondadas por 18h a 4 ºC com anticorpos primários específicos para fosfo-Akt (Ser473) ou fosfo-PKC-δ (Tyr155) (todos a 1:500 e produzidos em coelhos) ou para β-actina (1:10000 produzido em camundongo). Após lavagem com tampão Tris-salina, as membranas foram, então, sondadas por duas horas com os anticorpos secundários apropriados (anti-coelho a 1:40000 ou anti-camundongo a 1:80000) conjugados com peroxidase. Após nova lavagem, as bandas foram visualizadas em filmes fotográficos utilizando ECL (*enhanced chemiluminescence*). As densidades relativas das bandas foram determinadas por meio de análise densitométrica utilizando o software NIH Image.

3.8 Reagentes

Aloxana, RPMI-1640, ionomicina e anticorpos secundários conjugados com peroxidase (anti-coelho e anti-camundongo) adquiridos da Sigma-Aldrich (St. Louis, MO-EUA), IgG anti-SRBC da Cappel Organon Teknika (Durham, NC-EUA); soro fetal bovino da JCR Biosciences (Lenexa, KS-EUA); insulina cristalina da Biobrás (São Paulo, SP-Brasil); zileuton da Abbott Laboratories (Chicago, IL-EUA); LTB_4, LTD_4 e MK571 da Biomol International (Plymouth Meeting, PA-EUA); hemácias de carneiro da bioBoaVista (Valinhos, SP-Brasil); CP105.696 da Pfizer (Groton, CT-EUA); kits EIA para dosagem de LTB_4 e LTC_4 da Cayman Chemical (Ann Arbor, MI-EUA), ECL (*enhanced chemiluminescent*) da GE Healthcare (Waukesha, WI-EUA).

3.9 Análise estatística

A análise estatística foi realizada utilizando-se o programa GraphPad (San Diego, CA, EUA), com comparação por análise de variância (ANOVA) e teste de Bonferroni.

Resultados

4 RESULTADOS

4.1 Fagocitose via FcγR por MAs de ratos sadios e diabéticos e papel dos LTs

Os MAs de ratos sadios e diabéticos foram obtidos por lavagem broncoalveolar, distribuídos sobre lamínulas e incubados com hemácias de carneiro não-opsonizadas ou opsonizadas com IgG (IgG-SRBC) por uma hora para determinação do índice fagocítico (IF). O IF de MAs de ratos diabéticos foi significativamente menor (30%) que o de ratos sadios (Figura 1), e não houve fagocitose de hemácias não-opsonizadas.

Para avaliar o papel dos leucotrienos na fagocitose via FcγR de MAs de ratos sadios e diabéticos, utilizamos zileuton como inibidor da enzima 5-LO, CP105.696 como antagonista do receptor de LTB_4 e MK571 como antagonista do receptor de cisteinil-LTs. Estas drogas foram adicionadas às culturas de MAs antes da adição dos alvos fagocíticos. Em MAs de ratos sadios, a inibição da síntese de LTs pelo zileuton reduziu a fagocitose de IgG-SRBC em cerca de 40% (Figura 2A). Inibição semelhante foi observada ao se adicionar antagonistas de receptores de LTB_4 e de cisteinil-LTs. Já em MAs de ratos diabéticos, o IF não sofreu alterações significativas após inibição da síntese de LTs ou antagonismo de seus receptores (Figura 2B). Tendo em vista que os LTs aumentam a fagocitose em MAs de ratos sadios, é possível que a fagocitose diminuída nos diabéticos seja devida a produção deficiente de LTs ou a uma menor resposta aos mesmos.

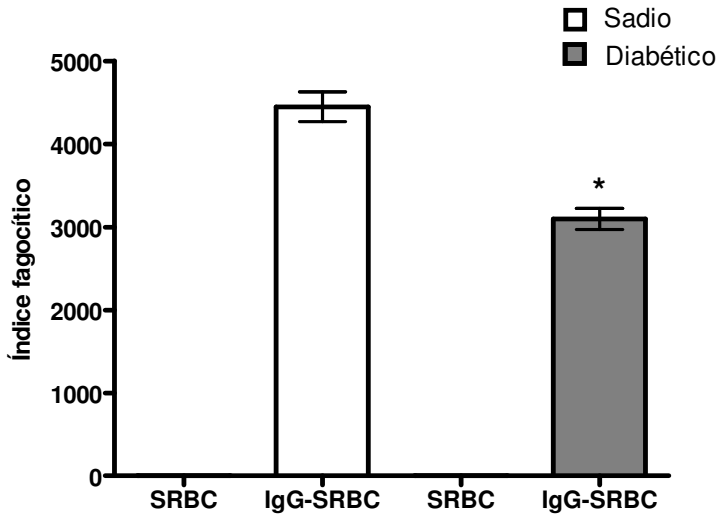

FIGURA 1– Fagocitose via FcγR por MAs de ratos sadios e diabéticos.
MAs (5 x 10^5) de ratos sadios e diabéticos foram obtidos por lavagem broncoalveolar e incubados por uma hora com suspensão de IgG-SRBC (proporção de 30 IgG-SRBC por MA) para fagocitose. O índice fagocítico (produto do número de MAs que fagocitaram ao menos uma IgG-SRBC pelo número de IgG-SRBC fagocitadas, num total de 100 MAs observados) foi determinado por contagem em microscópio óptico (1000x). Os valores correspondem a média±EPM de cinco experimentos independentes (*$p<0,01$ *vs* IgG-SRBC dos sadios).

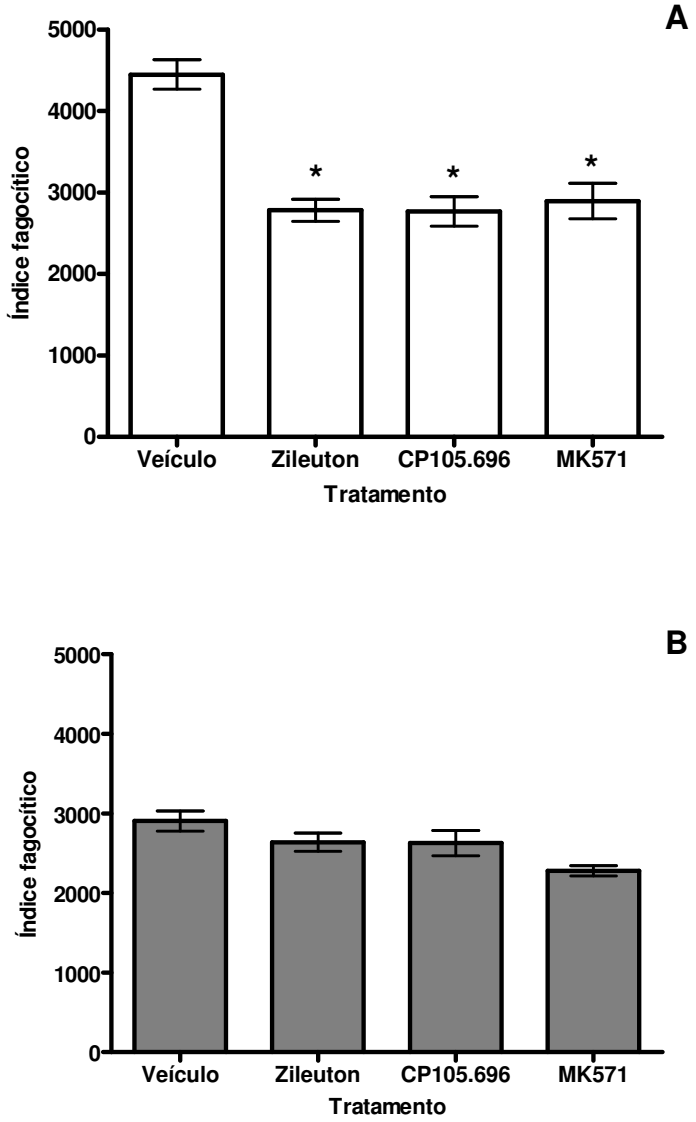

FIGURA 2– Papel dos LTs na fagocitose via FcγR por MAs de ratos sadios e diabéticos.

MAs (5 x 10^5) de ratos sadios (A) e diabéticos (B) foram obtidos por lavagem broncoalveolar e pré-tratados, ou não, com inibidor da 5-LO (zileuton, 10 μM, 30 min) ou antagonista de receptor de LTB_4 (CP105.696, 10 μM, 20 min) ou antagonista de receptor de cisteinil-LTs (MK571, 10 μM, 20 min) e incubados com suspensão de IgG-SRBC (proporção de 30 IgG-SRBC por MA) para fagocitose. O índice fagocítico (produto do número de MAs que fagocitaram ao menos uma IgG-SRBC pelo número de IgG-SRBC fagocitadas, num total de 100 MAs observados) foi determinado por contagem em microscópio óptico (1000x). Os valores correspondem a média±EPM de cinco experimentos independentes (*$p<0,01$).

4.2 Efeito da adição de LTs na fagocitose via FcγR por MAs de ratos sadios e diabéticos

Para avaliar se a capacidade fagocítica reduzida observada nos MAs de ratos diabéticos se deve a uma diminuída resposta aos LTs, determinamos o IF na presença de doses crescentes de LTs. Os LTB_4 ou LTD_4 foram adicionados às culturas de MAs nas doses de 10, 100 ou 1000 nM, 5min antes da adição de IgG-SRBC. Ambos os LTs foram capazes de aumentar o IF de MAs de ratos sadios. O pico de fagocitose, que aumentou o IF em cerca de três vezes, ocorreu com adição de 100 nM de LTB_4 ou LTD_4 (Figura 3A). Em MAs de ratos diabéticos, LTs a 100 nM também resultaram em maior aumento da fagocitose dentre as diluições (Figura 3B). Esse aumento foi de cerca de três vezes, semelhante ao aumento induzido por esta dose em MAs de ratos sadios. Os resultados mostram que MAs de ratos diabéticos respondem aos LTs adicionados a cultura de modo similar aos MAs de ratos sadios.

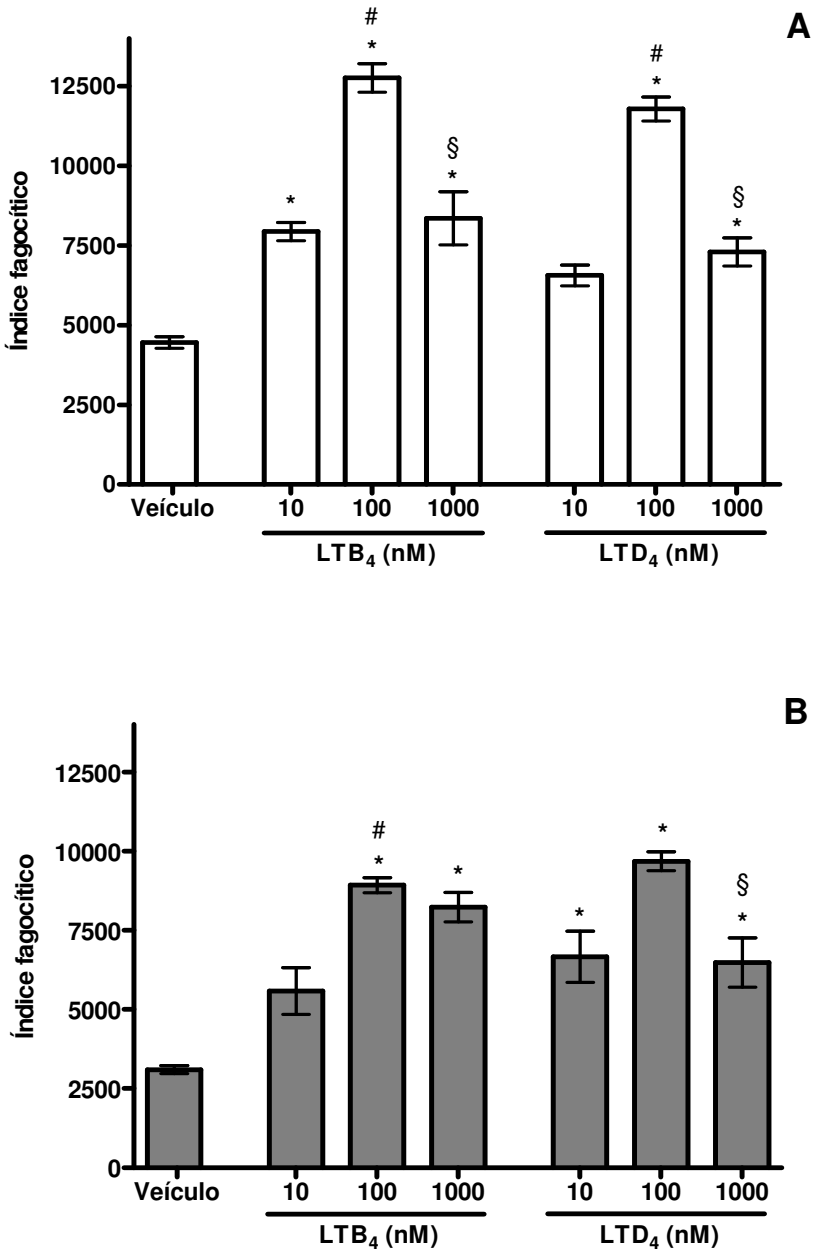

FIGURA 3– Efeito da adição de leucotrienos na fagocitose por macrófagos alveolares de ratos sadios e diabéticos.

MAs de ratos sadios (A) e diabéticos (B) foram obtidos por lavagem broncoalveolar e pré-tratados com LTB_4 (10, 100 ou 1000 nM, 5 min) ou LTD_4 (10, 100 ou 1000 nM, 5 min) e incubados com suspensão de IgG-SRBC (proporção de 30 IgG-SRBC por MA) por uma hora para fagocitose. O índice fagocítico (produto do número de MA que fagocitaram ao menos uma IgG-SRBC pelo número de IgG-RBC fagocitadas, num total de 100 MAs observados) foi determinado por contagem em microscópio óptico (1000x). Os valores correspondem a média±EPM de cinco experimentos independentes (*$p<0,01$ *vs* veículo; # e §$p<0,01$ *vs* LT em concentração imediatamente menor).

4.3 Produção de LTs durante a fagocitose via FcγR por MAs de ratos sadios e diabéticos

Para verificar se a capacidade diminuída de fagocitar via FcγR dos MAs de ratos diabéticos está relacionada com a produção de LTs, medimos a concentração de LTB_4 e LTC_4 no sobrenadante de culturas de MAs incubados por uma hora com IgG-SRBC ou SRBC não-opsonizada. Os dados apresentados na tabela 1 mostram que a fagocitose de IgG-SRBC foi capaz de induzir a produção de LTB_4 e LTC_4, e que ratos sadios e diabéticos produzem quantidades similares de ambos os tipos de LTs. Quando estimulados com ionomicina, os MAs de ratos sadios e diabéticos também produziram quantidades equivalentes de LTs (Tabela 1).

Tabela 1– Produção de LTs durante fagocitose via FcγR por MAs de ratos sadios e diabéticos.

	SRBC		**IgG-SRBC**		**IgG-SRBC + insulina**		**ionomicina**	
	Sadio	***Diabético***	***Sadio***	***Diabético***	***Sadio***	***Diabético***	***Sadio***	***Diabético***
LTB_4 (pg/mL)	301±109	208±085	878±359	1124±0462	1059±0326	862±290	34360±11564	31739±04727
LTC_4 (pg/mL)	17±08	45±03	548±116	642±140	516±134	553±203	3834±1393	2558±0814

MAs de ratos sadios e diabéticos foram obtidos por lavagem broncoalveolar e incubados por uma hora com suspensão de IgG-SRBC (proporção de 30 IgG-SRBC por MA) contendo ou não insulina (10 mU/mL) para fagocitose. Um controle-positivo da produção de LTs foi realizado tratando-se MAs com ionomicina (1 μM, 1 h). O sobrenadante das culturas foi coletado, centrifugado e submetido a determinação de LTB_4 e LTC_4 por imunoensaio enzimático. Os valores correspondem a média±EPM de três experimentos independentes.

4.4 Vias de sinalização (Akt e PKC-δ) ativadas durante a fagocitose de IgG-SRBC por MAs de ratos sadios e diabéticos

Com base na observação de que os LTs não afetaram o IF em MAs, apesar destes MAs responderem aos LTs adicionados nas culturas, fomos investigar as vias de sinalização acionadas durante a fagocitose e se os LTs endógenos afetam a sinalização. Assim, avaliamos a fosforilação de Akt e PKC-δ estimulada via FcγR na presença ou não de inibidor da síntese de LTs. Para tanto, a fosforilação das quinases Akt e PKC-δ de MAs de ratos sadios e diabéticos foram determinadas por *immunoblotting*.

A figura 4ª mostra que a adição de IgG-SRBC aos MAs de ratos sadios aumentou em mais de quatro vezes a fosforilação de Akt, e a inibição da síntese de LTs aboliu este aumento, sugerindo que a ativação desta quinase estimulada via FcγR é dependente de LTs. Porém, em MAs de ratos diabéticos, observamos que a adição de IgG-SRBC não foi capaz de aumentar a fosforilação de Akt de modo significativo, e que a inibição da síntese de LTs não teve qualquer efeito.

A figura 4B mostra que, em MAs de ratos sadios, o estímulo via FcγR aumenta a fosforilação de PKC-δ (cerca de sete vezes) de modo dependente de LTs, já que a inibição da síntese destes mediadores reverte este aumento. Em MAs de ratos diabéticos, a adição de IgG-SRBC também aumentou a fosforilação da PKC-δ, porém a inibição da síntese de LTs não foi capaz de reverter este fenômeno de modo significativo, sugerindo que em diabéticos os LTs não tenham papel fundamental na fosforilação de PKC-δ estimulada via FcγR nestas células.

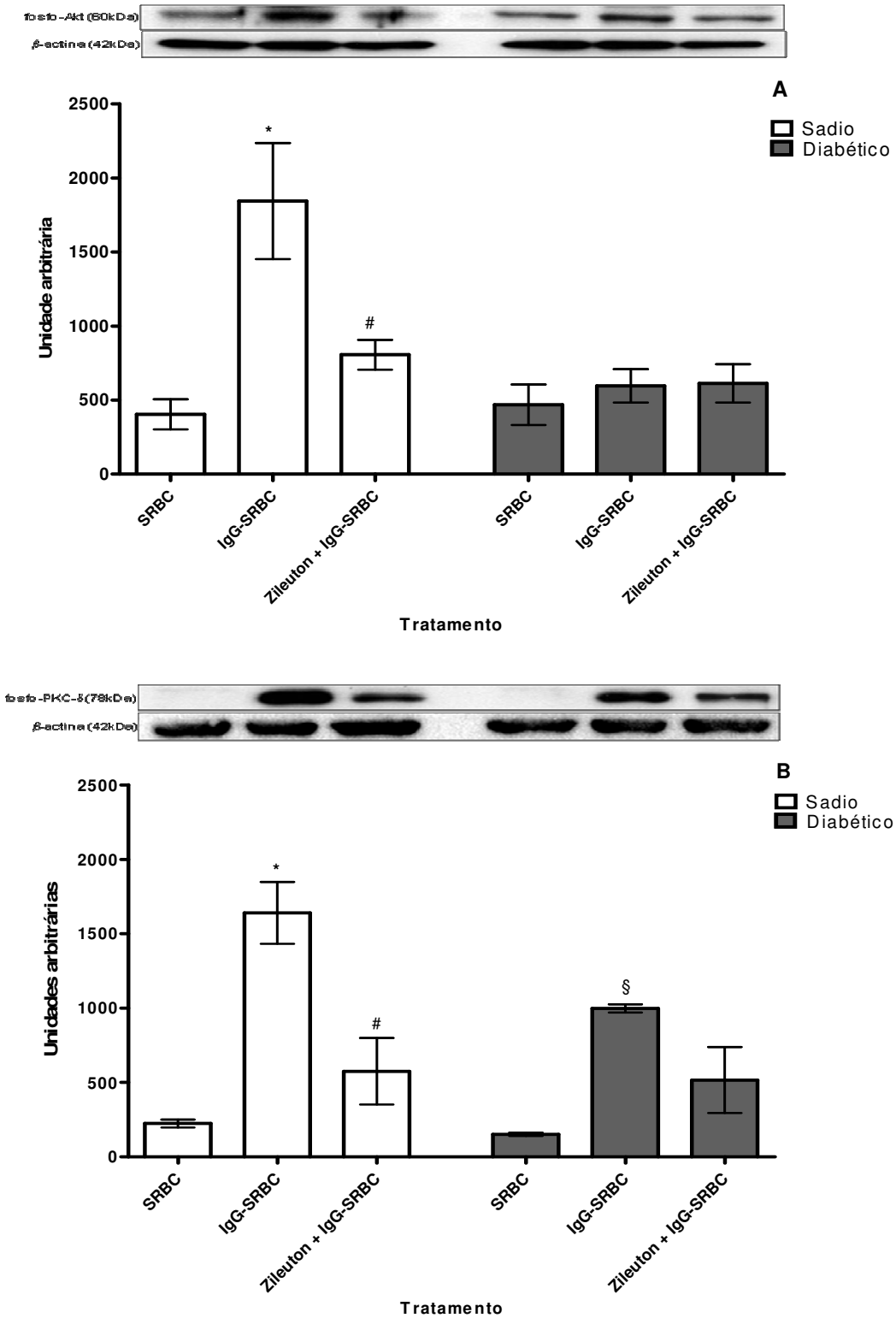

FIGURA 4- Vias de sinalização (Akt e PKC-δ) ativadas durante a fagocitose de IgG-SRBC por MAs de ratos sadios e diabéticos.

MAs de ratos sadios e diabéticos foram obtidos por lavagem broncoalveolar e pré-tratados, ou não, com zileuton (10 μM, 30 min) e incubados por quinze minutos com suspensão de IgG-SRBC ou SRBC não-opsonizadas. Lisados das culturas foram submetidos a *immunoblotting* para detecção de fosfo-Akt (A) e fosfo-PKC-δ (B). As ilustrações das bandas representam um de três experimentos independentes realizados (*$p<0,05$ vs SRBC sadios, $^{\#}p<0,05$ vs IgG-SRBC sadio e $^{\S}p<0,05$ vs SRBC diabético).

4.5 Efeito da insulina na fagocitose via FcγR por MAs de ratos sadios e diabéticos

Determinamos o IF de MAs de ratos sadios e diabéticos após uma hora de fagocitose de IgG-SRBC na presença de insulina em concentrações crescentes. A figura 5 mostra que a insulina aumentou o IF de MAs de ratos sadios e diabéticos. O IF de MAs de ratos diabéticos se igualou ao basal de ratos sadios — ou seja, foi restaurado — com a dose mínima utilizada (1 mU/mL) com aumento de cerca de 70% em relação ao veículo. As curvas formaram um platô nos valores de IF com concentração de insulina a 10 mU/mL, aumentando em duas vezes a fagocitose por MAs de ambos os grupos. Doses maiores do hormônio (200 mU/mL) limitaram o platô com queda gradual da fagocitose. Além disso, as curvas mantiveram as proporcionalidades entre si, ou seja, os valores de IF entre os grupos não variaram (não se igualaram numa mesma dose de insulina). Tais dados mostram que a insulina tem efeito potencializador sobre a fagocitose via FcγR tanto em MAs de ratos sadios como diabéticos.

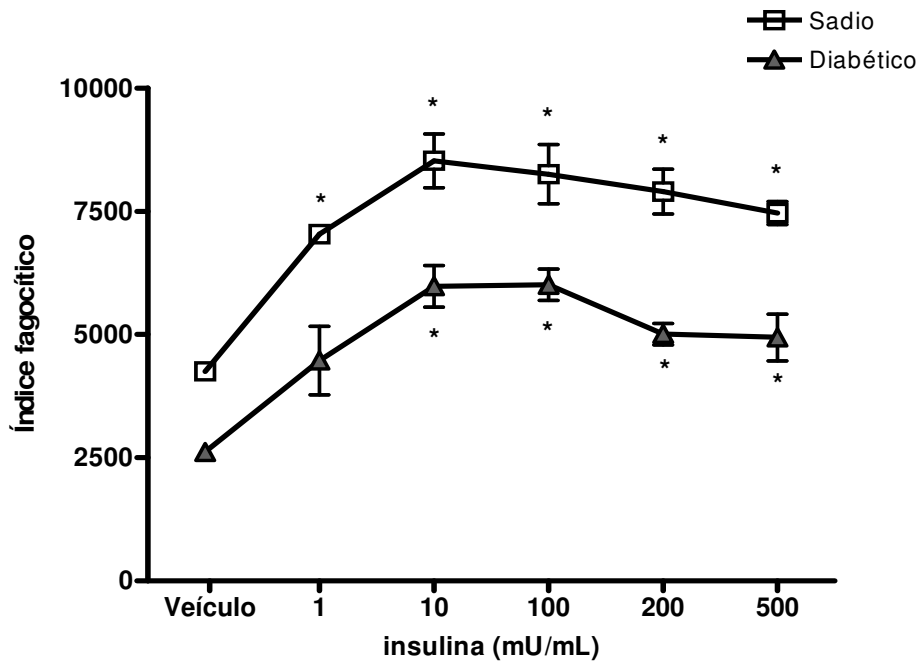

FIGURA 5– Efeito da insulina na fagocitose via FcγR por macrófagos alveolares de ratos sadios e diabéticos.

MAs (5 x 10^5) de ratos sadios e diabéticos foram obtidos por lavagem broncoalveolar e incubados por uma hora com suspensão de IgG-SRBC (proporção de 30 IgG-SRBC por MA) para fagocitose contendo ou não insulina a 1, 10, 100, 200 ou 500 mU/mL. O índice fagocítico (produto do número de MAs que fagocitaram ao menos uma IgG-SRBC pelo número de IgG-SRBC fagocitadas, num total de 100 MAs observados) foi determinado por contagem em microscópio óptico (1000x). Os valores correspondem a média±EPM de cinco experimentos independentes (*$p<0,01$ *vs* veículo dentro de cada grupo).

4.6 Papel dos LTs no efeito potencializador da insulina sobre a fagocitose via FcγR por MAs de ratos sadios e diabéticos

Para avaliar o papel dos LTs no aumento da fagocitose induzido por insulina, inibimos a síntese de LTs com zileuton antes da adição de IgG-SRBC contendo insulina a 10 mU/mL. Conforme mostrado na figura 4, a adição de insulina junto com a suspensão de IgG-SRBC na cultura de MAs de ratos sadios e diabéticos aumentou o IF. Porém, a inibição da síntese de LTs impediu o aumento da fagocitose estimulado por insulina em MAs de ratos sadios (Figura 6), sugerindo que este efeito potencializador da fagocitose pela insulina seja dependente de LT. Já os MAs de ratos diabéticos não foram inibidos do aumento da fagocitose promovido pela insulina após tratamento com zileuton. Os valores de IF de MAs de ratos diabéticos tratados com zileuton e insulina não diferem dos valores correspondentes aos MAs tratados somente com insulina (Figura 6), indicando que, em MAs de ratos diabéticos, a insulina promove aumento da fagocitose de modo independente da síntese de LTs.

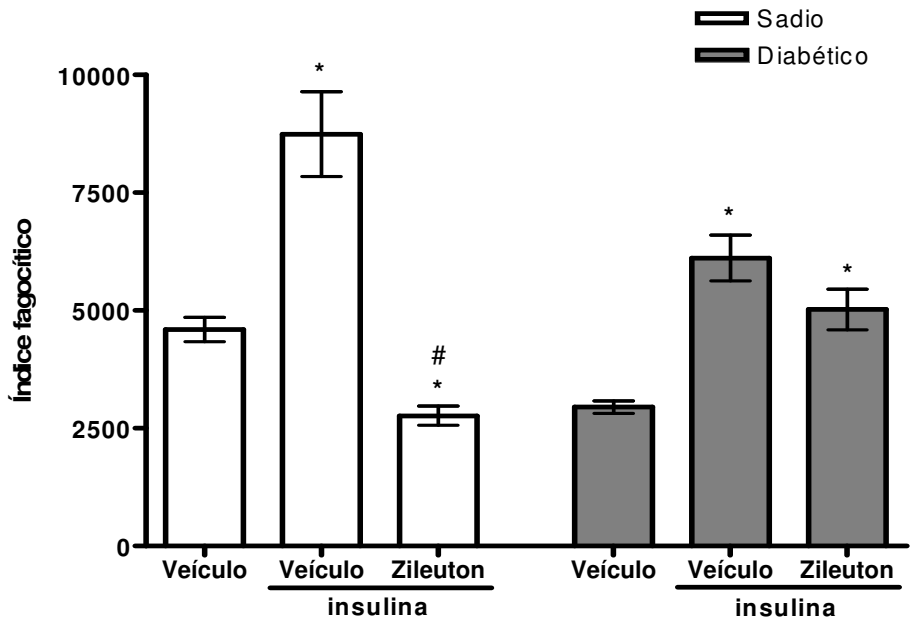

FIGURA 6– Papel dos leucotrienos no efeito potencializador da insulina sobre a fagocitose via FcγR por macrófagos alveolares de ratos sadios e diabéticos.

MAs de ratos sadios e diabéticos foram obtidos por lavagem broncoalveolar e pré-tratados, ou não, com zileuton (10 µM, 30 min) e incubados por uma hora com suspensão de IgG-SRBC contendo, ou não, insulina (10 mU/mL). O índice fagocítico (produto do número de MAs que fagocitaram ao menos uma IgG-SRBC pelo número de IgG-SRBC fagocitadas, num total de 100 MAs observados) foi determinado por contagem em microscópio óptico (1000x). Os valores correspondem a média±EPM de cinco experimentos independentes (*p<0,01 *vs* veículo dentro do grupo analisado; #p<0,01 *vs* veículo + insulina).

4.7 Efeito da insulina na potencialização da fagocitose via FcγR por LTs em macrófagos alveolares de ratos sadios e diabéticos

Para avaliar se existe alguma interação entre os efeitos potencializadores da insulina e dos LTs exógenos, os MAs de ratos sadios e diabéticos foram pré-tratados com LTB_4 ou LTD_4 (100 nM) cinco minutos antes da adição de IgG-SRBC na presença de insulina (10 mU/mL). Na figura 7A, observamos que a potencialização da fagocitose pelos LTs nas concentrações utilizadas é maior que a promovida pela insulina, e que o tratamento dos MAs de ratos sadios com os dois agentes não alterou significativamente o maior IF detectado (promovido pelos LTs). No caso dos diabéticos, semelhante aos sadios, encontramos que o tratamento dos MAs com LTs e insulina naõ alterou a potencialização mais intensa da fagocitose promovida pelos LTs. Estes dados mostram que não existe efeito sinérgico ou aditivo da ação potencializadora da fagocitose entre os LTs e a insulina, ou então, que os níveis máximos de IF determinados aqui representem MAs com nível máximo possível de fagocitose (saturados).

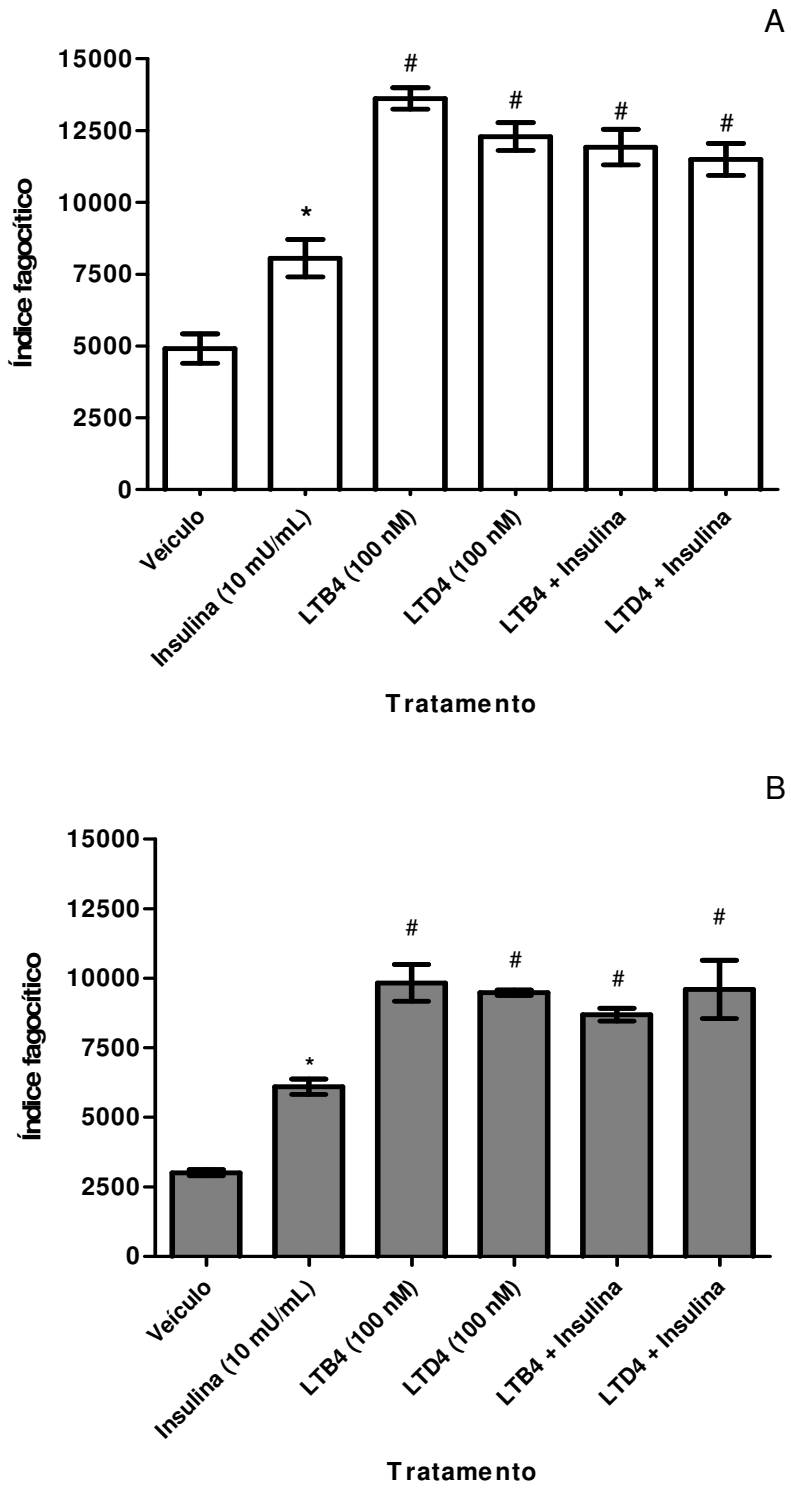

FIGURA 7– Efeito da insulina na potencialização da fagocitose via FcγR por LTs em macrófagos alveolares de ratos sadios e diabéticos.

MAs de ratos sadios (A) e diabéticos (B) foram obtidos por lavagem broncoalveolar e pré-estimulados, ou não, com LTB_4 ou LTD_4 (ambos a 100 nM, 5 min) e incubados por uma hora com suspensão de IgG-SRBC contendo, ou não, insulina (10 mU/mL). O índice fagocítico (produto do número de MAs que fagocitaram ao menos uma IgG-SRBC pelo número de IgG-SRBC fagocitadas, num total de 100 MAs observados) foi determinado por contagem em microscópio óptico (1000x). Os valores correspondem a média±EPM de cinco experimentos independentes (*$p<0,05$ *vs* veículo; $^{\#}p<0,05$ *vs* veículo e insulina).

Discussão

5 DISCUSSÃO

Neste trabalho, comparamos a capacidade fagocítica de MAs de ratos sadios e diabéticos e os resultados obtidos mostram que: a) MAs de ratos diabéticos fagocitam menos que os de ratos sadios; b) a fagocitose é dependente de LTs endógenos em sadios, mas não em diabéticos; c) a adição de LTB_4 ou LTD_4 aos MAs em cultura aumenta a fagocitose em sadios e diabéticos; d) MAs de sadios e diabéticos produzem quantidades equivalentes de LTB_4 e LTC_4; e) a adição de insulina aos MAs aumenta a capacidade fagocitica em sadios e diabéticos. Ainda, em ratos sadios, a fagocitose via FcγR induz fosforilação de Akt e PKC-δ que é amplificada por LTs endógenos, enquanto que, em ratos diabéticos, ocorreu fosforilação da PKC-δ, mas não da Akt.

Trabalhos anteriores mostraram que a fagocitose por neutrófilos e macrófagos peritoneais de ratos diabéticos é deficiente e sugeriram que esta deficiência poderia ser uma das razões da alta susceptibilidade a infecções que diabéticos apresentam (POZZILLI e LESLIE, 1994; MCMAHON e BISTRIAN, 1995; COSTA ROSA *et al.*, 1996; PANNEERSELVAM e GOVINDASAMY, 2003; ALBA-LOUREIRO *et al.*, 2006). Nossos resultados mostram que esta deficiência também ocorre em macrófagos alveolares que representam a primeira linha de defesa contra infecções das vias aéreas. Nós vimos que a fagocitose via FcγR é cerca de 30% menor em MAs de diabéticos comparada com MAs de sadios.

O estudo dos mecanismos responsáveis por esta deficiência este foi um dos objetivos do nosso trabalho. Para isto, nos valemos de trabalhos anteriores do laboratório do Dr. Marc Peters-Golden que demonstraram que a fagocitose de alvos opsonizados com IgG por MAs de ratos é potencializada por LTs, pois quando se inibe a síntese de LTs ou quando se antagonizam seus receptores a fagocitose é significativamente reduzida. Além disso, a adição de LTs aos MAs em cultura aumentou a fagocitose (MANCUSO *et al.*, 1998).

Embora haja poucos estudos sobre LTs e diabetes na literatura, Roth *et al.* (1984) mostraram que o pulmão de ratos diabéticos estimulado com ionóforo de cálcio produziam menos cisteinil-LT que o de ratos sadios. Jubiz *et al.* (1984) relataram menor produção de LTB_4 por neutrófilos de indivíduos diabéticos

comparados com sadios. Nós mostramos neste trabalho que a concentração de LTB_4 e LTC_4 nas culturas de MAs fagocitando IgG-SRBC foi semelhante entre ratos sadios e diabéticos. Além disso, a adição de LTs nas culturas de MAs aumentou marcantemente a fagocitose tanto em sadios como em diabéticos. Entretanto, o tratamento dos MAs de diabéticos com inibidores da síntese ou antagonistas de LTs, ao contrario do que foi observado em MAs de sadios, não foi inibida. Portanto, enquanto em MAs de ratos sadios a fagocitose é dependente de LTs, nos MAs de ratos diabéticos ela é independente destes mediadores.

Por outro lado, nós observamos que MAs de sadios e diabéticos respondem de modo semelhante quando adicionamos LTs ao meio de cultura. Tanto o LTB_4 como os cisteinil-LTs aumentaram a fagocitose de IgG-SRBC. Isto indica que tanto os receptores de membrana para os LTs como as vias de sinalização acionadas por eles não estão comprometidas nos diabéticos.

Portanto, embora os MAs de diabéticos respondam normalmente aos LTs exógenos, a fagocitose via FcγR por estas células não é dependente de LTs. Esta aparente contradição pode ser explicada pelo fato de existirem receptores tanto de membrana plasmatica como intracelulares para os LTs (BANDEIRA-MELO *et al.*, 2002), com funções não obrigatoriamente iguais. Uma ação intrácrina já foi descrita para outros mediadores lipídicos (ZHU *et al.*, 2006), mas muito pouco se conhece sobre a ação intracelular deste grupo de mediadores.

Os mecanismos moleculares deste efeito dos LTs já haviam sido estudados em MAs de ratos sadios, e constatou-se que a amplificação da fagocitose via FcγR por LTs é dependente de PKC (MANCUSO e PETERS-GOLDEN, 2000) . Além disso, este grupo mostrou que o efeito amplificador do LTB_4, mas não dos cisteinil-LTs, depende da ativação de *spleen tyrosine kinases* (*Syk*) e Ca^{2+} intracelular (CANETTI *et al.*, 2003). Mais recentemente, Campos e col 2009, mostraram que a potencialização da fagocitose via FcγR por LTs é também dependente da ativação de MAP kinases e de PI3Kinase, e que LTB_4 e LTD_4 agem por vias de sinalização distintas para amplificar a fagocitose. Fomos, então, analisar o papel dos LTs na ativação de quinases em MAs de diabéticos. Encontramos que a fagocitose via FcγR aumenta a fosforilação de PKC-δ em MAs de ratos diabéticos, a semelhança do que ocorre nos sadios. Entretanto, em diabéticos a fagocitose não ativou a Akt.

O fato de não ocorrer ativação da Akt em MAs de ratos diabéticos poderia explicar porque a fagocitose por MAs destes animais não foi dependente de LTs endógenos, visto que, segundo Campos et al. (2009), o aumento da fagocitose via FcγR tanto pelo LTB_4 quanto cisteinil-LTs é dependente da ativação da PI3K, cujo substrato, ou seja, alvo a ser fosforilado, é a própria Akt. Os mesmos autores acreditam que a PI3K seja um ponto de convergência entre as distintas vias de sinalização do LTB_4 e cisteinil-LTs e que, por isso, a inibição da mesma é capaz de abolir o efeito de ambos os LTs.

Podemos especular que o fato da PI3K não se ativar nos MAs de diabéticos seja porque estas células estão carentes da ação da insulina. Isto implica que a insulina seja fundamental para a ativação desta via de sinalização.

A ativação de PKC é necessária para o aumento da fagocitose causada por LTs (MANCUSO e PETERS-GOLDEN, 2000). Porém, é importante ressaltar que a ação de um mediador não depende somente da fosforilação de uma quinase ou mesmo da ativação de apenas uma via. Os receptores de LTs, por serem acoplados a proteínas G, também desencadeiam alterações na concentração de Ca^{2+} intracelular, ativação de segundos mensageiros, como o AMPc, etc (PERES *et al.*, 2007). Assim como para Akt, outras quinases envolvidas na resposta de MAs de ratos diabéticos aos LTs podem apresentar alterações no padrão de fosforilação após estímulo do FcγR.

Neste trabalho, avaliamos ainda o efeito da adição de insulina sobre a fagocitose via FcγR. Observamos que a adição de insulina nas culturas de macrófagos aumentou a fagocitose via FcγR por MAs tanto de ratos sadios como diabéticos. Estes resultados concordam com os de Lima *et al.* (1979), que mostraram que a insulina em concentrações fisiológicas aumenta a fagocitose via FcγR por macrófagos peritoneais de camundongos. Outros estudos mostram que a insulina é capaz de aumentar não só a fagocitose de partículas de zymosan, mas também a produção de H_2O_2 por macrófagos peritoneais de ratos (COSTA ROSA *et al.*, 1996). Por outro lado, Muschel *et al.* (1977) demonstraram a capacidade da insulina em diminuir a fagocitose, porém isto foi encontrado em células de linhagem macrófago-símile (J774.2) e, embora o estímulo fosse o mesmo, o tempo de tratamento e a dose de insulina utilizados eram diferentes.

Nós encontramos que o efeito potencializador da insulina foi abolido quando se impediu a síntese de LTs nos MAs de ratos sadios. Já nos MAs de ratos diabéticos, a inibição da síntese de LTs não teve efeito na fagocitose e nem na sua potencialização pela insulina. Com isso, constatamos que o efeito potencializador da insulina nos MAs de ratos sadios é dependente de LTs, enquanto que em MAs de ratos diabéticos é independente destes mediadores.

É interessante notar que a Akt é uma quinase-chave da sinalização do receptor de insulina para sua função de aumento no transporte de glicose (HAN *et al.*, 2006). Mostramos que a insulina é capaz de aumentar a fagocitose via FcγR em MAs de ratos sadios. Poderíamos especular que este hormônio é essencial para que ocorra a fosforilação desta quinase e, com isso, resultar em uma resposta mais intensa dos MAs aos LTs produzidos. No caso dos MAs de ratos diabéticos, nos quais não ocorreu a fosforilação da Akt pelo estímulo do FcγR, a insulina também aumentou a fagocitose talvez por uma ação direta sobre a Akt. Ainda, observamos que, enquanto que em MAs de sadios a potencialização da fagocitose pela insulina foi inibida por zileuton, sendo portanto dependente de LTs, nos MAs de diabéticos o efeito potencializador da insulina não foi inibido pelo zileuton. Estes resultados mostram que MAs de ratos diabéticos tem menor capacidade de fagocitar alvos opsonizados com IgG e sugerem que isto se deve a incapacidade de ativação da PI3K por acoplamento do FcγR nos MAs de diabéticos.

A ação deste hormônio também é descrita como anti-inflamatória. A administração de insulina em pacientes em estado crítico tem sido bastante discutida. Em revisão, Das (2003) defende a ação da insulina como anti-inflamatório para uso em pacientes com sepse. Van den Berghe *et al.* (2006), em estudo clínico, mostraram que a administração contínua de insulina em doses baixas reduziu a morbidade entre pacientes na UTI médica por prevenir o desenvolvimento de novas complicações. Também foi descrito que em indivíduos sadios que receberam insulina, os neutrófilos têm capacidade microbicida e fagocítica aumentadas (WALRAND *et al.*, 2006). Em trabalho paralelo, mostramos que a insulina é capaz de inibir a ativação de vias de sinalização acionadas por LPS em MAs (MARTINS *et al.*, 2008a). Além disso, a insulina também inibe a translocação de p65NFκB para o núcleo e a expressão de iNOS e COX-2 e seus produtos óxido nítrico e

prostaglandina E_2 (MARTINS *et al.*, 2008b). Estes trabalhos (ANEXO) ratificam um efeito anti-inflamatório da insulina sobre MAs de ratos sob estímulo por LPS.

É interessante observar o efeito modulador da insulina na imunidade. Em MAs, ela foi capaz de inibir a produção de mediadores estimulada por LPS, mostrando, portanto, ação anti-inflamatória. Além disso, a insulina aumentou a capacidade fagocítica dos MAs, sugerindo um papel relevante deste hormônio na defesa contra infecções.

Tomados em conjunto, nossos resultados mostram que a fagocitose de alvos opsonizados por IgG por MAs é dependente de LTs produzidos pela ativação do FcγR, os quais amplificam a fosforilação da PKC e da PI3K . MAs provenientes de ratos diabéticos fagocitam menos, a fagocitose não é dependente de LTs endogenos e não se observa ativação de PI3K. Podemos especular que em condições de carência de insulina não ocorre ativação da PI3K em MAs fagocitando via FcγR e, assim, não se manifesta o efeito potencializador dos LT sobre esta via. Nosso trabalho contribui para um melhor conhecimento dos mecanismos envolvidos na ação dos LTs e da insulina sobre a resposta imune.

Conclusões

6 CONCLUSÕES

Enquanto que o estímulo do FcγR em MAs de ratos sadios induz a produção de LTs que potencializam a fagocitose, MAs de ratos diabéticos são deficientes dessa potencialização dependente de LTs. Este fato não está relacionado à falha na capacidade de MAs de ratos diabéticos em sintetizar LTs após estímulo pelo FcγR e nem a sua capacidade de responder aos LTs.

Nossos resultados analisando as vias de sinalização acionadas por estimulação dos FcγR nos permite especular que, em MAs de ratos diabéticos, devido a carência dos efeitos da insulina, o estímulo do FcγR não induz ativação da PI3K. Como esta quinase é é um dos alvos da ação potencializadora dos LTs endógenos na fagocitose, isto explicaria a fagocitose diminuída dos MAs de ratos diabéticos.

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Anexos

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Insulin Inhibits LPS-Induced Signaling Pathways in Alveolar Macrophages

Joilson O. Martins, Matheus Ferracini, Natalia Ravanelli, Richardt G. Landgraf and Sonia Jancar

Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil

Key Words
Insulin • LPS • Macrophages • ERK • p38 • Akt • PKC • TNF

Abstract
The systemic inflammatory response syndrome (SIRS) is triggered by lipopolysaccharide (LPS) from Gram-negative bacteria. Insulin was shown to have a protective role in SIRS related to sepsis. Lungs are particularly affected in this condition and provide a second wave of mediators/cytokines which amplifies SIRS. The aim of the present study was to investigate the effect of insulin on the signaling pathways elicited by LPS in alveolar macrophages (AMs) and its consequence in cellular response to LPS measured as production of tumor necrosis factor (TNF). To this purpose, resident AMs from male Wistar rats were obtained by lung lavage and stimulated by LPS (100 ng/mL). Insulin (1 mU/mL) was added 10 min before LPS. Activation (phosphorylation) of signaling molecules by LPS was analyzed by western blot, 30 min after LPS stimulation. TNF was measured in the AMs culture supernatants by bioassay using L-929 tumor cells. Relative to controls, LPS induced a significant increase in the activation of ERK (3.6-fold), p38 (4.4-fold), Tyr-326 Akt (4.7-fold), Ser-473 Akt (6.9-fold), PKCα (4.7-fold) and PKCδ (2.3-fold). Treatment of AMs with insulin before LPS stimulation, significantly reduced the activation of ERK (54%), p38 (48%), Tyr-326 Akt (64%), Ser-473 Akt (41%), PKCα (62%) and PKCδ (39%). LPS induced TNF production in AMs which was also inhibited by insulin (60%). These results show that insulin down-regulates MAPK, PI3K and PKCs and inhibits a downstream effect of LPS, TNF production, in rat AMs stimulated with LPS and suggest that the protective effect of insulin in sepsis could be through modulation of signal transduction pathways elicited by LPS in lung macrophages.

Introduction

The systemic inflammatory response syndrome (SIRS) is triggered by lipopolysaccharide (LPS) from Gram-negative bacteria that affects many organs and

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Joilson O. Martins, PhD
Department of Immunology, Institute of Biomedical Sciences
University of São Paulo, Av. Prof. Lineu Prestes, 1730
05508-900 São Paulo, SP (Brazil)
Phone/FAX: +55-11-3091-7744, E-Mail martinsj@usp.br

may lead to death. Insulin was shown to attenuate SIRS in endotoxemic rats [1], to modulate inflammatory response in diabetic rats [2-4] and to decrease mortality and incidence of sepsis in critically ill patients [5]. Lungs are particularly affected by SIRS related to sepsis and provide a second wave of mediators/cytokines amplifying SIRS and the mortality associated to this condition. Thus, one possibility to explain the protective effect of insulin in SIRS would be that it reduces the release of mediators/ cytokines by the lungs. This could be achieved by an effect of insulin on LPS-induced signaling pathways in lung macrophages. It is well established that LPS/LBP (LPS-binding protein) complex acts on toll-like receptor (TLR)-4 through CD14 [6] to activate inflammatory genes expression in macrophages and release of a plethora of mediators/cytokines which are involved in SIRS such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF), nitric oxide (NO), leukotrienes and platelet activating factor (PAF). These effects of LPS are consequent to activation of intracellular signaling cascades among them the mitogen-activated protein kinases (MAPK), which comprises the extracellular signal-regulated kinase (ERK) and p38. In macrophages, LPS was shown to activate both pathways [7, 8]. LPS also induces activation of PKCα and PKCδ in mouse peritoneal macrophages, both involved in macrophage functions such as phagocytosis, respiratory burst and cytokines secretion [9]. Akt is a downstream regulator of phosphotidylinositol 3'-kinase (PI3K) and is implicated on PI3K-mediated regulation of NF-kB [10] an important transcription factor for pro-inflammatory mediators. Insulin has the potential to interfere with these pathways: its effects at cellular level includes glucose transport, glycogen synthesis, mitogenesis [11-13]; stimulation of protein kinases and activation or repression of genes transcription [14].

In the present study we investigated the effect of insulin on the signaling pathways induced by LPS in alveolar macrophages focusing on ERK, p38, Akt and PKC proteins activation. The effect of insulin on a downstream effect of LPS, production of TNF, was also investigated.

Materials and Methods

Animals

Male Wistar rats weighing 200 +/- 20g (about 9 weeks of age) were obtained from Central Laboratory Animal of the Biomedical Sciences Institute of University of São Paulo. The animals were maintained at 23 +/- 2°C under a cycle of 12 hours light: 12 hours darkness and were allowed access to food and water *ad libitum.* Animal care and research protocols were in accordance the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by The Ethical Committee for Animal Research of the Biomedical Sciences Institute, University of São Paulo.

Cell isolation and culture

Resident AMs from rats were obtained by *ex vivo* lung lavage, as previously described [15] and were resuspended in RPMI-1640. Cells were allowed to adhere in culture-treated plates for 1 hour (37°C, 5% CO_2); this was followed by one wash with warm RPMI-1640, resulting in more than 99% of adherent cells identified as AMs by staining with a modified Wright-Giemsa stain. Cells were cultured overnight in RPMI-1640 supplemented 10% FBS (fetal bovine serum) and were washed twice the next day with warm medium to remove the non-adherent cells.

Cell treatments

AMs ($1x10^6$) plated in 4-well tissue culture dishes were pre-treated or not with crystalline insulin at a final concentration of 1 mU/mL, 10 minutes before LPS stimulus *in vitro* [16]. Then, AMs were stimulated for 30 minutes with 100 ng/mL LPS and were placed on ice for 10 minutes to stop the reaction. After that, AMs were washed three times in ice-cold PBS and lysed by sonication in ice-cold lyses buffer containing 150 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 µg/mL leupeptin, followed by ultracentrifugation at 100 000 g for 20 minutes at 4°C, the supernatant was frozen -70ºC for immunobloting [15, 17].

Insulin treatment

AMs ($1x10^6$) plated in tissue culture dishes were treated with insulin 10 min before, concomitantly or 10 min after LPS stimulation. Different doses of crystalline insulin (0.1, 1, 10 mU/mL) were used. Then, AMs were stimulated for 30 minutes with 100 ng/mL LPS from *Escherichia coli* (serotype 055:B5). After that, the supernatant was frozen -70ºC for measuring of TNF production.

TNF biossay

The TNF levels were determined in the supernatants of macrophage cultures by a biossay with L929 tumor cells [18, 19]. Briefly, L929 cells were plated in 96-well flat-bottom microplates in a concentration of $5x10^4$ cells/100 mL of complete medium and incubated for 24 h until obtaining a monolayer. The samples were serially diluted and added to the L929 monolayers in presence of actinomycin D (2 µg/mL). After 24 h of incubation at 37ºC in humidified 5% CO_2 incubator, the L929 monolayers were stained with crystal violet (0.5% in acetic acid 30%) for 10 min. After this, the plates were washed with distilled water and left to dry at room temperature. A volume of 100 µL of absolute methanol was added to dissolve the stain and the absorbance was read at 620 nm in a Dynatech microplate reader. One unit of TNF was referred to as the reciprocal of the dilution that induces 50% of L-929 cell lyses.

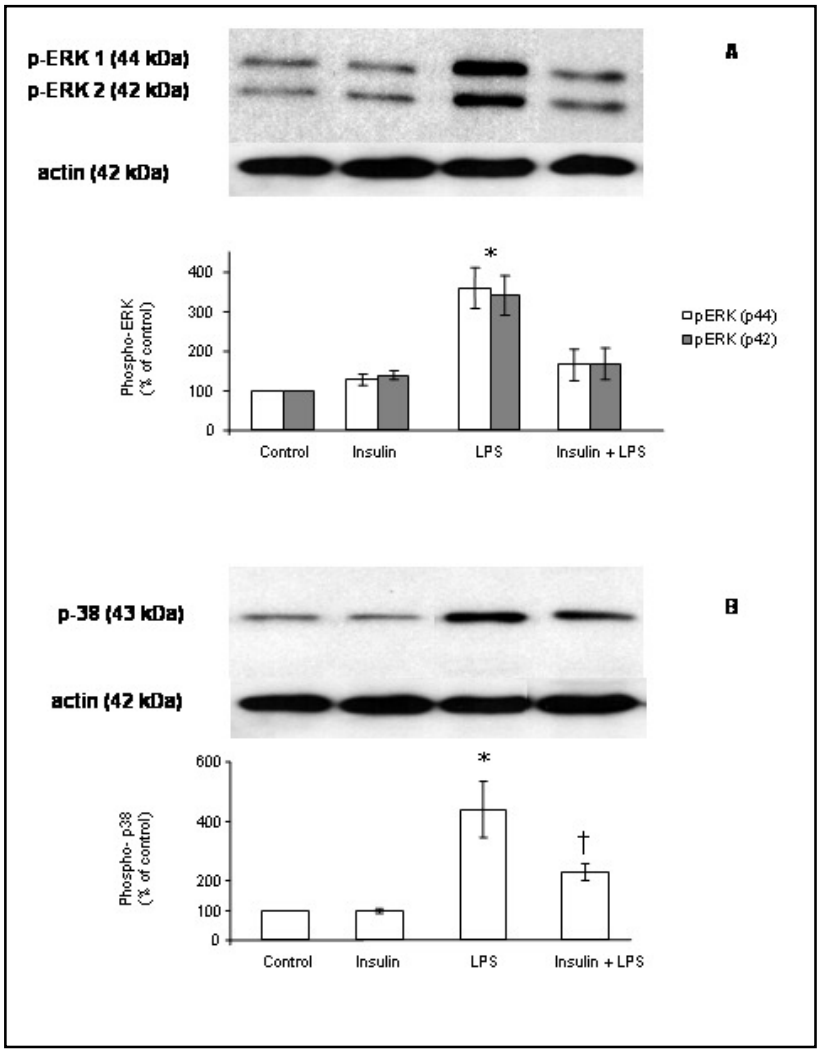

Fig. 1. Insulin inhibition of LPS-induced ERK (A) and p38 MAPK (B) activation. Alveolar macrophages ($1x10^6$) were obtained by *ex vivo* lung lavage from male Wistar rats and incubated with insulin (1 mU/mL) 10 minutes before LPS stimulation (100 ng/mL) for 30 minutes. ERK and p38 MAPK phosphorylation were assessed by western blot analysis. The antibodies recognized phosphorylated residues of Thr183/Tyr185 of p44/42 rat MAP Kinase and the Thr180/Tyr182 of p38 MAP Kinase. Illustrations of the western blot represent one out of 6 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 6 animals per group. *P<0.001 *vs* other groups; †P<0.05 *vs* LPS group.

Western blotting

Protein concentration was determined by BCA protein assay reagent kit. Samples containing 20 µg protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membrane using the Biorad Mini-Gel system and trans-blot® SD-semidry Transfer cells. For immunobloting, the nitrocellulose membranes were incubated in TSB-T buffer (150 mM NaCl, 20 mM Tris, 1% Tween 20, pH 7,4) containing 5% non-fat milk dried milk, for 1 h. After that, the blots were washed with TSB-T buffer three times for 5 min and were probed with antibodies (1:500 dilution) directed against phospho-ERK 1/2 MAP Kinase (Thr183/Tyr185), phospho-p38 MAP Kinase (Thr180/Tyr182), phospho-Akt (Tyr326), phospho-Akt (Ser473), phospho-PKCα (Thr638/641) and phospho-PKCδ (Thr505), for 90 min, followed by anti-rabbit secondary antibody (1:2000; for 1 h). Blots were developed using enhanced chemiluminescence (ECL) detection and exposed to photographic film. Finally, blots were stripped with 200 mM glycine, pH 3.0, for 10 min, washed with TBS-T three times for 30 min each, and reprobed with β-actin (1:10.000), followed by anti-mouse secondary antibody (1:2000). The band densities were determined by densitometric analysis using the AlphaEaseFC™ program. Density values of bands were normalized to the total β-actin present in each lane and expressed in % of control.

Drugs and reagents

RPMI-1640 and FBS (Gibco-Invitrogen, Carlsbad, CA, USA); Wright-Giemsa stain (Diff-Quik; American Scientific Products, McGraw Park, IL, USA); Methanol (Merck, São Paulo, Brazil); Cristalline insulin (Biobrás, São Paulo, Brazil); LPS, actinomycin D, crystal violet and β -actin (Sigma, Chemical Co, St Louis, Mo, USA); BCA protein assay reagent kit (Pierce Chemical, Rockford, IL, USA); chemiluminescence (ECL) detection (Amersham, Piscataway, NJ, USA); Antibodies all from Cell Signaling technology, INC. Beverly, MA, USA.

Statistical analysis

Data are presented as mean ± SEM of at least four experiments, statistical analysis was performed using the GraphPad Software (San Diego, CA, USA), and compared by analysis of variance (ANOVA) followed by the Bonferroni test. A p values lower than 0.05 were considered statistically significant. TNF data are presented as median of the titles and differences of at least two dilutions were considered significant.

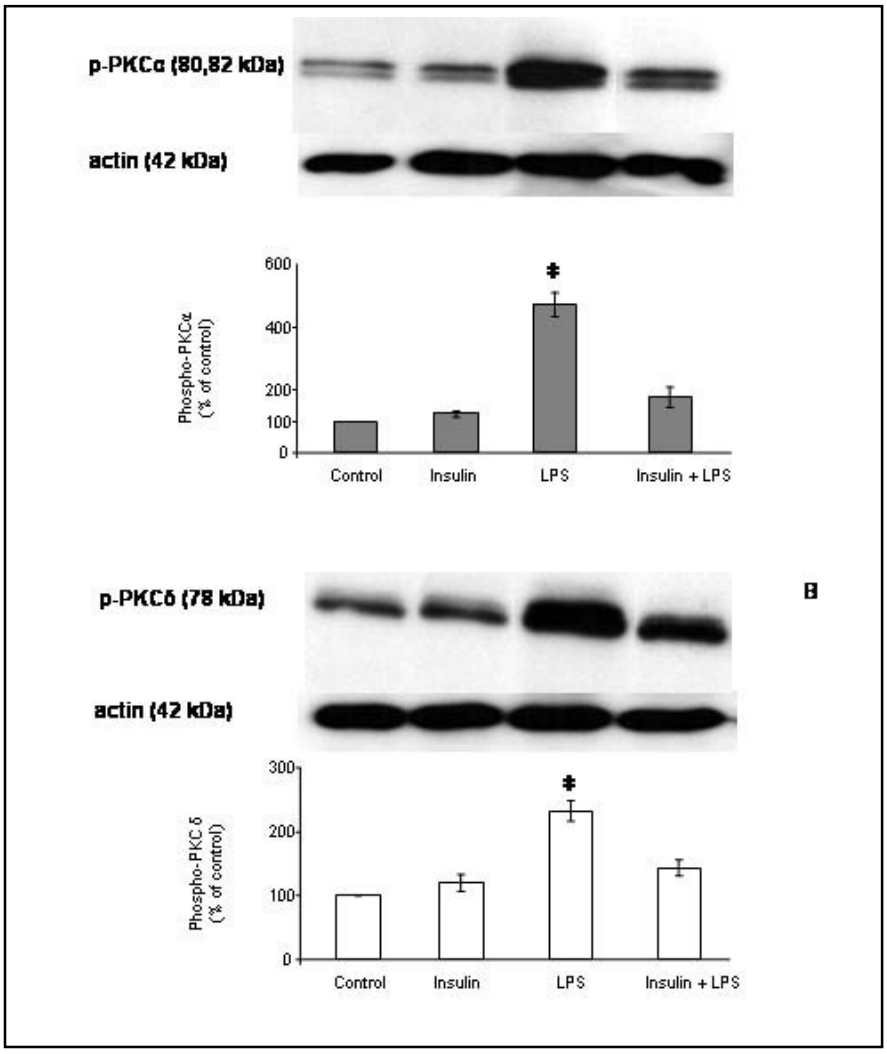

Fig. 2. Insulin inhibition of LPS-induced PKCα (A) and PKCβ (B) activation. Alveolar macrophages ($1x10^6$) were obtained by *ex vivo* lung lavage from male Wistar rats and incubated with insulin (1 mU/mL) 10 minutes before LPS stimulation (100 ng/mL) for 30 minutes. PKCα and PKCδ phosphorylation were assessed by western blot analysis. The antibodies recognized phosphorylated residues of Thr638/641 of PKCα and the Thr505 of PKCδ. Illustrations of the western blot represent one out of 6 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 6 animals per group. *P<0.001 *vs* other groups.

Results

Insulin inhibits LPS-induced ERK and p38 MAPK activation

Resident AMs from male Wistar rats were obtained by ex vivo lung lavage and stimulated with LPS (100 ng/mL) for 30 min. Activation (phosphorylation) of signaling of MAPK by LPS was analyzed by western blot. Figure 1 shows that, relative to controls, LPS induced a significant increase in the activation of ERK (3.6 fold) and p38 (4.4 fold). A group of AMs was pre-treated with insulin (1 mU/mL) 10 minutes before LPS stimulation. This dose of insulin was taken from previous publication from our group [16]. Insulin significantly reduced the activation of ERK (54%) and p38 (48%). These data show that insulin down-regulates the MAPK cascade in AMs stimulated with LPS.

Insulin inhibits LPS-induced PKCα and PKCδ activation

Activation (phosphorylation) of PKCα and PKCδ by LPS was analyzed by western blot. Figure 2 shows that, relative to controls, LPS induced a significant increase in the activation of PKCα (4.7 fold) and PKCδ (2.3 fold) in AMs. Treatment of AMs with insulin (1 mU/mL), 10 minutes before LPS stimulation, significantly reduced the activation of PKCα (62%) and PKCδ (39%). These data suggest that the insulin down-regulates PKC´s activation in AMs activated with LPS.

Insulin inhibits LPS-induced Akt activation

Activation (phosphorylation) of Akt in AMs stimulated with LPS was analyzed by western blot. Figure 3 shows that LPS increased phosphorylation of both Ser-473 and Tyr-326 Akt residues. Relative to the

Fig. 3. Insulin inhibition of LPS-induced Akt activation. Alveolar macrophages ($1x10^6$) were obtained by *ex vivo* lung lavage from male Wistar rats and incubated with insulin (1 mU/mL) 10 minutes before LPS stimulation (100 ng/mL) for 30 minutes. Akt phosphorylation was assessed by western blot analysis. The antibodies used recognize phosphorylated residues of the Tyr-326 or Ser-473 of Akt. Illustration of the western blot represents one out of 6 (for Tyr-326) or 3 (for Ser-473) of Akt independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 6 or 3 animals per group. *P<0.001 *vs* other groups; †P<0.05 *vs* LPS group; ±P<0.05 *vs* control.

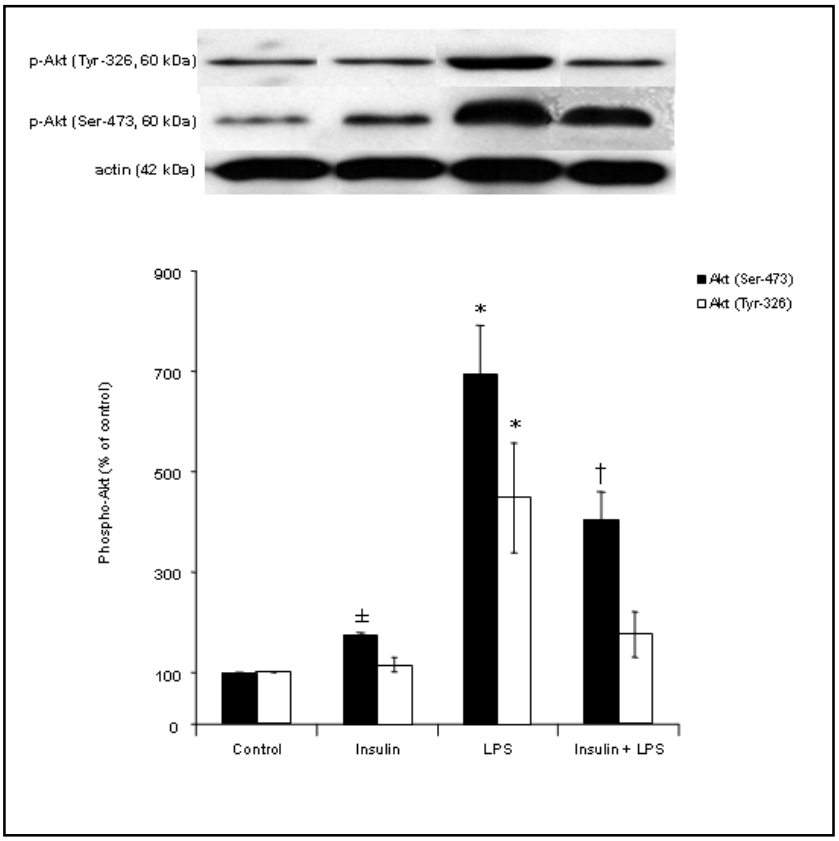

Table 1. Insulin inhibition of LPS-induced TNF production. Alveolar macrophages ($1x10^6$) were obtained by *ex vivo* lung lavage from male Wistar rats. Insulin (0.1; 1; 10 mU/mL) was given 10 minutes before, together or 10 minutes after LPS stimulation. TNF levels were measured in supernatants by bioassay using L929 cells 30 minutes after LPS. The median of TNF (U/mL) and standard deviation (SD) of each group are shown. Differences of two or more dilutions were considered as significant. *P<0.001.

Groups	Median of TNF (U/mL)	SD
Control	4 *	0
LPS	32	18.5
Insulin before LPS		
0.1	32	9.2
1	4 *	2.3
10	6	0
Insulin together LPS		
0.1	32	9.2
1	4 *	2.3
10	4 *	2.3
Insulin after LPS		
0.1	32	0
1	8 *	2.3
10	8 *	0

controls, the Tyr-326 phosphorylation was 4.7-fold and of Ser-473 was 6.9-fold. At the concentration of 1 mU/ml insulin did not increase significantly the phosphorylation of the Tyr-326 residue although it did cause a small increase (1.8-fold, p<0.05 relative to control) in the Ser-473 residue phosphorylation. Treatment of AMs with insulin (1 mU/mL), 10 minutes before LPS stimulation, significantly reduced the activation of Tyr-326 Akt (64%) and of Ser-473 Akt (41%). These data show that insulin exerts an inhibitory effect on PI3K activation in AMs stimulated with LPS as verified by phosphorylation of both Tyr-326 and Ser-473 residues of Akt.

Insulin inhibits LPS-induced TNF production

TNF release was measured by a cytotoxicity assay using L-929 tumor cells. AMs were pre-treated with insulin (0.1, 1, 10 mU/mL) 10 min before stimulation with LPS and TNF levels determined in the culture supernatants 30 min later. We found that 0.1 mU/mL has no effect,

and that 1 and 10 mU/mL of insulin abolished TNF release. We also assayed the effect of adding insulin concomitantly with LPS and after 10 min. There is no difference between pre and concomitant treatment on LPS-induced TNF. The post treatment with insulin was also inhibitory but relative to pre and concomitant treatments, showed a tendency to increase but the difference was not significant (table 1). These data show that insulin is able to markedly affect a macrophage function and confirm that the lowest concentration of insulin to achieve this effect was 1 mU/mL.

Discussion

Data presented here show that LPS added to rat AMs enhance phosphorylation of ERK 1/2, p38 MAPK, Akt, PKCα PKCδ and that the phosphorylation of these molecules decreased when insulin was given before LPS stimulation. Insulin alone did not activate these pathways, in rat AMs at the experimental condition used in this study. We also showed a downstream effect of insulin e.g. inhibition of TNF release. Insulin is a key hormone regulating the control of metabolism and maintenance of normoglycemia and normolipidemia. Our results are consistent with an inhibitory effect of insulin and corroborate previous findings showing a protective effect of insulin on systemic inflammation related the sepsis [1]. In this study, we focused on the ERK 1/2, p38 MAPK, Akt, PKCα and PKCδ, which are kinases involved with the regulation of a number of macrophage functions including phagocytosis, respiratory burst, and secretion of several cytokines, such as TNF.

The presence of insulin receptors has been demonstrated in monocytes/macrophages [20, 21] but not in alveolar macrophages. However, there are several studies showing that insulin exerts clear effects on these macrophages [22, 23], which suggests that they do express insulin receptors. In the present study we wanted to use the lower dose of insulin able to interfere with LPS-induced signaling without having a significant effect by itself on the molecules analyzed. At 1mU/ml, insulin alone did not increase basal levels of phosphorylation of most molecules analyzed but it clearly interfered with the LPS-induced signaling cascades. Although it is well known that insulin activates Akt [reviewed in 24] in our experimental conditions it only caused a small increase in the phosphorylation of Ser-473 but not Tyr-326 residues of Akt. We believe that higher doses of insulin will have more marked effect. Grupe et al. [25] showed that insulin receptor phosphorylation was dose-dependent; the dose of 1 mM was minimally active and the dose of 10 mM induced a sharp increase. Taken together these results suggest that the inhibitory effect of insulin on LPS-induced signaling in AMs is a receptor-dependent phenomenon.

The MAPK pathway is involved in the activation of several transcription factors that control gene activity and expression. Activation of $p21^{ras}$ leads to activation of all members of the MAP kinase family, and this effect is mediated by specific MEK kinases [26, 27], including ERK and p38. Our results show that LPS-induced increase in ERK and p38 phosphorylation was down-regulated by insulin, which could suggest that insulin acts directly on $p21^{ras}$. Insulin was shown to activate the $p21^{ras}$/MAP kinase pathway through the binding of growth factor receptor-bound protein (GRB)-2 to tyrosine-phosphorylated insulin receptor substrate (IRS)-1 [28]. In addition, several IRS-1-associated proteins have already been identified including PI3K [29]. Our results show that LPS increased Akt phosphorylation at both Tyr-326 and Ser-473 residues. The increase was more prominent at the Ser-473 residue (6.9-fold relative to control) than on Tyr-326 (4.7-fold relative to control).

PI3K is composed of two subunits, a 100 kDa (p110) catalytic subunit and an 85 kDa (p85) regulatory subunit [30]. A direct interaction between $p21^{ras}$ and p110, and the demonstration that $p21^{ras}$ regulates PI 3'-kinase activity [31], suggests that activation of $p21^{ras}$ via IRS-1 could initiate both the MAP kinase and PI3K signaling pathway.

Jeschke et al. [1] reported that in endotoxemic rats, insulin treatment increased the serum level of anti-inflammatory cytokines and decreased the proinflammatory ones, helping to restore systemic homeostasis. TNF is a well-characterized pro-inflammatory cytokine and may contribute to complex clinical problems encountered in the sepsis syndrome [32]. MAPKs are a group of signaling molecules that appear to play important roles in inflammatory processes and LPS has been reported to activate MAPK pathways [8]. We decided to measure TNF production in order to evaluate a downstream effect of insulin in LPS-induced signaling and found that insulin abolished TNF production. We have no direct evidence that this effect of insulin is related to inhibition of the signaling molecules studied. However, others have demonstrated that selective pharmacological inhibitors of p38 and ERK pathways reduced TNF production by murine macrophages [33]. In our experiments, insulin down-regulated both p38 and ERK pathways and TNF production elicited by LPS.

These observations favor the hypothesis that the protective effect of insulin in sepsis is due to modulation of cellular signal transcription factors rather than to changes in metabolism and blood glucose. In addition, although the insulin receptor and the enzymes whose activity is altered by insulin treatment have been well characterized, the initial steps following insulin binding to its receptor that leads to a change in the activity of a specific enzyme remains to be elucidated [34].

PKC is a family of multifunctional serine/threonine kinases also involved with the regulation of macrophage functions. Treatment of mouse peritoneal macrophages with LPS stimulates activation of PKCα and PKCδ in a dose- and time-dependent manner [9]. In agreement, our results show that increase of both, PKCα and PKCδ phosphorylation in LPS-activated AMs was down-regulated by pre-treatment of AMs with insulin. Since PKC plays a role in macrophage functions such as phagocytosis and insulin inhibits PKC, thus insulin should reduce phagocytosis and consequently, the host defense against infection. However, host defense during sepsis, is dependent primarily on circulating neutrophils and it is not known what effect has insulin on phagocytosis and killing by neutrophils. Also we do not know what is the effect of the low dose of insulin that we used (1 mU/mL) on the macrophages microbicidal activity (reactive oxygen intermediate - ROI, NO production, etc). At much higher concentration (200 mU/mL), Costa Rosa et al. [22], found that insulin enhanced phagocytosis and production of H_2O_2 by macrophages. Thus, the effect of insulin seems to be dependent on the dose, time and type of cell.

In conclusion, this report shows that insulin inhibits the LPS-induced ERK 1/2, p38, Akt, PKCα and PKCδ phosphorylation and TNF release in AMs *in vitro*. Thus, it is possible that the protective effect of insulin in sepsis reported by others is due to inhibition of the secondary wave of mediators released by the lungs during this condition.

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Insulin Suppresses LPS-induced iNOS and COX-2 Expression and NF-κB Activation in Alveolar Macrophages

Joilson O. Martins, Matheus Ferracini, Natalia Ravanelli, Richardt G. Landgraf and Sonia Jancar

Department of Immunology, Institute of Biomedical Sciences, University of São Paulo

Key Words
Insulin • LPS • Macrophages • NO • iNOS • PGE_2 • COX-2 • NF-κB • IκBα

Abstract
The development of septic shock is a common and frequently lethal consequence of gram-negative infection. Mediators released by lung macrophages activated by bacterial products such as lipopolysaccharide (LPS) contribute to shock symptoms. We have shown that insulin down-regulates LPS-induced TNF production by alveolar macrophages (AMs). In the present study, we investigated the effect of insulin on the LPS-induced production of nitric oxide (NO) and prostaglandin (PG)-E_2, on the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, and on nuclear factor kappa B (NF-κB) activation in AMs. Resident AMs from male Wistar rats were stimulated with LPS (100 ng/mL) for 30 minutes. Insulin (1 mU/mL) was added 10 min before LPS. Enzymes expression, NF-κB p65 activation and inhibitor of kappa B (I-κB)α phosphorylation were assessed by immunobloting; NO by Griess reaction and PGE_2 by enzyme immunoassay (EIA). LPS induced in AMs the expression of iNOS and COX-2 proteins and production of NO and PGE_2, and, in parallel, NF-κB p65 activation and cytoplasmic I-κBα phosphorylation. Administration of insulin before LPS suppressed the expression of iNOS and COX-2, of NO and PGE_2 production and Nuclear NF-κB p65 activation. Insulin also prevented cytoplasmic I-κBα phosphorylation. These results show that in AMs stimulated by LPS, insulin prevents nuclear translocation of NF-κB, possibly by blocking I-κBα degradation, and supresses the production of NO and PGE_2, two molecules that contribute to septic shock.

Introduction

The development of septic shock is a common and highly lethal consequence triggered by lipopolysaccharide (LPS) from gram-negative bacteria that affects many organs and may lead to death. Insulin was shown to modulate inflammatory response in diabetic rats [1-3] and to decrease mortality and incidence of sepsis in critically

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Joilson O. Martins, PhD
Department of Immunology, Institute of Biomedical Sciences
University of São Paulo, Av. Prof. Lineu Prestes, 1730
05508-900 São Paulo, SP (Brazil)
Phone/Fax +55-11-3091-7744, E-Mail martinsj@usp.br

ill patients [4]. Lungs are particularly affected during sepsis and provide a second wave of mediators/cytokines, amplifying the systemic inflammatory response and the mortality associated to this condition. LPS activates several signaling pathways in macrophages by acting on toll-like receptor (TLR)-4 through CD14 [5] to activate inflammatory gene expression and release of mediators/ cytokines such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF), nitric oxide (NO), leukotrienes and platelet activating factor (PAF).

Activated macrophages produce a vast array of bioactive molecules, among them is NO, derived from the amino acid L-arginine by enzymatic activity of nitric oxide synthase (iNOS). Prostaglandins (PGs) are product of arachidonic acid metabolism via cyclooxygenases (COX). After stimulation with LPS, macrophages express the inducible forms of these enzymes (iNOS and COX-2), which are responsible for the production of large amounts of NO and PGs [6]. The promoter region of COX-2 genes in mice [7], rats [8], and humans [9] has been cloned and sequenced. This promoter region contains various putative transcriptional regulatory elements such as cyclic AMP response element (CRE), GATA box, nuclear factor-kappa B (NF-κB) and NF-IL-6. Amongst these elements, NF-κB and NF-IL-6 act as positive regulatory elements for the COX-2 transcription in some cell lines [10].

NF-κB is an inducible transcription factor that mediates signal transduction between cytoplasm and nucleus in many cell types [11]. In resting cells, NF-κB is localized in the cytoplasm as a heterodimer composed of two polypeptides of 50 kDa (p50) and 65 kDa (p65), which are non-covalently associated with cytoplasmic inhibitory proteins, including inhibitor of kappa B (I-κB)α. Upon cell stimulation by a variety of agents, the NF-κB complex migrates into the nucleus and binds DNA recognition sites in the regulatory regions of the target genes [12]. Activation of NF-κB by LPS induces a cascade of events leading to the phosphorylation of I-κBα and its futher proteolytic degradation [13].

We have recently shown that insulin down-regulates mitogen-activated protein kinases (MAPK), phosphotidylinositol 3'-kinase (PI3K) and protein kinase C (PKC)-α and PKC-δ and inhibits TNF production, in rat alveolar macrophages (AMs) stimulated with LPS [14].

In the present study we investigated the effect of insulin on the NF-κB activation, expression of iNOS and COX-2, and production of NO and PGE_2, in AMs stimulated with LPS.

Materials and Methods

Animals

Male Wistar rats weighing 200 +/- 20g (about 9 weeks of age) were obtained from Central Laboratory Animal of the Biomedical Sciences Institute of University of São Paulo. The animals were maintained at 23 +/- 2°C under a cycle of 12 hours light: 12 hours darkness and were allowed access to food and water *ad libitum*. Animal care and research protocols were in accordance the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by The Ethical Committee for Animal Research of the Biomedical Sciences Institute, University of São Paulo.

Cell isolation and culture

Resident AMs from rats were obtained by *ex vivo* lung lavage, as previously described [15] and were resuspended in RPMI-1640. Cells were allowed to adhere in culture–treated plates for 1 hour (37°C, 5% CO_2); this was followed by one wash with warm RPMI-1640, resulting in more than 99% of adherent cells identified as AMs by staining with a modified Wright-Giemsa stain. Cells were cultured overnight in RPMI-1640 supplemented 10% FBS (fetal bovine serum) and were washed twice the next day with warm medium to remove the non-adherent cells.

Cell treatments

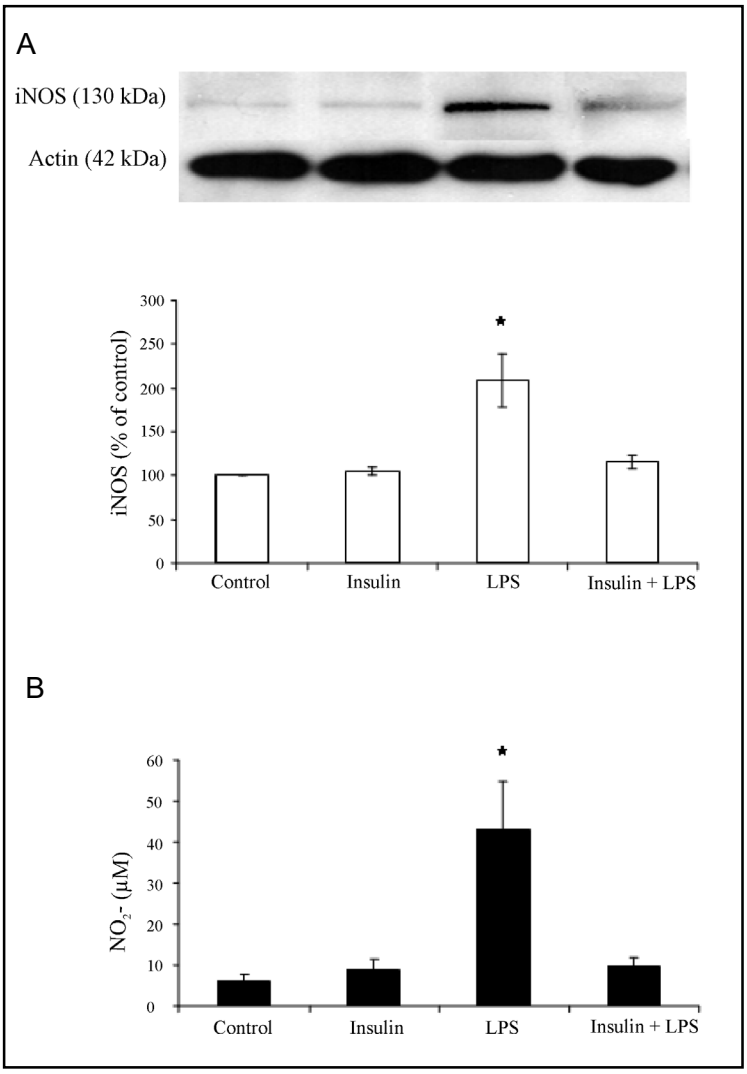
AMs ($1x10^6$) plated in 4-well tissue culture dishes were pre-treated or not with crystalline insulin at a final concentration of 1 mU/mL, 10 minutes before LPS from *Escherichia coli* (serotype 055:B5) stimulus *in vitro* [14]. Then, AMs were stimulated for 30 minutes with 100 ng/mL LPS and were placed on ice for 10 minutes to stop the reaction. After that, AMs were washed three times in ice-cold PBS and lysed by sonication in ice-cold lyses buffer containing 150 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 µg/mL leupeptin, followed by ultracentrifugation at 100 000 *g* for 20 minutes at 4°C, the supernatant was frozen -70ºC for immunobloting and measuring of NO and PGE_2 production [15, 16].

Measurement of nitrite and PGE_2

To evaluate NO production, nitrite concentration in the supernatants of AMs cultures was measured using the standard Griess reaction. Briefly, 50 µl of the culture supernatant was mixed with 50 µl of Griess Reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% H_3PO_4 for 10 min at room temperature. The absorbance was measured at 540 nm using a 620 nm reference filter in a Dynatech microplate reader, and the nitrite concentration was calculed using a standard curve of sodium nitrite. All assays were done in triplicate.

PGE_2 levels in the supernatants of the AMs culture were measure by enzyme immunoassay (EIA) using a commercial kit from Cayman Chemical. Briefly, dilutions of the supernatants were incubated with the conjugated eicosanoid-acetylcholi-

Fig. 1. Insulin suppresses LPS-induced iNOS expression (A) and NO production (B). Alveolar macrophages ($1x10^6$) were obtained by *ex vivo* lung lavage from male Wistar rats and incubated with insulin (1 mU/mL) 10 minutes before LPS stimulation (100 ng/mL) for 30 minutes. iNOS expression was assessed by western blot analysis and NO levels measured by Griess reaction. Ilustration of the western blot represent one out of 4 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 4 animals per group. *P<0.001 *vs* other groups.



nesterase and with the specific antiserum in 96-well plates precoated with anti-rabbit immunoglobulin G antibodies. After overnight incubation at 4ºC, the plates were washed and the enzyme substrate (Ellman's reagent) was added for 60 to 120 min at 25ºC. The optical density of the samples was determined at 412 nm in a microplate reader, and the concentration of eicosanoids was calculated from standard curve. All assays were done in duplicate.

iNOS and COX-2 expression

Protein content in the supernatant of the lysed AMs was determined using the BCA protein assay reagent kit (Pierce), according to the manufacture´s protocol. Samples containing 20 µg protein were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membrane using the Biorrad Mini-Gel system and trans-blot® SD-semidry Transfer cells. For immunoblotting, the nitrocellulose membranes were incubated in TSB-T buffer (150 mM NaCl, 20 mM Tris, 1% Tween 20, pH 7.4) containing 5% non-fat milk dried milk, for 1 h. The blot was treated with 1:1000 dilution of rabbit polyclonal antibodies to COX-2 or rabbit antiserum iNOS for 2 h at room temperature, then were washed three times with TBS-T, and incubated with 1:2000 dilution of peroxidase-conjugated monoclonal anti-rabbit IgG for 1 h at room temperature. Protein bands at 72 kDa (COX-2) or at 130 kDa (iNOS) were identified by comparison with Rainbow™ protein molecular weight markers. The immunocomplexed peroxidase-labeled antibodies were visualized by an ECL chemiluminescence kit following manufacturer´s instruction (Amersham) and exposed to photographic film. Finally, blots were stripped with 200 mM glycine, pH 3.0, for 10 min, washed with TBS-T three times for 30 min each, and reprobed with β-actin (1:10.000), followed by anti-mouse secondary antibody (1:2000). The band densities were determined by densitometric analysis using the AlphaEaseFC™ program. Density values of bands were normalized to the total β-actin present in each lane and expressed in percentagem of control.

NF-κB western blot analysis (p65 and I-κBα)

To determine cytoplasmic I-κBα phosphorylation and NF-kB p65 subunit phosphorylation, the extraction of AMs cytoplasmic and nuclear proteins was perfomed using NE-PER nuclear and cytoplasmic extraction reagents containing 1% of

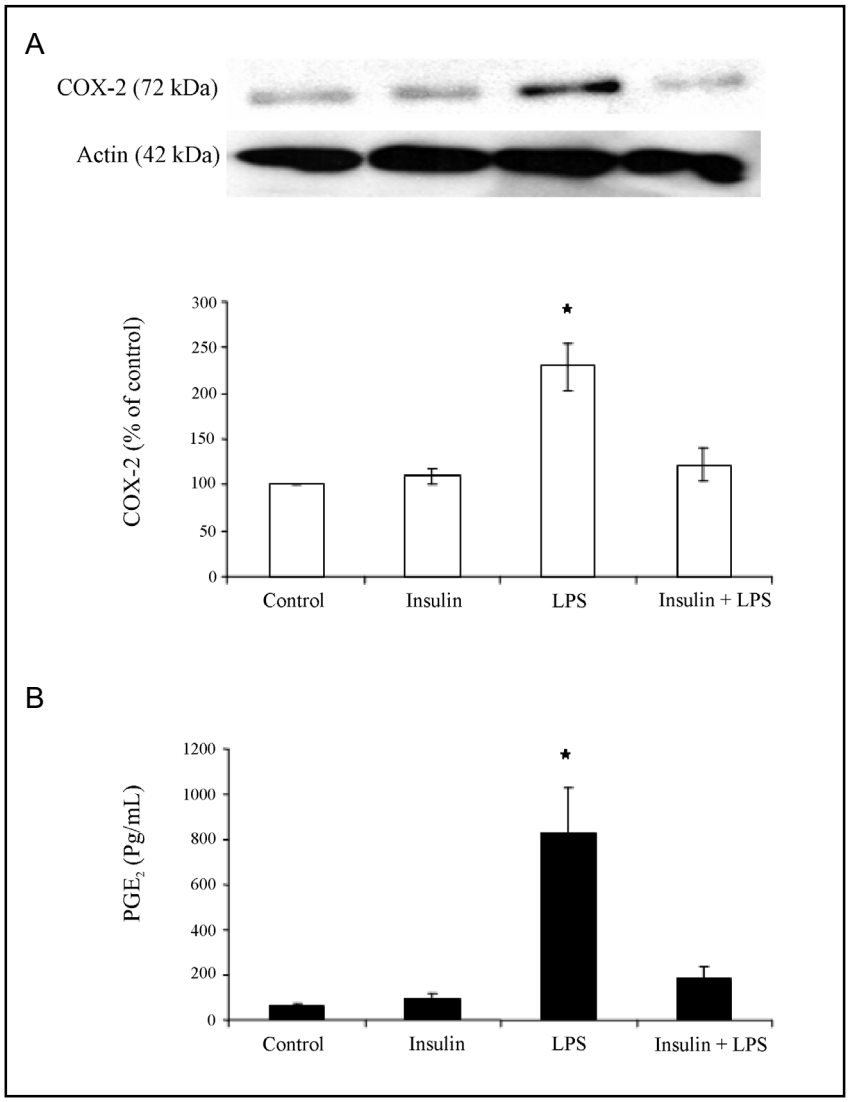


Fig. 2. Insulin suppresses LPS-induced COX-2 expression (A) and PGE_2 production (B). Alveolar macrophages ($1x10^6$) were obtained by *ex vivo* lung lavage from male Wistar rats and incubated with insulin (1 mU/mL) 10 minutes before LPS stimulation (100 ng/mL) for 30 minutes. COX-2 expression was assessed by western blot analysis and PGE_2 levels measured by EIA. Ilustration of the western blot represent one out of 4 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 4 animals per group. *P<0.001 *vs* other groups.

inhibitor cocktail per the manufacture´s instructions (Pierce). Protein content in the supernatant of the lysed AMs was determined as described above. In separate experiments, phosphorylation of I-κBα (cytoplasmic extracts) or activation of p65 NF-κB (nuclear extracts) containg 10 µg of protein per sample were suspended in SDS sample buffer (Invitrogen) and collected by boiling the sample at 100ºC for 5 min. Western blot was analysed as described above with primary antibodies specific for phosphorylated I-κBα (1:250) or phosphorylated NF-κB p65 (1:500). Data are expressed as pixel total or the percent of control after adjustment for the density of its respective control band.

Drugs and reagents

RPMI-1640 and FBS (Gibco-Invitrogen, Carlsbad, CA, USA); Cristalline insulin (Biobrás, São Paulo, Brazil); LPS and β-actin (Sigma, Chemical Co, St Louis, Mo, USA); NE-PER nuclear and cytoplasmic extraction reagents, inhibitor cocktail and BCA protein assay reagent kit (Pierce Chemical, Rockford, IL, USA); chemiluminescence (ECL) detection and Rainbow™ protein molecular weight markers (Amersham, Piscataway, NJ, USA); COX-2 and iNOS from Cayman Chemical, Ann Arbor, MI, USA. Phospho-I-κBα (Ser32), phospho-NF-kB p65 (Ser276), ani-rabbit and anti-mouse IgG antibody are from Cell Signaling technology, INC. Beverly, MA, USA.

Statistical analysis

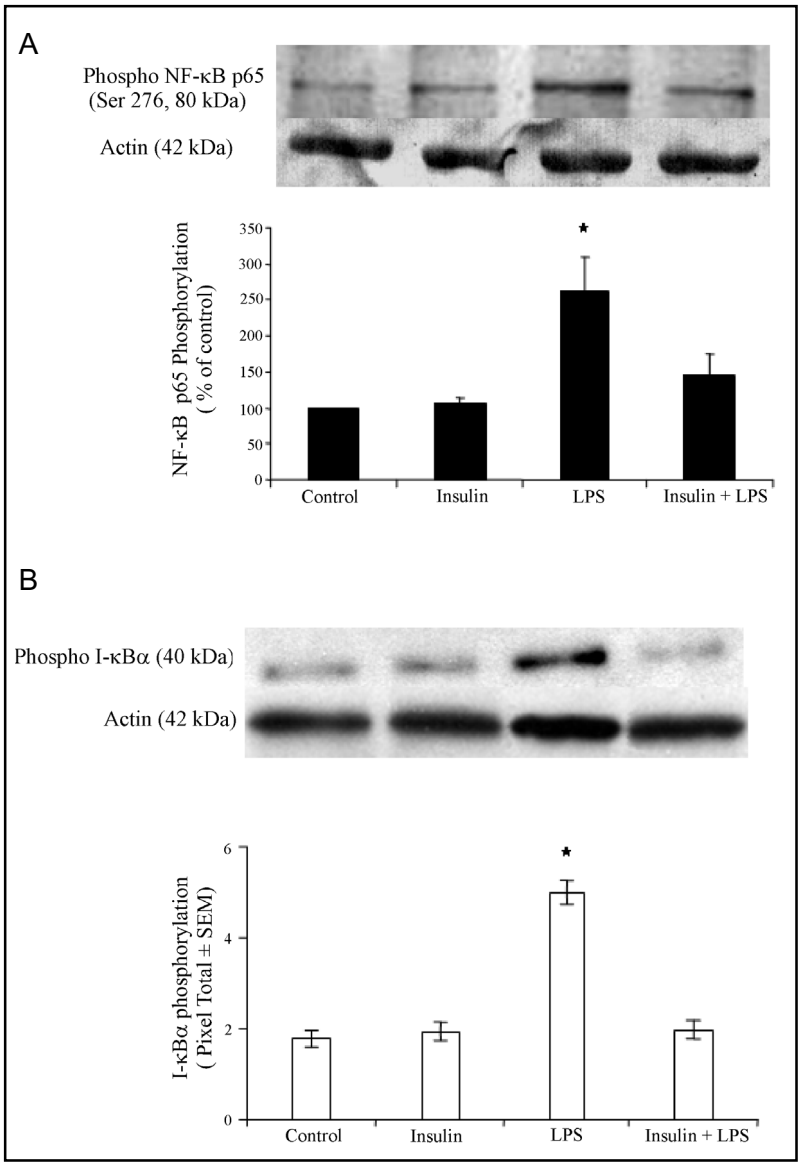
Data are presented as mean ± SEM of at least four experiments, statistical analysis was performed using the GraphPad Software (San Diego, CA, USA), and compared by analysis of variance (ANOVA) followed by the Bonferroni test. P< 0.05 were considered statistically significant.

Results

Insulin inhibits LPS-induced iNOS and COX-2 protein expression and NO and PGE_2 release

Resident AMs from male Wistar rats were obtained by *ex vivo* lung lavage and stimulated with LPS (100 ng/mL) for 30 min. Protein expression of iNOS was analyzed

Fig. 3. Insulin modulates LPS-induced NF-kB activation and I-κBα phosphorylation. Alveolar macrophages ($1x10^6$) were obtained by *ex vivo* lung lavage from male Wistar rats and incubated with insulin (1 mU/mL) 10 minutes before LPS stimulation (100 ng/mL) for 30 minutes. Nuclear NF-kB p65 subunit phosphorylation and cytoplasmic I-kBα phosphorylation were assessed by western blot analysis. The antibodies recognized phosphorylated residue of the Ser276 of NF-kB p65 subunit and the Ser32 of I-kBα. Ilustration of the western blot represents one out of 4 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 4 animals per group. *P<0.001 *vs* other groups.



by western blot and NO release by the standard Griess reaction. Figure 1 shows that LPS induced the expression of iNOS protein as well as the release of NO. A group of AMs was pre-treated with insulin (1 mU/mL), 10 minutes before LPS stimulation. This dose of insulin was taken from previous publication from our group using the same system [14]. Insulin alone did not induce iNOS but insulin supressed iNOS expression and NO generation in AMs stimulated with LPS.

COX-2 protein expression was analyzed by western blot and PGE_2 by EIA. Figure 2 shows that LPS induced the expression of COX-2 protein as well as the release of PGE_2. Insulin alone did not induce this protein or its product in rat AMs. Treatment of AMs with insulin (1 mU/mL), 10 minutes before LPS stimulation, suppressed the expression of COX-2 and release of PGE_2.

Insulin modulates LPS-induced NF-κB activation and I-κBα phosphorylation

Activation of NF-κB p65 subunit and phosphorylation of cytoplasmic I-κBα in AMs stimulated with LPS was analyzed by western blot. Figure 3 shows that, LPS induced the activation of NF-κB p65 subunit and phosphorylation of cytoplasmic I-κBα. Insulin alone did not induce activation of NF-κB or phosphorylation of its inhibitor in rat AMs. Treatment of AMs with insulin (1 mU/mL), 10 minutes before LPS stimulation, significantly suppressed the nuclear NF-κB p65 activation as well as the phosphorylation of cytoplasmic I-κBα. These results suggest that the suppression of NF-kB activation by insulin might be attributed to inhibition of nuclear translocation of NF-kB resulting from blockade of the phosphorylation of I-kBα in AMs activated with LPS.

Discussion

In the present work, we showed that activation of resident rat AMs with LPS *in vitro* induced the expression of iNOS and COX-2 proteins and release of the products of these enzymes, NO and PGE_2. Insulin given before LPS suppressed expression of these enzymes and mediator release. We also showed that LPS induced a significant increase in NF-κB p65 activation and in cytoplasmic I-κBα phosphorylation and that the treatment of AMs with insulin before LPS stimulation, significantly reduced these transcription factors activation. We recently showed that insulin inhibits the LPS-induced ERK 1/2, p38, Akt, PKCα and PKCδ phosphorylation and TNF release in AMs *in vitro* [14]. Our results are consistent with an inhibitory effect of insulin and corroborate previous findings showing a protective effect of insulin on systemic inflammation related to sepsis [17]. Taken together these results, suggest that the protective effect of insulin in sepsis reported by others could be, at least partially, attributed to inhibition of the secondary wave of mediators released by the lungs during this condition.

In a previous work from our group we performed a dose-response curve to insulin on LPS-induced TNF production [14]. We showed that at 1 mU/mL, insulin alone did not increase basal levels of phosphorylation of MAPK and PKC´s but it clearly interfered with the LPS-induced signaling cascades. So, in the present work we kept the same experimental conditions used in the previous work and employed the lower dose of insulin able to interfere with the LPS-induced signaling cascades and TNF production.

Intracellular levels of iNOS and COX-2 play a central role in determining NO and PGE_2 production rates in macrophages, two important mediators of the systemic inflammation during sepsis. We showed here that insulin suppressed LPS-induced iNOS and COX-2 protein expression as well as the release of the NO and PGE_2. These observations favor the hypothesis that the protective effect of insulin in sepsis could be due to modulation of cellular signal transcription factors and downstream effects of LPS on TNF [14] NO and PGE_2 production rather than to changes in metabolism and blood glucose.

One critical event that triggers sepsis/septic shock is the nuclear translocation of NF-kB and induction of NF-kB-dependent effector genes [18]. Böhrer *et al.* [19], observed that all patients with septic shock showed increased NF-kB binding activity in peripheral blood mononuclear cells (PBMC), and those in whom the binding activity exceed 200% of day 1, died. They also showed that somatic gene transfer with an expression plasmid coding for I-kBα reduced LPS-mediated NF-kB activation and increased mice survival after LPS administration. This suggests that NF-kB mediates mortality in animal models of sepsis. They also reported that gene transfer with I-kBα was not effective when given simultaneously with or after LPS, suggesting that gene transfer has to be done before the cells is stimulated to release mediator critical for the pathophysiology of sepsis. The central role of NF-kB in mediating inflammatory processes is evident from both the importance of its target genes and from the phenotypes of mice lacking the NF-kB p65 subunit [20]. Therefore, compounds inhibiting NF-kB are potentially of great interest for developing therapeutic agents for the treatment of acute and chronic inflammation. In our study LPS activation of AMs induced alterations in the activation of NF-kB p65 subunit and I-kBα phosphorylation. These alterations induced by LPS were reversed by insulin. Support for this finding comes from a study demonstrating that insulin has a potent acute anti-inflammatory effect including reduction in intranuclear NF-kB and increase in I-kB in mononuclear cells in obese subjects [21]. Thus, this acute anti-inflammatory effect, if demonstrated in the long term, as described by them, may be beneficial in sepsis.

NF-kB is also a crucial transcription factor for mRNA expression of iNOS [22] and COX-2 [23] and this report shows that insulin suppresses NF-kB activation possibly by inhibition of nuclear translocation of NF-kB resulting from blockade of phosphorylation of I-kBα. Regarding the intracellular mechanism of action mediated by insulin to decrease NF-kB activation, insulin has the potential to interfere with the stimulation of protein kinases and activation or repression of genes transcription [24]. We have shown in a previous paper [14] that insulin inhibits Akt and this molecule is a downstream regulator of PI3K and is implicated on PI3K-mediated regulation of NF-kB [25]. Another possibility is that insulin would act on the I-kB kinase (IKK), a protein kinase which activates the common pathways to NF-kB activation that is based on inducible I-kB degradation. In fact we showed here that insulin suppressed the nuclear NF-κB p65 activation as well as the phosphorylation of cytoplasmic I-κBα.

In Type II diabetes, the plasma levels of insulin are increased but the patient condition is rather associated with an inflammatory picture. In this condition, the presence of hyperglycemia is, at least in part, responsible for the proinflammatory state. In addition, obesity and type II diabetes presents insulin-resistance. Insulin exerts

an anti-inflammatory effect at the cellular and molecular level in vitro and in vivo [26]. A low dose infusion of insulin (2.5 IU/h) reduces reactive oxygen species (ROS) generation by mononuclear cells, suppresses NADPH oxidase expression and intranuclear NF-kB binding, induces I-kB expression and suppresses plasma intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) concentrations. It also suppresses the proinflammatory transcription factor early growth response gene-1 (Egr-1), plasminogen activator inhibitor-1 (PAI-1) and MCP-1 concentrations [21, 27]. Another possibility is that an interruption/ alteration of insulin signal transduction in diabetes type II would prevent the anti-inflammatory effect of insulin from being exerted.

Hyperglycemia and insulin resistance are common in severe illness and are associated with adverse outcomes [28]. Van den Berghe et al. [4] showed in a study conducted in an intensive care unit (ICU) that strict control of blood glucose levels with insulin reduced morbidity and mortality. More recently, they showed that intensive insulin therapy significantly reduced morbidity but not mortality among all patients in the ICU [29]. It has been discussed that in critically ill patients several mediators are involved, and a single drug is unlikely to be of significant benefit. However, in some particular situations or, in some patients, insulin is beneficial. In sepsis, Das [30] discussed that a combination of naturally occuring endogenous anti-inflammatory molecules such as insulin, is one additional tool that can be used in the management of patients with sepsis, a condition for which no adequate therapy is available at present.

In conclusion, the results presented here and in the previous paper [14] suggest that the protective effect of insulin in sepsis, reported by others, could be due to inhibition of the secondary wave of mediators released by the lung macrophages.

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SIGNALING PATHWAYS AND MEDIATORS IN LPS-INDUCED LUNG INFLAMMATION IN DIABETIC RATS: ROLE OF INSULIN

[1*]Joilson O. Martins, [1]Matheus Ferracini, [1]Denise B. C. Anger, [1]Daniel O. Martins, [1]Luciano F. Ribeiro-Jr, [2]Paulina Sannomiya, [1]Sonia Jancar,

[1]Department of Immunology, Institute of Biomedical Sciences, University of São Paulo; [2]Research Division, Heart Institute (INCOR), LIM-11, University of São Paulo Medical School, São Paulo, SP, Brazil.

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[*]**Corresponding author:**
Joilson O. Martins, PhD
Department of Immunology
Institute of Biomedical Sciences
University of São Paulo
Av. Prof. Lineu Prestes, 1730
05508-900 São Paulo, SP, Brazil
Phone/FAX: 55-11-3091-7744
e-mail: martinsj@usp.br

Abstract

Diabetic patients are more susceptible to infections and that their inflammatory response is impaired. This is restored by insulin treatment. In the present study we investigated the effect of insulin on LPS-induced signaling pathways and mediators in the lung of diabetic rats. Diabetic male Wistar rats (alloxan, 42 mg/Kg, i.v., 10 days) and control rats received intratracheal instillation of LPS (750 μg/0.4 mL) or saline. Some diabetic rats were given neutral protamine Hagedorn (NPH) insulin (4 IU, s.c.) 2 hours before LPS. After 6 hours, bronchoalveolar lavage (BAL) was performed for mediators release and lung tissue was homogenized for analysis of LPS-induced signaling pathways. Relative to control rats, diabetic rats exhibited a significant reduction in LPS-induced phosphorylation of ERK (64%), p38 (70%), Akt (67%), PKC-α (57%) and PKC-δ (65%) and in the expression of inducible nitric oxide synthase (32%) and cyclooxygenase-2 (67%) in the lung homogenates. The BAL fluid concentration of nitric oxide (47%) and IL-6 (49%) were also reduced in diabetic rats whereas the cytokine-induced neutrophil chemoattractant (CINC)-2 levels was increased 23% and CINC-1 was not different from control animals. Treatment of diabetic rats with insulin completely or partially restored all these parameters. In conclusion, data presented show that insulin regulates MAPK, PI3K, PKC pathways, the expression of the inducible enzymes, COX-2 and iNOS, and the levels of IL-6 and CINC-2 in LPS-induced lung inflammation in diabetic rats. These results suggest that the protective effect of insulin in sepsis could be due to modulation of cellular signal transduction factors.

INTRODUCTION

It has long been recognized that diabetic patients are more susceptible to infections and that their inflammatory response is impaired. Reversal of the impaired responses is attained by treatment of the animals with insulin [1-4]. There is evidence that insulin through direct or indirect effects regulates the inflammatory response [5-8].

The lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall, is known to induce systemic inflammation, which affects several organs [9] among them the lung, and in consequence acute lung injury (ALI) may develop [10-13]. Binding of LPS to toll-like receptors (TLRs) triggers a complex sequence of events leading to increased expression of specific genes through nuclear factor (NF)-κB [14] and release of a plethora of mediators which are involved in inflammatory response. These effects of LPS are consequent to activation of intracellular signaling cascades among them the mitogen-activated protein kinases (MAPK), which comprises the extracellular signal-regulated kinase (ERK) and p38. Protein kinase C (PKC) plays a central role in signal transduction and participates in diverse biological and biochemical functions [15, 16]. For example, PKC may participate in glycogen metabolism, release of neurotransmitters, and protein transactivation by phosphorylation [15-17]. Protein kinase B (Akt) is a downstream regulator of phosphotidylinositol 3`-kinase (PI3K) and is implicated on PI3K-mediated regulation of NF-kB [18] an important transcription factor for pro-inflammatory mediators.

Insulin has the potential to interfere with these pathways by its effects at cellular level which include glucose transport, glycogen synthesis, mitogenesis [19]; stimulation of protein kinases and activation or repression of genes transcription [20].

Previous studies from our group has established a protocol of insulin treatment of alloxan-induced diabetic rats in which the dose of insulin was chosen as the dose able to significantly

reverse inflammatory parameters that were reduced in diabetic rats. Although this dose of insulin only partially reduces the blood glucose, it maintains blood insulin levels elevated during the time of the experiment. It is plausible that the observed effects would be primarily due to the increased levels of insulin rather than to the reduction of glycemia. Using this protocol, we showed that insulin modulates the development of LPS-induced ALI in diabetic rats [1-3]. We found that in ALI caused by intratracheal instillation of LPS the neutrophil infiltration and concentration of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-10 in the airways are significantly reduced in diabetic rats compared to non-diabetic controls. All these parameters are restored after treatment of diabetic rats with the single dose of insulin. We also found that this effect of insulin on cytokines production is at the gene transcription level [3]. In addition, despite no significant differences in lung intercellular adhesion molecule (ICAM)-1 and E-selectin immune staining between LPS diabetic and LPS control rats, an increase in the expression of both adhesion molecules is observed after insulin treatment [1]. We also found that the mechanism of the increased expression of ICAM-1 by insulin involves the NF-κB signaling pathway [3]. The present study was desiged to investigate signaling pathways and mediators in the course of LPS-induced acute lung inflammation in alloxan-induced diabetic rats and the effect of insulin treatment. The hypothesis is that insulin may restore diabetes-induced immune hyporesponsiveness by modulation of cellular signal transduction factors.

MATERIALS AND METHODS

Animals

Specific-pathogen free male Wistar rats weighing 200 ± 20 g at the beginning of the experiments were used. The animals were maintained at 23 ± 2°C under a 12 hour light:dark cycle and were allowed access to food and water *ad libitum*. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by The Ethical Committee for Animal Research of the Biomedical Sciences Institute, University of São Paulo.

Alloxan-induced diabetes

Diabetes mellitus was induced by an intravenous injection of 42 mg/Kg of alloxan monohydrate (Sigma Chemical Co., St. Louis, MO, USA) dissolved in physiologic saline (0.9% NaCl). Control rats were injected with physiologic saline only. Ten days thereafter the presence of diabetes was verified by blood glucose concentrations above 200 mg/dL determined with the aid of blood glucose monitor (Eli Lilly, São Paulo, SP, Brazil) in samples obtained from the cut tip of the rat tail. The concentration of serum insulin was determined by Enzyme-Linked Immunosorbent Assay (ELISA, SPIbio, Massy Cedex, France) in blood samples collected from abdominal aorta of the animals. Results are presented as pg/mL.

Insulin treatment

A group of diabetic rats received 4 IU of neutral protamine Hagedorn (NPH) insulin (Eli Lilly, São Paulo, SP, Brazil) subcutaneously, 2 hours before LPS instillation. As the maximum serum concentration (Cmax) of NPH insulin is reached between 6 and 8 hours

after subcutaneous administration, we decided to give insulin to the animal to 2 hours before LPS instillation as in previous studies in this line [1,3]. The dose of insulin injection was chosen based on previous studies from our group [1-3] as the dose able to restore inflammatory parameters that are suppressed in diabetic rats. Although this dose was not sufficient to return glucose levels in diabetics to normal values, the serum insulin was above normal levels during the time of the experiment (6 hours after LPS instillation).

LPS-induced acute lung injury

The animals were anesthetized by an intraperitoneal injection (150 mg/Kg) of ketamine hydrochloride (Ketamin-S(+), Cristalia, SP, Brazil), the trachea was exposed, and 750 μg of *Escherichia coli* LPS (serotype 055:B5, Sigma Chemical Co., St. Louis, MO, USA) in saline solution was instilled. Control animals received physiologic saline only by the same route. The incision was closed, and the animals were returned to their cages and were allowed access to food and water *ad libitum*. Six hours after LPS instillation the animals were anesthetized, as describe above, and exsanguinated from the abdominal aorta. For bronchoalveolar lavage (BAL), 10 mL of phosphate buffered saline (PBS) was instilled intratracheally and the recovered sample was centrifuged (500 g for 15 min). The pellet was ressuspended in PBS and total cell counts were performed in an automatic hemacytometer (CELM, SP, Brazil). Differential cell counts were carried out on Hematoxylin/eosin stained slides under oil immersion microscopy. The supernatants were kept at -70ºC until use for nitrite and cytokines measurement. In another set of experiments, the lungs were removed 6 hours after LPS instillation, rinsed and immediately immersed in liquid nitrogen, and stored at -70ºC until use for Western blot analysis.

Western blotting

Lungs were homogenized in PBS containing 1% of Protease Inhibitor Cocktail according to manufacture´s instructions (Sigma Chemical Co.). Samples containing 20 μg protein were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE 10%) and transferred to nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA). The membranes were incubated in TSB-T (150 mM NaCl, 20 mM Tris, 1% Tween 20, pH 7.4) containing 5% non-fat dried milk for 60 min. After that, the blots were washed with TSB-T and probed with antibodies (Cell Signaling Technology, INC., Beverly, CA, USA; 1:500 dilution) directed against phospho-ERK 1/2 MAPK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-Akt (Ser473), phospho-PKCα (Thr638/641) and phospho-PKCδ (Thr505) (Cell Signaling Technology), and against COX-2 or iNOS (Cayman Chemical, Ann Arbor, MI, USA, 1:500) for 120 min at room temperature. The membranes were washed with TBS-T and incubated with peroxidase-conjugated monoclonal anti-rabbit IgG (1:2000) for 60 min at room temperature. The immunocomplexed peroxidase-labeled antibodies were visualized by an ECL chemiluminescence kit following manufacturer´s instruction (Amersham, Piscataway, NJ, USA) and exposed to photographic films. Finally, blots were stripped and reprobed for β-actin. The band densities were determined by densitometric analysis using the AlphaEaseFC™ program. Density values of bands were normalized to the total β-actin present in each lane and expressed in % of control.

Cytokines and nitrite assays-

IL-6, CINC-1 and CINC-2 concentrations in the BAL supernatant were determined by ELISA using commercially available kits according to the manufacturer´s instructions (R & D Systems Inc., Minneapolis, MN, USA). The sensitivity of the assay was of 15 pg/mL.

Nitrite concentration in the BAL was measured using the standard Griess reaction. Briefly, 50 µl of the BAL was mixed with 50 µl of Griess Reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% H_3PO_4) for 10 min at room temperature. The absorbance was measured at 540 nm using a 620 nm reference filter in a Dynatech microplate reader, and the nitrite concentration was calculed using a standard curve of sodium nitrite. All assays were done in triplicate.

Statistical analysis

Data are presented as means ± SEM and analyzed by Student's t-test or ANOVA followed by the Tukey-Kramer multiple comparisons test when appropriate. $P < 0.05$ was considered significant.

RESULTS

General characteristics of the experimental model

Relative to controls, alloxan-treated diabetic rats exhibited a significant reduction in body weight gain (values, mean ± SEM, control: 60 ± 2 g, n=12; diabetic: 21 ± 9 g, n=12, p<0.001) during 10-days period, sharply elevated blood glucose levels (control: 82 ± 1 mg/dL, n=12; diabetic: 449 ± 34 mg/dL, n=12, p<0.0001), and a significant reduction in serum insulin concentrations (control: 4974 ± 227 pg/mL; diabetic: 1215 ± 74 pg/mL, n=6, P<0.001). After treatment with a single dose of NPH insulin diabetic rats exhibited a significant reduction in blood glucose levels, from 474 ± 15 mg/dL to 256 ± 16 mg/dL (n=7, p<0.0001). This amount of insulin was not sufficient to reduce glycemia to control values, but the serum levels of insulin increased above normal values (6975 ± 240 pg/mL, n=7) after treatment of diabetic rats with a single dose of NPH insulin. The acute lung injury caused by LPS instillation into airways was characterized by increased number of cells in the BAL, 6 times higher than in the saline group. Neutrophils accounted for 89% of total cells. Leukocyte counts in the BAL fluid of LPS-treated diabetic rats were significantly lower (11.1 ± 0.3 x 10^6 cells, n=5) compared with non-diabetic LPS-treated control animals (28.2 ± 1.5 x 10^6 cells, n=7). Treatment of diabetic animals with a single dose of NPH insulin, 2 hours before LPS exposure, restored the capacity of the animals to respond to LPS (50.8 ± 4.1 x 10^6 cells, n=7).

Insulin modulates LPS-induced phosphorylation of signaling pathways

ERK and p38 MAPK

Six hours after intratracheal instillation of LPS, the lungs were removed, as described in material and methods, and phosphorylation of MAPK was analyzed by Western blot. Figure 1 shows that LPS was able to phosphorylation of MAPK in the lung of non-diabetic rats as

shown by the increased levels of phosphorylated ERK (3.0 fold) and p38 (3.3 fold) compared to the control group that received instillation of saline. In contrast, in diabetic rats there was a significant reduction in the LPS-induced phosphorylation of ERK (64%) and p38 (70%) compared to LPS-treated control rats. Treatment of diabetic rats with a single dose of NPH insulin 2 hours before LPS exposure completely restored lung ERK and p38 phosphorylation.

***Akt* phosphorylation**

Figure 2 shows that in non-diabetic rats, LPS increased the phosphorylation of Akt (3.3 fold) compared to the control, saline instilled group. In contrast, diabetic rats exhibited a 67% reduction in the levels of phosphorilated Akt compared to the LPS-treated control rats. Treatment of diabetic rats with a single dose of NPH insulin, 2 hours before LPS instillation, partially restored phosphorylation of Ser-473 residues of Akt.

***PKC-α and PKC-δ* phosphorylation**

Figure 3 shows that LPS induced in non-diabetic rats a significant increase in the phosphorylation of PKC-α (3.0 fold) and PKC-δ (3.2 fold) in the lung, compared to saline-instilled rats. In contrast, diabetic rats presented a significant reduction in the phosphorylation of lung PKC-α (57%) and PKC-δ (65%) after LPS instillation compared to LPS-treated control rats. Treatment of diabetic rats with a single dose of NPH insulin 2 hours before LPS exposure completely restored lung PKC-α and PKC-δ phosphorylation.

Insulin modulates IL-6 and CINC-2 release

Results illustrated in Figure 4 showed that, in non-diabetic rats, intratracheal instillation of LPS increased BAL concentrations of CINC-1 (3.0-fold), CINC-2 (3.8-fold) and IL-6 (46.5-

fold), compared to the control group, which received saline. In diabetic rat, the concentration of IL-6 was reduced (49%) whereas CINC-2 levels were increased 23% above values attained in LPS-treated control rats. CINC-1 levels did not differ between LPS-treated groups (Figure 4A). Interestingly, CINC-2 and IL-6 concentrations in the BAL fluid were normalized after treatment of diabetic rats with insulin (Figure 4B and 4C).

Insulin modulates LPS-induced COX-2 and iNOS protein expression

Lung COX-2 and iNOS protein expression was analyzed by Western blot, 6 hours after intratracheal instillation of LPS. Figure 5 shows that LPS induced the expression of COX-2 protein and iNOS in non-diabetic rats. In diabetic rats the expression of lung COX-2 and iNOS protein following LPS instillation was significantly reduced (67% and 32%, respectively) compared to the LPS-treated control rats. Treatment of diabetic rats with insulin 2 hours before LPS exposure completely restored lung COX-2 and iNOS expression. The LPS also increased the BAL levels of NO (figure 5C) in non-diabetic rats, measured by the standard Griess reaction, but in diabetic rats levels were reduced (47%) compared to the LPS-treated control rats. BAL NO concentration was normalized by insulin treatment (Figure 5C).

DISCUSSION

The instillation of LPS into the airways of rats causes acute inflammation characterized by increased number of neutrophils in the alveolar space, increased expression of vascular adhesion molecules and mediators [1]. We found that the increased cytokine levels in the BAL fluid of rats were associated with increased expression of their transcripts in the lung and mesenteric lymph nodes which suggests that intratracheal LPS not only induces local but also systemic inflammation. These local and systemic effects of LPS were impaired in diabetic rats but were restored by insulin treatment. We also found that insulin restores the activation of NF-κB pathway, which is reduced in diabetic rats [3]. In the present study we further investigated the effect of insulin on the LPS-induced signaling pathways in the lung by measuring phosphorylation of ERK, p38, AKT, PKCα and PKCδ. The Western blots in this study were referenced to beta-actin. Although it would have been more adequate to reference the phosphorylation blots to the total protein it is acceptable to reference them to beta- actin as we done here.

Data presented herein show that (i) relative to LPS-treated control rats, LPS-treated diabetics exhibited reduced phosphorylation of ERK, p38, Akt, PKC-α and PKC-δ; (ii) reduced expression of iNOS and COX-2; (iii) reduced concentration of NO and IL-6 in the BAL fluid; (iv) and increased CINC-2 levels. Treatment of diabetic rats with insulin restored the phosphorylation of these signaling molecules, enzyme expressions and cytokine levels.

Abnormalities in the course of the inflammatory response in diabetes mellitus might contribute to the increased susceptibility and severity of infections in the diabetic host. Critical evaluations of the topic show that infection is more serious and possibly more difficult to eradicate in the diabetic host [21,22]. Van den Berghe et al. [23] showed in a study conducted in an intensive care unit (ICU) that strict control of blood glucose levels with

insulin reduced morbidity and mortality. Nevertheless, other clinical studies are in marked contrast to the results of this study, and establish that intensive insulin therapy has no consistent benefit in critically ill patients in a medical ICU and that this therapy increases the risk of hypoglycemic episodes [24,25]. Thus the role of intensive insulin therapy in critically ill patients is uncertain as well as the controversy as to whether the beneficial effects are due to insulin per se or to tight glycemic control. This issue has been extensively discussed in the Editorial of a recent issues of The New England Journal of Medicine [26].

As general rule, insulin regulates many cellular processes, such as glucose transport, glycogen synthesis, and gene transcription [20]. Through different signaling pathways insulin exerts a negative effect on the transcription of a subset of genes, and for others the effect is positive [20]. In the present study we found reduced phosphorylation of MAPK, PI3K and PKC pathways in diabetic rats and that insulin restored the phosphorylation of these signaling molecules.

MAPK are a group of signaling molecules that appear to play important roles in inflammatory processes and LPS has been reported to activate MAPK pathways [27, 28]. This pathway is involved in the activation of several transcription factors that control gene activity and expression. Activation of $p21^{ras}$ leads to activation of all members of the MAPK family, and this effect is mediated by specific MEK kinases [29,30], including ERK and p38. Results presented herein showed that in diabetic rats, the LPS-induced ERK and p38 phosphorylation in the lung is reduced compared to non-diabetic rats. However, the treatment of diabetic rats with insulin restored the phosphorylation of these signaling molecules to the levels of the LPS-treated control animals. Insulin has been shown to activate the $p21^{ras}$/MAPK pathway through the binding of growth factor receptor-bound protein (GRB)-2 to tyrosine-phosphorylated insulin receptor substrate (IRS)-1 [31]. Several IRS-1-associated

proteins have already been identified including PI3K [32]. PI3K is composed of two subunits, a 100 kDa (p110) catalytic subunit and an 85 kDa (p85) regulatory subunit [33]. A direct interaction between $p21^{ras}$ and p110, and the demonstration that $p21^{ras}$ regulates PI3`-kinase activity [34], suggests that activation of $p21^{ras}$ via IRS-1 could initiate both the MAPK and PI3K signaling pathway. Data presented herein showed that insulin restored the phosphorylation of Ser-473 Akt that was reduced in diabetic rats. This suggests that insulin could act directly on $p21^{ras}$, which favors the hypothesis that the protective effect of insulin in critically ill patients [23] is due to modulation of cellular signal transcription factors [7,8] rather than to changes in metabolism and blood glucose levels [1-4].

PKC is a family of multifunctional serine/threonine kinases, which is involved in insulin action, resistance and secretion [35]. LPS induces leukocyte transmigration and changes in permeability of the airway epithelium via PKC and ERK [36]. Results presented herein showed that phosphorylation of PKCα and PKCδ were reduced in the lung of LPS-treated diabetic rats compared to LPS-treated control rats. Treatment of diabetic rats with insulin restored the phosphorylation of these signaling molecules. We have previously shown that the number of leukocytes that migrate to the lung in response to LPS stimulation is remarkably increased after treatment of diabetic rats with insulin, when compared with values observed in LPS-treated control animals [1]. It can be speculated that insulin increases leukocyte transmigration in the lung via activation of PKC and ERK pathways.

It has been shown that in LPS-induced lung inflammation, the early recruitment of neutrophils is accompanied by production and release of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) and chemokines (IL-8, macrophage inflammatory protein-2) [37]. Moreover, it was shown that lung neutrophil migration and production/release of proinflammatory (IL-1β, TNF-α) and anti-inflammatory (IL-10) cytokines depend on the

availability of insulin [1]. Results presented herein showed that, compared to control animals, alloxan-induced diabetic rats exhibited reduced IL-6 in the BAL fluid after intratracheal instillation of LPS. The concentration of this cytokine was normalized by treatment of diabetic rats with insulin. Taken together, these data indicate that insulin regulates proinflammatory cytokines release into the lungs of diabetic rats. These cytokines amplify the inflammatory response by stimulating the release of chemoattractant factors by alveolar macrophages and airways epithelial cells, and the expression of adhesion molecules by leukocytes and the endothelium [38]. In the present study we analyzed CINC-1 and CINC-2 in BAL fluid and found that, relative to control rats, diabetic rats exhibited increased levels of CINC-2 and that treatment with insulin reduced the concentration of this chemokine to values attained in LPS-treated control rats. On the other hand, CINC-1 levels were similar in LPS-treated diabetic and control rats and were not affected by treatment with insulin. This discrepancy between CINC-1 and CINC-2 results might be explained by the time-course in these chemokines release. Shibata et al. [39] have demonstrated increases in the concentrations of CINC-1 and CINC-2 in inflammatory exudate induced by LPS in rats. The CINC-1 concentration in the exudate reaches a maximum at 4 hours after LPS injection, whereas the CINC-2 level steadily increases up to 8 hours. The number of infiltrated cells in the exudate increases linearly until 6 hours and gradually up to 8 hours. Increasing cell numbers correlate with the total concentrations of CINC-1 and CINC-2. The authors suggest that CINC-2 as well as CINC-1 play an important role in the accumulation of neutrophils in LPS- induced inflammation in rats. Indeed, concentrations of both chemokines increased 3 to 4 times after LPS instillation of control rats, which presented elevated numbers of leukocytes in the BAL fluid. Despite an equivalent increase in CINC-1 levels and even higher

concentrations of CINC-2, leukocyte infiltration into the airway of diabetic rats was markedly reduced.

Intracellular levels of COX-2 and iNOS determine PGE_2 and NO production by inflammatory cells, which are important mediators of the systemic inflammation during sepsis. In addition, it has been shown that insulin regulates the generation of PGE_2 during the course of ALI induced by LPS [2]. Here we found that the lung expression of COX-2 and iNOS as well as the levels of NO in the BAL fluid were significantly lower in diabetic compared to LPS-treated control rats. Treatment of diabetic rats with insulin restored both enzyme expressions and the concentration of NO, which suggest that insulin also regulates the expression of COX-2 and iNOS in LPS-induced inflammation in diabetic rats.

The normal course of the inflammatory process seems to depend on the availability of insulin [1-6]. Therefore, we suggest that insulin exerts its restorative effect through MAPK, PI3K, PKC pathways and the expression of inducible enzymes, COX-2 and iNOS. It is important here to discuss the choice of the dose and timing of insulin treatment used in the present and previous studies of our group [1-3]. As the maximum serum concentration (Cmax) of NPH insulin is reached between 6 and 8 hours after subcutaneous administration, we decided to give insulin to the animal to 2 hours before LPS instillation as in previous studies in this line [1,3]. The dose of insulin was choosen based on its ability to restore LPS-induced inflammatory parameters in the lung of diabetic rats. This dose was not sufficient to normalize glycemia. However, serum insulin concentrations were above normal levels during the time of the experiment (6 hours after LPS instillation). Thus we believe that the effects seen in insulin treated rats are primarily due to the increased levels of insulin rather than to normalization of glycemia.

In conclusion, data presented show that insulin regulates MAPK, PI3K, PKC pathways, the expression of inducible enzymes, COX-2 and iNOS, and the levels of IL-6 and CINC-2 in LPS-induced lung inflammation in diabetic rats. These results suggest that the protective effect of insulin in sepsis could be due to modulation of cellular signal transduction factors.

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FIGURE LEGENDS

Fig.1. Effect of insulin on LPS-induced ERK (A) and p38 MAPK (B) activation in the lung tissue of control (C, open bars) and diabetic rats (D, black bars) 6 hours after saline or LPS instillation. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, grey bars) 2 hours before LPS instillation. ERK and p38 MAPK phosphorylation was assessed by Western blot analysis. The antibodies recognized phosphorylated residues Thr183/Tyr185 of p44/42 MAPK and Thr180/Tyr182 of p38 MAPK. Illustrations of the Western blot represent one out of 4 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 4 animals per group. $^{\dagger}P<0.001$ *vs* other groups.

Fig. 2. Effect of insulin on LPS-induced Akt activation in the lung tissue of control (C, open bars) and diabetic rats (D, black bars) 6 hours after saline or LPS instillation. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, grey bars) 2 hours before LPS instillation. Akt phosphorylation was assessed by Western blot analysis. The antibody used recognize phosphorylated residues Ser-473 of Akt. Illustrations of the Western blot represent one out of 4 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 4 animals per group. $^{\dagger}P<0.001$ *vs* other groups. $^{*}P<0.05$ *vs* DI group.

Fig. 3. Effect of insulin on LPS-induced PKCα (A) and PKCδ (B) activation in the lung tissue of control (C, open bars) and diabetic rats (D, black bars) 6 hours after saline or LPS instillation. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, grey bars) 2 hours before LPS instillation. The antibodies recognized phosphorylated residues Thr638/641 of PKCα and Thr505 of PKCδ. Illustrations of the Western blot represent one out of 4 independent

experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM of 4 animals per group. †P<0.001 *vs* other groups.

Fig. 4. Effect of insulin on CINC-1 (A), CINC-2 (B) and IL-6 (C) concentrations in the BAL fluid of control (C, open bars) and diabetic rats (D, black bars) 6 hours after saline or LPS instillation. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, grey bars) 2 hours before LPS instillation. Values are means ± SEM for 5 to 7 animals in each roup. †P<0.001 *vs* control saline group. **P*<0.05 *vs* LPS control and DI groups. ‡*P*<0.001 *vs* LPS diabetic group.

Fig. 5. Effect of insulin on LPS-induced COX-2 (A) and iNOS (B) protein expression in the lung tissue and NO concentration in the BAL fluid (C) of control (C, open bars) and diabetic rats (D, black bars) 6 hours after saline or LPS instillation. Insulin (NPH, 4 IU/rat) was given to diabetic rats (DI, grey bars) 2 hours before LPS instillation. COX-2 and iNOS expressions were assessed by Western blot analysis and NO levels measured by Griess reaction. Ilustration of the Western blot represent one out of 4 independent experiments. Expression of protein was quantified by AlphaEaseFC™ software. Values are means ± SEM for 4 to 7 animals in each group. ‡P<0.001 *vs* other groups. †P<0.001 *vs* control saline group. **P*<0.05 *vs* LPS control and DI groups.

Figure 1

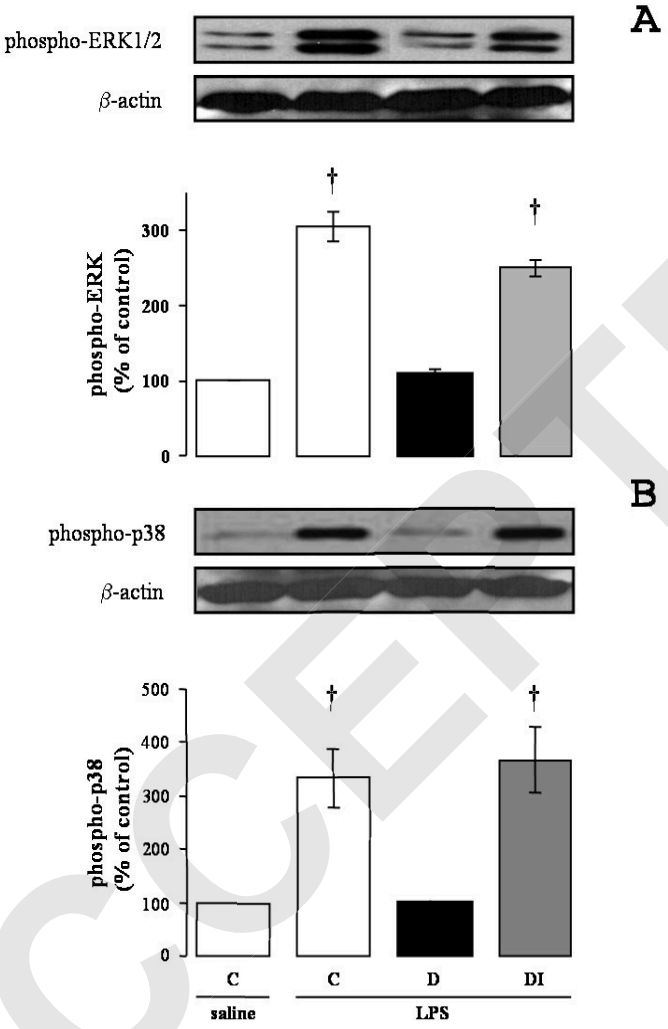


Figure 2

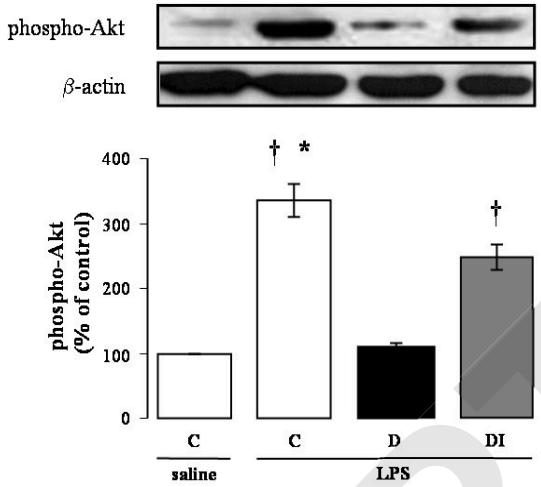


Figure 3

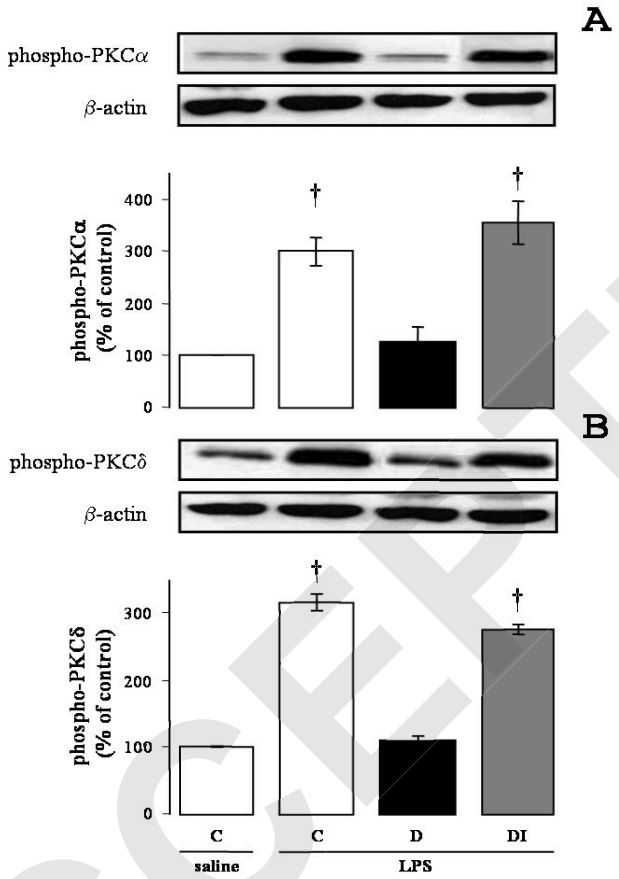


Figure 4

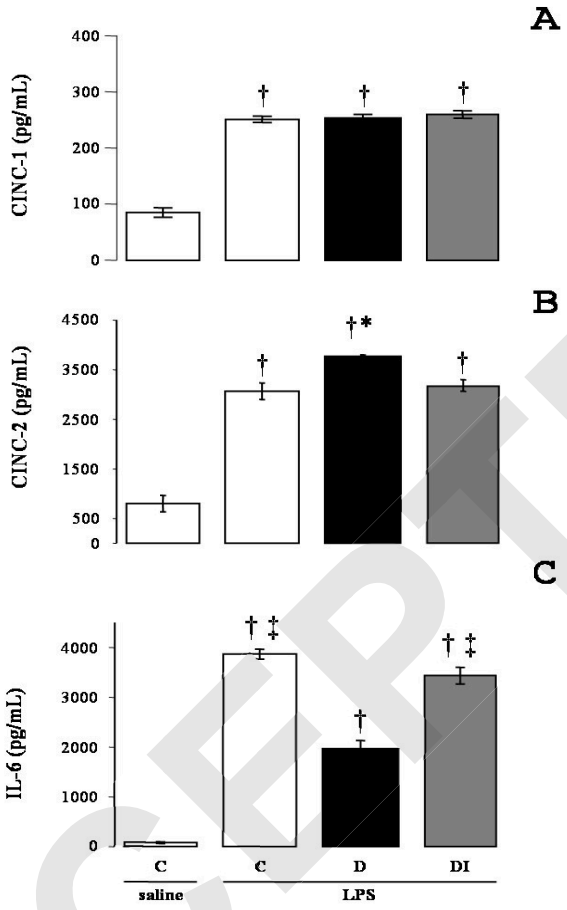
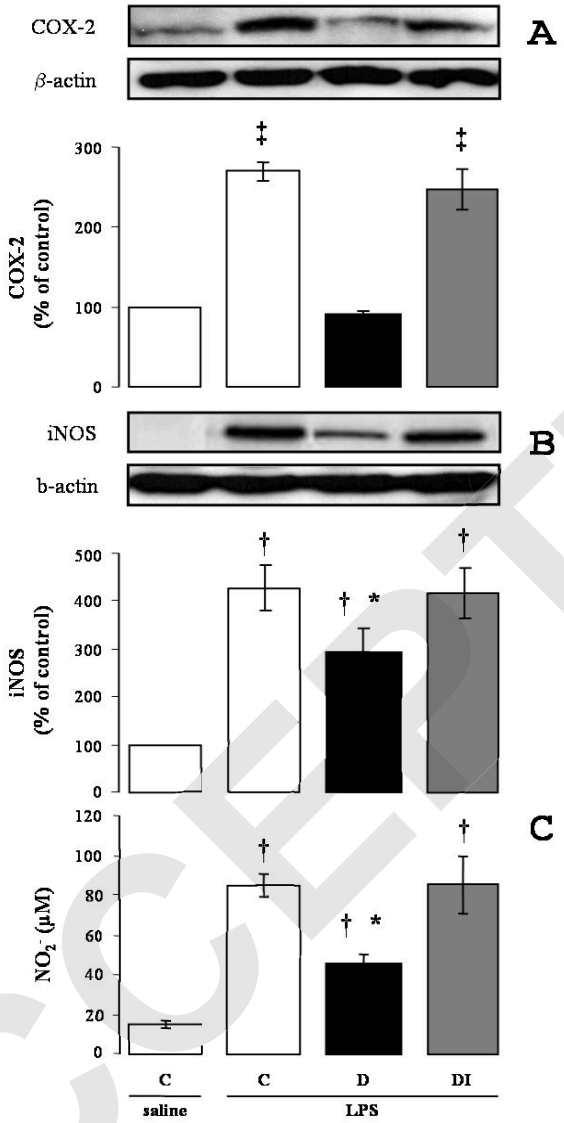


Figure 5



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