ALEXANDRE GABARRA DE OLIVEIRA

# EFEITO DO EXERCÍCIO FÍSICO AGUDO E CRÔNICO NA EXPRESSÃO E ATIVAÇÃO DO TLR4 EM ROEDORES.

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**FACULDADE DE CIÊNCIAS MÉDICAS**
**UNIVERSIDADE ESTADUAL DE CAMPINAS**

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

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Data: 29/02/2012

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e amada esposa Mayra, aos meus
pais, Edimar e Cristina e aos
meus sogros, Mario e Graça.

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**RESUMO**

Evidências recentes têm apontado para estreita correlação entre resistência à insulina, inflamação e obesidade. Nesse contexto, o toll-like receptor 4 (TLR4) parece exercer papel importante, pois sua ativação leva a aumentos na fosforilação de IκB kinase (IKKβ) e c-Jun NH2-terminal kinase (JNK) e na expressão de citocinas inflamatórias como o fator de necrose tumoral alfa (TNF-α), substâncias capazes de atenuar a sensibilidade à insulina.. Recentemente, nosso laboratório demonstrou que animais com TLR4 não funcional ficam protegidos da resistência à insulina e do ganho de peso, induzidos por dieta hiperlipídica, sugerindo papel de destaque do TLR4 na interface entre sistema imune inato e metabolismo energético. Por outro lado, o exercício físico pode melhorar a sensibilidade à insulina e a inflamação na obesidade, entretanto os mecanismos são pouco compreendidos. A relação entre TLR4 e exercício físico é um tópico ainda pouco investigado, no entanto há indícios de que o exercício físico possa levar à redução na sua expressão gênica. Assim buscamos analisar o efeito do exercício físico sobre a expressão e ativação do TLR4 e de suas consequências sobre a sinalização e sensibilidade à insulina em ratos obesos. O presente estudo demonstrou que apenas o exercício crônico foi capaz de reduzir a expressão de RNA mensageiro e de proteína do TLR4 em músculo, fígado e tecido adiposo. Contudo, tanto o exercício crônico quanto o agudo atenuaram a sinalização do TLR4 nesses tecidos, como evidenciado pela redução da fosforilação de JNK e IKKβ. Observamos também importante redução nos níveis circulantes de

lipopolissacarídeo (LPS) nos dois protocolos de exercício, e menores níveis de ácidos graxos livres (AGL) apenas no exercício crônico. Em paralelo, demonstramos melhora na fosforilação em tirosina do IRβ e do IRS-1 e da Akt em serina após ambos os protocolos. Dessa forma, podemos concluir que o exercício físico promoveu importante atenuação sobre a sinalização do TLR4 em músculo, fígado e tecido adiposo; possivelmente mediada pela redução dos níveis de LPS circulante, que resultou em melhora na sinalização e sensibilidade à insulina em animais com obesidade induzida por dieta hiperlipídica. Esses dados promoveram considerável progresso no entendimento dos eventos moleculares que ligam o exercício físico com a melhora na inflamação e na resistência à insulina.

**ABSTRACT**

Recent evidences have suggested a strong correlation among insulin resistance, inflammation and obesity. In this context, the toll-like receptor 4 (TLR4) seems to play an important role in the development of insulin resistance, since the activation of this receptor increases IκB kinase (IKKβ) and c-Jun NH2-terminal kinase (JNK)activity and pro-inflammatory cytokines expression such as tumor necrosis factor-alpha (TNF-α), substance known by its negative effects over insulin sensitivity. Previous results from our laboratory demonstrated that mice with loss-of-function mutation in TLR4 are protected from both weight gain and insulin resistance induced by high-fat diet, suggesting an important role of TLR4 in the link between the innate immune system and energy metabolism. In parallel, life style interventions involving exercise clearly improve insulin sensitivity, and possibly inflammation, in obese individuals, yet the mechanisms for these effects are not well understood. There is evidence that exercise leads to decreased gene expression of TLR4, however the relationship between TLR4 and exercise is still poorly investigated. Thus we aimed to analyze the effect of physical exercise on TLR4 expression and activation in obese rats and its consequences in insulin signaling and sensitivity. The present study demonstrated that chronic exercise was able to reduce the TLR4 expression of both mRNA and protein in muscle, liver and adipose tissue. However, the acute and chronic exercise blunted the TLR4 signaling in these tissues, as evidenced by reducing phosphorylation levels of JNK and IKKβ. We also observed a significant reduction in lipopolysaccharide (LPS)

circulating levels in both exercise protocols and lower levels of free-fat acids (FFA) only in the chronic exercise. In parallel, we demonstrated improved insulin-induced IR, IRS-1 tyrosine phosphorylation, and Akt serine phosphorylation after both exercise protocols. Thus, we concluded that physical exercise promotes a significant attenuation of the TLR4 signaling pathway in muscle, liver and adipose tissue; possibly by reducing LPS circulating levels, that resulted in an improved insulin signaling and sensitivity diet-induced obese animals. These data provided considerable progress in our understanding of molecular events that link exercise with to an improvement in inflammation and insulin resistance.

## LISTA DE ABREVIATURAS

| | | |
|---|---|---|
| AGL | | Ácidos graxos livres |
| Akt | Serine/threonine protein kinase | Proteína quinase serina/treonina |
| CD14 | Cluster ofdifferentiation 14 | |
| CTL | Control group | Grupo controle |
| DIO | Diet-induced obesity | Obesidade induzida por dieta |
| DIO+AE | Obese acute exercised group | Grupo obeso exercitado agudamente |
| DIO+CE | Obese chronic exercised group | Grupo obeso exercitado cronicamente |
| DNA | Deoxyribonucleic acid | Ácido desoxirribonucleico |
| ELISA | Enzyme-Linked Immunosorbent Assay | Teste imunoenzimático |
| ERK1/2 | Extracellular signal-regulated kinases 1/2 | |
| FFA | Free fatty acids | |
| IkBα | Inhibitory Subunit Of NF Kappa B Alpha | Subunidade alpha inibidora de NFκB |
| IkBβ | Inhibitory Subunit Of NF Kappa B Beta | Subunidade beta inibidora de NFκB |
| IKKβ | IκB kinase | |
| IL-1β | Interleukin-1 beta | Interleucina- 1 beta |
| IL-6 | Interleukin- 6 | Interleucina- 6 |

| | | |
|---|---|---|
| IRAK-1 | Interleukin-1 receptor-associatedkinase1 | |
| IRβ | Insulin receptor beta | Receptor de insulina beta |
| IRS-1 | Insulin receptor substrate- 1 | Substrato do receptor de insulina- 1 |
| IRS-1ser307 | Insulin receptor substrate phosphorylated in serine 307 | |
| JNK | c-JunNH2-terminal kinase | |
| LBP | Lipopolysaccharide-binding protein | |
| LPS | Lipopolysaccharide | |
| MAPK | Mitogen-activated protein kinase | |
| MCP-1 | Monocyte chemotactic protein-1 | |
| MD2 | Myeloid differentiation factor-2; | |
| mRNA | Messenger ribonucleic acid | |
| MyD88 | Myeloid differentiation primary response protein 88 | |
| NFkB | Nuclear Factor-KappaB | |
| RNA | Ribonucleic acid | Ácido ribonucleico |
| RNAm | | Ácido ribonucleico mensageiro |
| RT-PCR | Reverse transcript-polymerase chain | |

| | | |
|---|---|---|
| | reaction | |
| TACE/TIMP3 | TNF-alpha converting enzyme/tissue inhibitor of metalloproteinase 3proteolytic system | |
| TG | Triglyceride | Triglicerídeo |
| TLR | Toll-like receptor | |
| TLR2 | Toll-like receptor 2 | |
| TLR4 | Toll-like receptor 4 | |
| TNF-a | Tumor necrosis fator- alpha | Fator de necrose tumoral alfa |

**SUMÁRIO**

## INTRODUÇÃO

Evidências recentes têm apontado para estreita correlação entre resistência à insulina e inflamação subclínica principalmente em indivíduos obesos (1-5). Entretanto, os mecanismos para a ativação das vias inflamatórias induzidas pela obesidade não foram totalmente elucidados.

Esse processo inflamatório crônico parece funcionar como mecanismo de ativação para o desenvolvimento da resistência à insulina, através do aumento crônico da secreção de citocinas pró-inflamatórias, como TNF-αe IL-6, substâncias com conhecidos efeitos negativos sobre a atividade da via de sinalização da insulina; e provavelmente também através de alterações no metabolismo dos ácidos graxos livres (AGL), como por exemplo, a redução na captação de AGL pelo tecido adiposo, possivelmente e relacionada com redução na expressão do transportador de ácido graxo e com comprometimento do aumento pós prandial da atividade da lipoproteína lipase induzida (5-12). Condição que promoveria um aumento na disponibilidade de AGL para outros tecidos.

Na mesma linha, o tecido adiposo branco pode exercer papel de destaque, visto não ser ele mais identificado apenas como um tecido acumulador de gordura, e sim como órgão metabolicamente ativo que recebe e envia sinais que podem modular o gasto energético, apetite, sensibilidade à insulina, função endócrina, reprodução, inflamação e imunidade; sendo desse modo capaz de produzir e liberar inúmeros peptídeos ativos, hormônios e citocinas na circulação sistêmica, no intuito de promover esse papel regulatório (13; 14). Contudo, essas propriedades não são exclusividades do tecido adiposo, visto que o músculo

esquelético recentemente também foi associado à produção e liberação de citocinas, especialmente a IL-6, que parece ser ainda mais aumentada em decorrência da infiltração de macrófagos como resposta a fatores exógenos ou mesmo endógenos, lipopolissacarídeo (LPS) e exercício físico de alta intensidade, respectivamente (15; 16).

Os AGL representam uma importante fonte energética mobilizada a partir dos triglicerídeos armazenados no tecido adiposo (17-19). Na obesidade, os níveis de AGL apresentam-se anormalmente elevados devido ao aumento de sua liberação pelo tecido adiposo em constante expansão, e podem desempenhar papel de destaque no desenvolvimento da resistência à insulina (18; 19). Estudo com a utilização de infusão de ácidos graxos demonstrou que eles podem induzir a redução na capacidade da insulina em suprimir a produção hepática de glicose e promover a captação de glicose pelo músculo esquelético, características que indicam diminuição na sensibilidade à insulina (20). Essa relação com a resistência à insulina possivelmente tem ligação com a ativação de mediadores inflamatórios (21).

Estudos anteriores demonstraram que o tecido adiposo branco de obesos é caracterizado por aumento na infiltração de macrófagos, que podem ser uma importante fonte de inflamação e, dessa forma, contribuir para a redução na sensibilidade à insulina nesse tecido (3; 22). É importante destacar que a elevação da concentração de TNF-α, proveniente desses macrófagos além de exercer papel crítico na resistência à insulina na obesidade, pode ainda estimular a lipólise pelos adipócitos, contribuindo assim para o aumento dos níveis de AGL (23; 24). Assim,

os ácidos graxos saturados e o TNF-α,provenientes respectivamente do metabolismo energético e da imunidade, parecem estabelecer um circulo vicioso de alterações metabólicas e inflamatórias como, por exemplo, aumento nas citocinas séricas e redução nos níveis de adiponectina, proteína antiinflamatória capaz de atenuar as propriedades inflamatórias do TNF-α (19; 25). Esses achados em conjunto podem levar a especulações de que os macrófagos infiltrados nos adipócitos sejam capazes de promover a liberação de citocinas pró-inflamatórias e de ácidos graxos saturados, via lipólise, que podem conduzir a um estado inflamatório tendo como consequência o aumento do risco de defeitos no metabolismo da glicose (26; 27).

Receptores *toll* são glicoproteínas tipo I com domínio extracelular, transmembrana e intracelular de sinalização; encontradas em insetos, que se conservaram ao longo filo do animal aparecendo também em mamíferos (28; 29). Essas proteínas foram originalmente descobertas em drosófilas, onde exercem papel na ontogênese e também na defesa antifúngica (28). Nos mamíferos as substâncias homologas ao *toll,* presentes nas drosófilas, foram denominadas de receptores *toll-like* (TLR) (30). Aproximadamente 13 tipos de TLR foram identificados em mamíferos, os quais funcionam principalmente como receptores de reconhecimento a padrões moleculares associados à patógenos, desempenhando assim papel fundamental no sistema imune inato através da ativação das vias de sinalização pró-inflamatórias em resposta a esses padrões, tais como bactérias gram-positiva e gram-negativa, DNA e RNA de vírus, fungos e protozoários (29; 31-33).

Cada TLR possui seus ligantes específicos, dentre os quais se pode destacar o LPS, componente presente em membranas de bactérias grã-negativa, que se liga a dois membros dessa família, os receptores *toll-like receptor 4* (TLR4) e *toll-like receptor 2* (TLR2), entretanto seu principal ligante é o TLR4 (29; 34; 35). Através da estimulação com LPS o TLR4, em conjunto com a *proteína LBP* e os co-receptores CD14 (denominado de receptor de LPS) e MD-2, regula a produção de citocinas inflamatórias e a ativação do sistema imune inato através da sinalização das vias inflamatórias NF-κB/IKKβ e AP1/MAPKs, principalmente a JNK (29; 36).

O modelo de sinalização do TLR4 através da estimulação com LPS pode ser descrito resumidamente da seguinte forma: o LPS é transferido para a membrana do CD14 pela LPB, formando assim o complexo LPS-CD14, o qual é reconhecido pelo complexo TLR4-MD-2 presente na superfície celular, formando um novo complexo capaz de transmitir a sinalização via domínio intracelular do TLR4, através do recrutamento da proteína adaptadora MyD88, que ativa as vias inflamatórias das serinas quinases IKKβ e JNK as quais induzem numerosos mediadores inflamatórios (37; 38). A ativação moderada desses receptores se faz necessária para garantir uma proteção contra doenças infecciosas, contudo sua ativação excessiva pode levar a efeitos negativos, como alergias e algumas doenças autoimunes (39; 40).

Existe uma importante interação entre os AGL em excesso e a inflamação, pois como possuem estrutura molecular similar ao lipídio A, porção toxica do LPS, podem também ativar o TLR4, e consequentemente induzir resposta inflamatória

(20; 41; 42). Nesse contexto, alguns estudos demonstraram que o TLR4 parece apresentar papel central no link entre resistência à insulina, inflamação e obesidade, e que camundongos knockout para TLR4 ou com uma mutação inativadora no gene que codifica essa proteína, ficam protegidos contra a resistência à insulina e a ativação das serinas quinases, IKKβ e JNK, normalmente induzidas pela dieta hiperlipídica, sugerindo que o TLR4 seja provavelmente o modulador chave no cross-talk entre a via metabólica e inflamatória (3; 21; 43-48). Ainda nessa linha, estudos mostraram que a administração exógena de AGL exerce efeitos inflamatórios em certos tipos de células através da ativação do TLR4 (44; 49). Mais especificamente, foi demonstrado que a exposição de células a LPS e AG simultaneamente amplificava a resposta inflamatória, quando comparada aos tratamentos com essas substâncias de forma isolada (50).

Song et al. (44), demonstraram em seu experimento, com cultura de adipócitos, que a ativação do TLR4 pelo tratamento com LPS atenuava a captação de glicose estimulada pela insulina, contribuindo dessa forma para um quadro de resistência à insulina. Além disso, outras pesquisas relataram que as concentrações plasmáticas de LPS aumentavam significativamente após a ingestão de uma dieta rica em gordura (51; 52), sugerindo que o LPS em excesso seja proveniente do trato gastrointestinal, uma vez que ele é lipossolúvel. Nesse sentido, tem sido demonstrado que ingestão de gorduras aumenta a permeabilidade intestinal para LPS (53; 54). Dessa forma, os TLRs ganham ainda mais importância, visto sua capacidade de regular a flora intestinal e assim contribuir para o metabolismo (55).

A célula muscular esquelética apresenta TLR4 funcional (56-58), ou seja, pode induzir a ativação de vias inflamatórias em resposta ao LPS, sendo assim é possível que a resistência à insulina nesse tecido esteja relacionada, ao menos em parte, com a interação deste receptor com o LPS e os AGL elevados na obesidade (59; 60). Assim, indivíduos obesos podem estar sujeitos a uma constante ativação do TLR4, contribuindo para a patogênese da resistência à insulina (2; 61). Nesse contexto, foi demonstrado que tanto a infusão de LPS quanto de ácido graxo saturado promovia o aumento sérico de TNF-$\alpha$ e IL-6 e redução da captação de glicose no músculo sóleo de camundongos e que seus pares com defeito para a sinalização de TLR4 não apresentavam qualquer alteração na circulação dessas citocinas e tão pouco na captação de glicose pelo tecido muscular (62). Portanto, se torna importante investigar os possíveis mecanismos que levem a reduções na expressão e ativação do TLR4, incluindo entre esses o papel do exercício físico.

Paralelamente, torna-se cada vez mais evidente que o exercício físico crônico moderado possui efeitos antiinflamatórios e o que é ainda melhor, sem importantes efeitos colaterais como aqueles normalmente observados com a utilização de tratamentos farmacológicos. Esse efeito benéfico do exercício já foi demonstrado em alguns tecidos como, o muscular (63), o adiposo (64), e provavelmente ocorra também no tecido hepático. Em ratos, o exercício físico crônico é capaz de reduzir de forma significativa a inflamação no tecido adiposo (65), sugerindo seu uso como terapia alternativa. Dessa forma, intervenções no estilo de vida de obesos, que envolvem exercício físico, melhoram o perfil

inflamatório, entretanto os mecanismos precisos para esses efeitos não foram totalmente elucidados.

Em relação à resistência insulínica, melhoras na tolerância à glicose já podem ser observadas após uma sessão aguda de exercício físico (66). Nessa linha, Ropelle et al. (67), demonstraram que uma sessão prolongada de natação, praticada de forma isolada, pode reverter, mesmo que parcialmente, a resistência à insulina induzida pela dieta hiperlipídica. Essa melhora na sensibilidade a insulina, comumente relacionado ao exercício físico, pode estar associada com a melhora no perfil inflamatório, provavelmente com participação da redução na expressão e/ou ativação de TLR4.

A relação entre TLR4 e exercício físico é um tópico ainda pouco explorado. Lancaster et al (68), demonstraram redução na expressão e função do receptor TLR4 em monócitos após uma sessão prolongada de exercício físico, que consistia em uma hora e meia em ciclo ergômetro a 65% do consumo máximo de oxigênio. Contudo, esse estudo possuía importante viés devido a alteração um pouco acima do normal da temperatura corporal, possivelmente imposta pela temperatura do ambiente se encontrar em 34°C; problema esse solucionado em um trabalho posterior de protocolo semelhante utilizando ambientes de temperaturas diferentes, no qual não foram encontradas alterações significativas dos TLR em virtude dessas variações na temperatura (32). Em conjunto, os dados desses estudos indicam que o exercício físico agudo pode culminar na redução da expressão de TLR4 em monócitos, sendo esse efeito independente da temperatura ambiental. No entanto, mais estudos são necessários para comprovar

essa redução nos níveis de TLR4 após uma única sessão de exercício prolongada, e também se essa ocorre em outros tecidos.

Quando o foco é colocado na influência efeito crônico do exercício físico sobre a expressão e atividade de TLR4, ainda menos estudos foram realizados. O exercício físico, quando praticado de forma regular pode exercer um importante efeito antiinflamatório através de redução da produção de citocinas e também diminuir os níveis basais de TNF-$\alpha$ e IL-1$\beta$ e IL-6 no músculo esquelético (69; 70), possivelmente com participação da redução da expressão ou mesmo apenas da ativação do TLR4.

Em mulheres idosas, Flynn et al. (69), encontraram menor expressão gênica de TLR4 em mulheres treinadas em exercícios resistidos, quando comparadas com as sedentárias. Na mesma linha, um estudo longitudinal relatou ser aproximadamente 2,5 vezes menor a expressão de TLR4 em mulheres idosas fisicamente ativas em relação a suas congêneres sedentárias (26). Em acordo com estes estudos, outro trabalho relacionou a expressão de TLR4 com o exercício físico encontrando dados semelhantes, porém com participação de jovens, onde após 12 semanas de treinamento concomitante de *endurance* e de força, foram encontradas reduções significativas na expressão de TLR4 (71). Constatou-se também que a produção de IL-6 estimulada pela presença de LPS esteve significativamente menor nas mulheres treinadas.

Tendo em vista que os estudos apresentados até o momento se mostraram apenas descritivos na relação entre os TLR e o exercício físico sendo, portanto necessário investigar se realmente ocorrem reduções significativas nos TLR em

decorrência do exercício físico, quais os mecanismos pelos quais se dão essas alterações e consequentemente o efeito que a redução de TLR4 pode exercer em longo prazo no organismo. Assim, o presente trabalho teve como objetivo principal investigar o efeito do exercício físico (agudo e crônico) sobre a expressão e a ativação do TLR4 em animais com obesidade induzida por dieta hiperlipídica.

# OBJETIVOS

## Objetivo Geral

Investigar o efeito do exercício físico agudo e crônico na expressão e sinalização (ativação da IKKβ e JNK) de TLR4 em tecido adiposo, fígado e músculo esquelético em roedores alimentados com dieta hiperlipídica.

## Objetivos Específicos

- Investigar o efeito do exercício físico sobre os parâmetros fisiológicos (peso corporal, glicemia de jejum, tolerância à insulina, etc).
- Investigar o efeito do exercício físico sobre a expressão de RNA mensageiro de TLR4, TNF-α e IL-6.
- Investigar o efeito do exercício sobre a expressão e fosforilação dos efetores da via do TLR4 (JNK e IKKβ).
- Investigar o efeito do exercício físico sobre a expressão e fosforilação das proteínas da via de sinalização da insulina (IRβ, IRS-1 e Akt).
- Investigar o efeito da dieta hiperlipídica e do exercício físico sobre a concentração de LPS circulante e de AGL.

## CAPÍTULO

O presente trabalho resultou em um artigo científico publicado no *Diabetes* 2011 Mar; 60 (3):784-96.

ORIGINAL ARTICLE

# Physical Exercise Reduces Circulating Lipopolysaccharide and TLR4 Activation and Improves Insulin Signaling in Tissues of DIO Rats

**Alexandre G. Oliveira, Bruno M. Carvalho, Natália Tobar, Eduardo R. Ropelle, José R. Pauli, Renata A. Bagarolli, Dioze Guadagnini, José B.C. Carvalheira, and Mario J.A. Saad**

**OBJECTIVE**—Insulin resistance in diet-induced obesity (DIO) is associated with a chronic systemic low-grade inflammation, and Toll–like receptor 4 (TLR4) plays an important role in the link among insulin resistance, inflammation, and obesity. The current study aimed to analyze the effect of exercise on TLR4 expression and activation in obese rats and its consequences on insulin sensitivity and signaling.

**RESEARCH DESIGN AND METHODS**—The effect of chronic and acute exercise was investigated on insulin sensitivity, insulin signaling, TLR4 activation, c-Jun $NH_2$-terminal kinase (JNK) and IκB kinase (IKKβ) activity, and lipopolysaccharide (LPS) serum levels in tissues of DIO rats.

**RESULTS**—The results showed that chronic exercise reduced TLR4 mRNA and protein expression in liver, muscle, and adipose tissue. However, both acute and chronic exercise blunted TLR4 signaling in these tissues, including a reduction in JNK and IKKβ phosphorylation and IRS-1 serine 307 phosphorylation, and, in parallel, improved insulin-induced IR, IRS-1 tyrosine phosphorylation, and Akt serine phosphorylation, and reduced LPS serum levels.

**CONCLUSIONS**—Our results show that physical exercise in DIO rats, both acute and chronic, induces an important suppression in the TLR4 signaling pathway in the liver, muscle, and adipose tissue, reduces LPS serum levels, and improves insulin signaling and sensitivity. These data provide considerable progress in our understanding of the molecular events that link physical exercise to an improvement in inflammation and insulin resistance. ***Diabetes*** **60:784–796, 2011**

It has become increasingly evident that insulin resistance, induced by obesity, is associated with a chronic systemic low-grade inflammation (1–4). In this context, recent studies from our group and others show that the Toll–like receptor 4 (TLR4) may play a central role in the link among insulin resistance, inflammation, and obesity and that a point mutation in TLR4, which inactivates this receptor, prevents the diet-induced obesity (DIO) activation of IκB kinase (IKKβ) and c-Jun $NH_2$-terminal kinase (JNK), and insulin resistance, suggesting that TLR4 is a key modulator in the cross-talk between inflammatory and metabolic pathways (5–10). TLR4 is an essential receptor for the recognition of lipopolysaccharide (LPS) (11). Moreover, a recent study demonstrated that LPS plasma concentrations increase significantly after the intake of high-fat, high-carbohydrate meals (12), suggesting that this LPS comes from the gastrointestinal tract because LPS is fat-soluble. In addition, it has recently been shown that fat intake leads to increased intestinal permeability for LPS (13).

On the other hand, evidence has emerged that exercise training has anti-inflammatory effects, with minimal side effects, which have been shown to occur in several tissues, including skeletal muscle (14), adipose tissue (15), and probably liver. In rats, exercise training lowers adipose inflammation (16), suggesting that exercise may be a useful therapy. Lifestyle interventions involving exercise clearly improve insulin sensitivity, and possibly inflammation, in obese individuals; yet the mechanisms for these effects are not well understood.

On the basis of data from these studies, we hypothesize that suppression of TLR4 signaling may play an important role in the exercise-induced improvement of insulin sensitivity. Thus, the current study aimed to analyze the effect of exercise on TLR4 expression and activation in obese rats, and its consequences on insulin sensitivity and signaling. We report that DIO induces the expression and activation of TLR4 in muscle, adipose tissue, and liver. Furthermore, both acute and chronic exercise strongly reverse the activation of this pathway and improve insulin signaling, providing a new mechanism by which exercise improves insulin action in obesity and type 2 diabetes. In addition, we show that exercise, both acute and chronic, promotes a reduction in serum LPS in DIO rats.

## RESEARCH DESIGN AND METHODS

Male Wistar rats, C3H/HeJ mice, and C3H/HeN mice, their respective controls from the University of Campinas Central Animal Breeding Center, were used in the experiments. All antibodies were from Santa Cruz Technology (Santa Cruz, CA), with the exception of anti-Akt, anti–phospho-Akt, anti–phospho-IKKβ, anti–phospho-IRS-1 $Ser^{307}$, and anti-TLR4, which were obtained from Cell Signaling Technology (Beverly, MA). TAK-242 (ethyl(6*R*)-6-[*N*-(2-chloro-4-fluorophenyl)sulfamoyl] cyclohex-1-ene-1-carboxylate) was synthesized at the Chemistry Institute of University of Campinas. LPS and routine reagents were purchased from Sigma Chemical (St. Louis, MO), unless specified elsewhere.

**Animal care and experimental procedures.** All experiments were approved by the ethics committee at the State University of Campinas. Eight-week-old male Wistar rats were randomly divided into groups: control (C), fed standard rodent chow and water ad libitum; DIO-sedentary rats (DIO), fed a high-fat diet, as previously used (9), and water ad libitum for 20 weeks; DIO-chronic exercised rats (DIO+CE) and DIO-acute exercised rats (DIO+AE), fed a high-fat diet. Insulin and glucose tolerance tests were performed on these rats after 20 weeks of consumption of the diets and after both the exercise protocols, as described previously (17,18). Because individual rats can vary in their ability to perform swimming, some rats (10–15%) were rejected in the screening for the procedure.

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**Hyperinsulinemic-euglycemic clamps.** After an overnight fast (~12 h), a 2-h hyperinsulinemic-euglycemic clamp was conducted in anesthetized catheterized rats with [3-$^3$H]glucose and 2-deoxy-*d*-[1-$^{14}$C]glucose to assess glucose metabolism in muscle, as previously described (18–20).

**Assays.** Insulin and interleukin (IL)-6 concentrations were determined by an ELISA (Linco, St. Charles, MO). Serum free fatty acid (FFA) levels were analyzed using NEFA-kit-U (Wako Chemical, Neuss, Germany) with oleic acid as a standard. Glucose was measured from whole venous blood with a glucose monitor (Glucometer; Bayer Diagnostics, New York, NY).

**Chronic exercise protocol.** Rats were adapted to swimming for 10 min for 2 days to reduce water-induced stress. Animals swam in groups of three in plastic barrels of 45 cm in diameter that were filled to a depth of 60 cm, and the water temperature was maintained at ~34°C. The training consisted of daily swimming sessions (1 h/day, 5 days/week, for 8 weeks) with a progressive load increase up to 5% of body weight. These conditions were chosen on the basis of previous studies showing that swimming training with this load improved the physical condition of rats (21). The animals were killed with an overdose of anesthetic (sodium thiopental) at 24 and 36 h after the last session.

**Acute exercise protocol.** Under the same conditions imposed as chronic exercise, the acute exercise protocol consisted of two 3-h bouts, separated by a 45-min rest period, as described previously (18). The animals were killed at 2 and 16 h after this protocol was carried out.

**Tissue extraction and protein analysis by immunoblotting.** After exercise protocols, rats were anesthetized and used 10–15 min later, i.e., as soon as anesthesia was ensured by the loss of pedal and corneal reflexes, the abdominal cavity was opened, the portal vein was exposed, and 0.2 mL of normal saline was injected with or without insulin ($10^{-6}$ mol/L). At 30 and 90 s after insulin injection, liver and gastrocnemius and adipose tissue were removed, minced coarsely, and homogenized immediately in extraction buffer, as previously described (22). Extracts were used for immunoprecipitation with MyD88 and Protein A-Sepharose 6MB (Pharmacia, Uppsala, Sweden). The precipitated proteins or whole-tissue extracts were subjected to SDS-PAGE and immunoblotting, as previously described (17,20).

**LPS levels.** We diluted plasma samples to 20% with endotoxin-free water and then heated them to 70°C for 10 min to inactivate plasma proteins. We then quantified serum LPS with a commercially available Limulus Amebocyte assay from Cambrex (Walkersville, MD) according to the manufacturer's protocol. We ran samples in duplicate and subtracted the background (23).

**Real-time RT-PCR.** The mRNA was determined in the muscle, liver, and adipose tissue using RT-PCR, as previously reported (24). Primer sequences are shown in Supplementary Table 1. Results are expressed as relative expression values, as published previously (1).

**Statistical analysis.** Data are expressed as means ± SEM, and the number of independent experiments is indicated. The results of blots are presented as direct comparisons of bands or spots in autoradiographs and quantified by optical densitometry (Scion Image; Scion Corporation, Frederick, MD). For statistical analysis, the groups were compared using a two-way ANOVA with the Bonferroni test for post hoc comparisons. The level of significance adopted was $P < 0.05$.

## RESULTS

**Physiologic and metabolic parameters.** Figure 1 shows comparative data regarding the controls, DIO-sedentary rats, and DIO rats submitted to chronic exercise when investigated at 24 h (DIO+CE24h) or 36 h (DIO+CE36h) after the last training session. All DIO animals, submitted to chronic exercise or not, exhibited a higher body weight and epididymal fat than the age-matched C group (Fig. 1*A* and *B*). The fasting plasma glucose concentrations were similar among all groups (Fig. 1*C*). During the glucose tolerance test, glucose and insulin levels were higher in DIO rats, compared with controls, and chronic exercise improved glucose tolerance and reduced insulin levels at all time points (Fig. 1*C* and *D*). Serum insulin levels were higher in DIO rats, and chronic exercise was able to reduce this hyperinsulinemia (Fig. 1*D*). The glucose disappearance rate was lower in the DIO group, and exercise training reversed these alterations (Fig. 1*E*). A hyperinsulinemic-euglycemic clamp with tracer infusions was also performed to examine the effects of training on glucose metabolism in the skeletal muscle. The glucose infusion rate was lower in the DIO group than in the C group and reestablished to control levels in DIO-exercised rats (Fig. 1*F*). As shown in Fig. 1*G*, DIO rats presented a significant reduction in glucose uptake in the skeletal muscle when compared with the control group. In contrast, chronic exercise improved insulin-induced glucose uptake in the muscle of DIO rats (Fig. 1*G*). Serum levels of FFA were also higher in DIO rats and significantly decreased after swimming training (Fig. 1*H*).

**Effect of chronic exercise on TLR4 expression.** The TLR4 protein content in muscle, liver, and adipose tissue was higher in the DIO group than in the controls (Fig. 2*A–C*). Results show that, at 24 h after the last training, there was a marked decrease in TLR4 expression and, after 36 h, this was still decreased when compared with nonexercised obese rats (Fig. 2*A–C*). Furthermore, the TLR4 mRNA expression was significantly reduced after chronic exercise in muscle, liver, and adipose tissue, compared with DIO rats (Fig. 2*D–F*).

**Chronic exercise-mediated suppression of TLR4 activity in the skeletal muscle, adipose tissue, and liver of DIO Wistar rats.** We next investigated TLR4 pathway activation in two steps: *1*) as an early event, TLR4/MyD88 interaction was examined; and *2*) for downstream signaling, JNK, IKKβ, and ERK1/2 phosphorylation were studied (25). Skeletal muscle, adipose tissue, and liver of DIO rats exhibited significant increases in the TLR4/MyD88 interaction, compared with the C group (Fig. 2*G–I*). Conversely, in exercised groups, the TLR4/MyD88 interaction decreased significantly in all tissues studied compared with obese sedentary rats (Fig. 2*G–I*). IRAK-1, another protein of the TLR4 pathway, showed an increased expression in DIO animals compared with the C group. Conversely, chronic exercise did not have any effect on this protein (Fig. 2*J–L*).

We also investigated whether this reduction was reflected in IKKβ and JNK phosphorylation, which are downstream of TLR4. As expected, an increase in IKKβ and JNK phosphorylation was found in the muscle, liver, and adipose tissue of DIO rats, compared with controls, and this effect was attenuated in the tissues of chronic-exercised obese rats, compared with sedentary DIO rats (Fig. 3*A–F*). With regard to ERK1/2, DIO rats exhibited high phosphorylation levels in the three tissues analyzed, compared with control animals (Supplementary Fig. 1). In contrast, we detected a marked reduction in ERK activation after chronic exercise (Supplementary Fig. 1).

We then evaluated the effect of exercise training on an important substrate of these kinases of insulin signaling pathway, namely, IRS-1 Ser$^{307}$ phosphorylation. This phosphorylation was, on average, markedly upregulated in the muscle, liver, and adipose tissue of obese sedentary rats, compared with controls (Fig. 3*G–I*). In addition, exercise training was also able to reverse diet-induced IRS-1 Ser$^{307}$ phosphorylation in the muscle, liver, and adipose tissue of DIO+CE24h and DIO+CE36h rats (Fig. 3*G–I*).

**Chronic exercise improves insulin signaling in obese rats.** We then examined the consequences that this exercise-induced improved inflammatory profile exerts on the insulin signaling pathway. In the muscle, liver, and adipose tissue, insulin-induced IRβ, IRS-1 tyrosine phosphorylation, and Akt phosphorylation were reduced by 50–80% in DIO rats, compared with the C group (Fig. 4*A–I*). On the other hand, insulin-induced IRβ, IRS-1, and Akt phosphorylation were increased in the tissues of DIO+CE24h and DIO+CE36h rats, compared with obese sedentary rats, and approached levels of those found in the C group (Fig. 4*A–I*). No changes in basal phosphorylation or tissue protein levels were observed among groups (Fig. 4*A–I*).

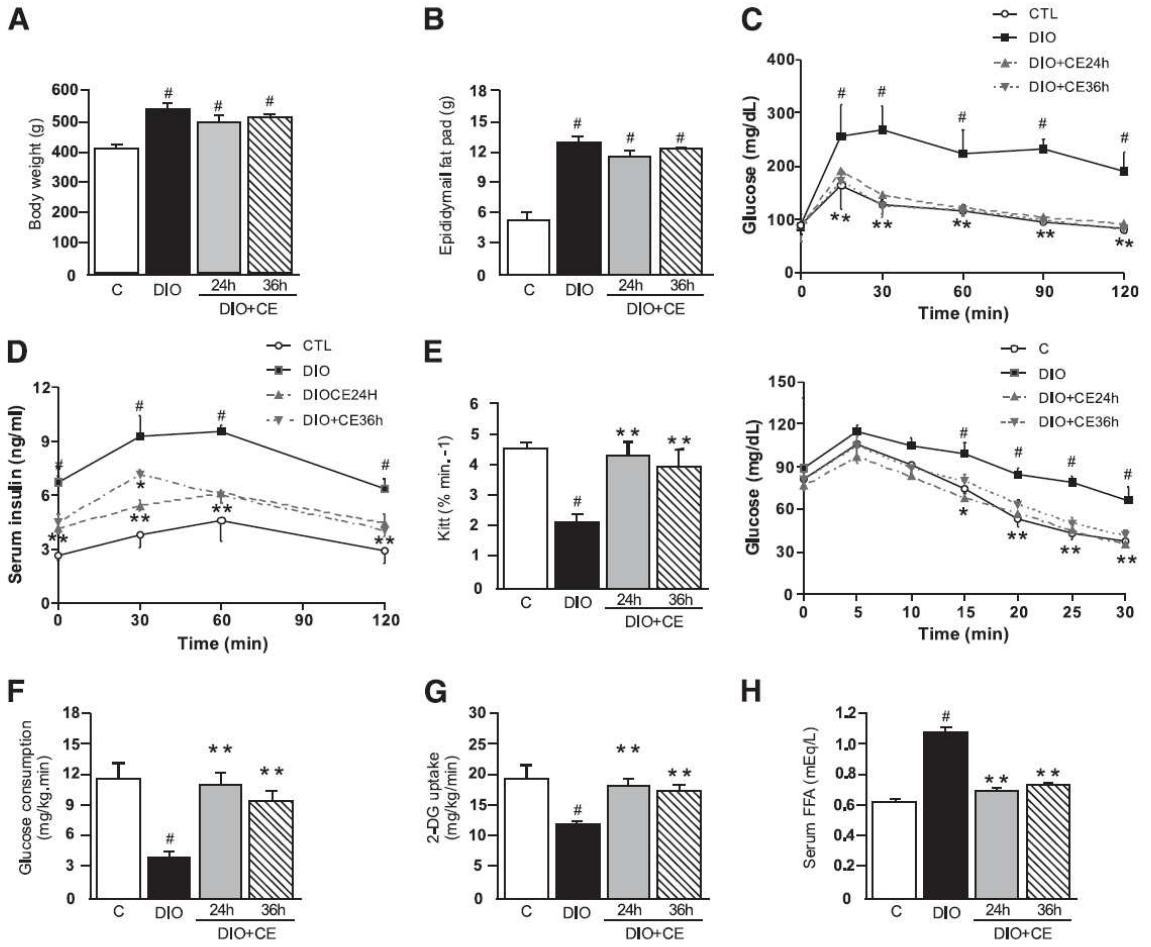


**FIG. 1. Physiologic, metabolic, and insulin tolerance parameters in control rats, obese rats, and obese rats submitted to a chronic exercise protocol. *A*: Body weight. *B*: Epididymal fat pad weight. *C*: Glucose tolerance test after 20 weeks of a high-fat diet. *D*: Serum insulin levels during the glucose tolerance test after 20 weeks of the diet. *E*: Rate constant for insulin tolerance test and glucose response curve during the insulin tolerance test after 20 weeks of a high-fat diet. *F*: Steady-state glucose infusion rates obtained from averaged rates of 90–120 min of 10% unlabeled glucose infusion during hyperinsulinemic-euglycemic clamp procedures in the control rats, DIO rats, and DIO rats submitted to chronic exercise. *G*: Glucose transport in gastrocnemius muscle was evaluated by 2-deoxy-D-glucose uptake during the last 45 min of the hyperinsulinemic-euglycemic clamp studies. *H*: Serum FFA concentrations. Data are presented as means ± SEM of 10 rats per group. #$P < 0.001$ vs. control. *$P < 0.05$ vs. DIO. **$P < 0.001$ vs. DIO.**

**Acute exercise and metabolic parameters.** Figure 5 shows comparative data for the DIO rats and DIO rats submitted to the acute exercise protocol (DIO+AE2h and DIO+AE16h). No significant variation was found in body weight, epididymal fat, and fasting blood glucose in DIO rats after a single session of exercise, when compared with sedentary obese rats (Fig. 5*A*–*C*). Acute exercise improved glucose tolerance and reduced serum insulin levels at all time points after the glucose load (Fig. 5*D* and *E*). The glucose disappearance rate was restored at both 2 h and 16 h after acute exercise (Fig. 5*E*). Acute exercise was also capable of restoring the glucose infusion rate during the hyperinsulinemic-euglycemic clamp (Fig. 5*F*), accompanied by a significant increase in glucose uptake in the skeletal muscle, compared with DIO rats (Fig. 5*G*). As expected, serum levels of FFA and IL-6 were higher in obese rats, and acute exercise induced a marked increase in the levels of this substrate, mainly in the DIO+AE2h group (Fig. 5*H* and *I*).

**Acute physical exercise reverses obesity-induced TLR4 activation in obese rats.** In DIO animals submitted to acute exercise, no changes in TLR4 protein expression were observed in muscle, liver, and adipose tissue (data not shown). On the other hand, the acute exercised animals showed lower TLR4 mRNA only in muscle (Supplementary Fig. 2*A*–*C*). In contrast, we observed significant reductions in the TLR4/MyD88 interaction in all studied tissues (Fig. 6*A*–*C*).

We also investigated the effect of acute exercise on TLR4 downstream signaling. As expected, significant decreases in IKKβ and JNK phosphorylation were found in the muscle, liver, and adipose tissue of acute exercised rats, compared with the DIO group (Fig. 6*D*–*I*). The same behavior was

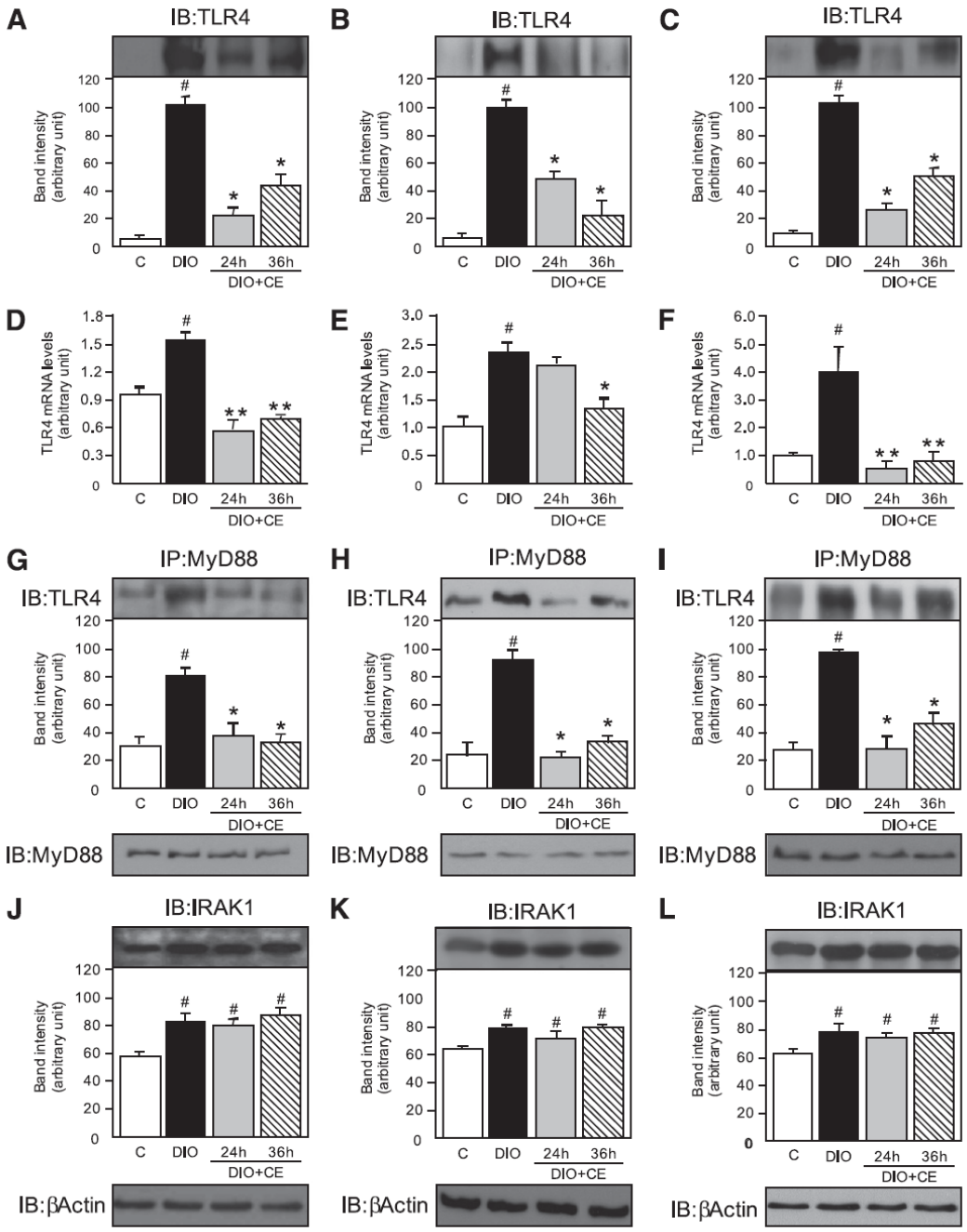


**FIG. 2. Effects of chronic exercise on TLR4 in obese Wistar rats. Representative blots show the expressions of TLR4 in muscle (*A*), liver (*B*), and adipose (*C*) of control, DIO, DIO+CE24h, and DIO+CE36h rats. Determination of the relative TLR4 mRNA expression by real-time PCR in muscle (*D*), liver (*E*), and adipose tissue (*F*) of control, DIO, DIO+CE24h, and DIO+CE36h rats. TLR4/MyD88 interaction in muscle (*G*), liver (*H*), and adipose (*I*) of control, DIO, DIO+CE24h, and DIO+CE36h rats (*G–I*, *top*). Total protein expression of MyD88 (*G–I*, *bottom*). IRAK-1 protein expression in muscle (*J*), liver (*K*), and adipose (*L*) of control, DIO, DIO+CE24h, and DIO+CE36h rats. Total protein expression of β-actin (*J–K*, *bottom*). Data are presented as means ± SE of 10 rats per group. #$P < 0.05$ vs. control group. *$P < 0.05$ vs. DIO. **$P < 0.001$ vs. DIO. IB, immunoblot; IP, immunoprecipitate.**

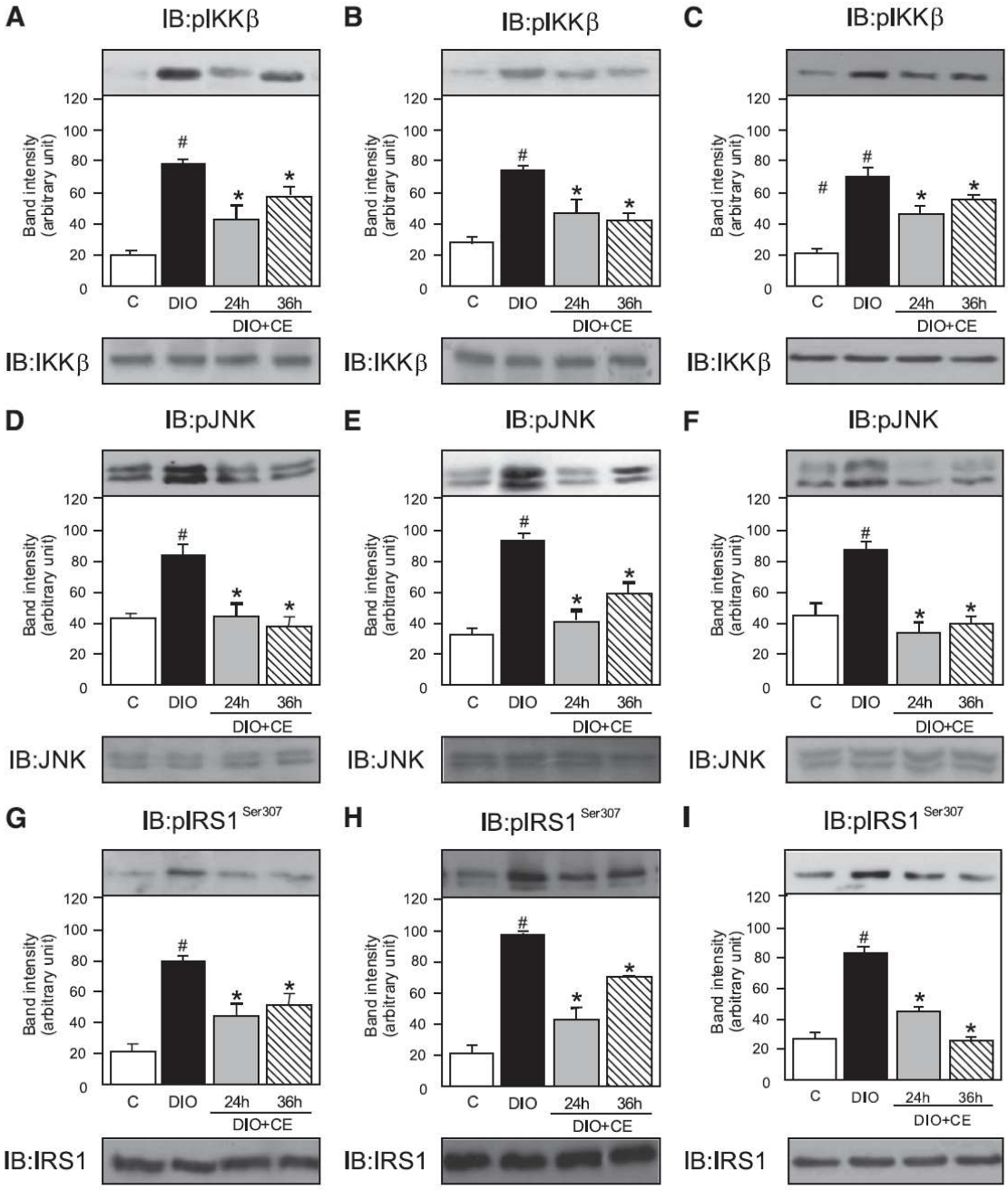


**FIG. 3. Effects of chronic exercise on modulators of insulin signaling. Representative blots show the expressions of IKKβ phosphorylation in muscle (*A*), liver (*B*), and adipose (*C*) of control, DIO, DIO+CE24h, and DIO+CE36h rats (*top*). Total protein expression of IKKβ (*A–C*, *bottom*). Expression of JNK phosphorylation in muscle (*D*), liver (*E*), and adipose (*F*) of control, DIO, DIO+CE24h, and DIO+CE36h rats (*D–F*, *top*). Total protein expression of JNK (*D–F*, *bottom*). IRS-1 serine 307 phosphorylation in muscle (*G*), liver (*H*), and adipose (*I*) of control, DIO, DIO+CE24h, and DIO+CE36h rats (*top*). Total protein expression of IRS-1 (*G–I*, *bottom*). Data are presented as means ± SE of 10 rats per group. #$P < 0.05$ vs. control group. *$P < 0.05$ vs. DIO. IB, immunoblot.**

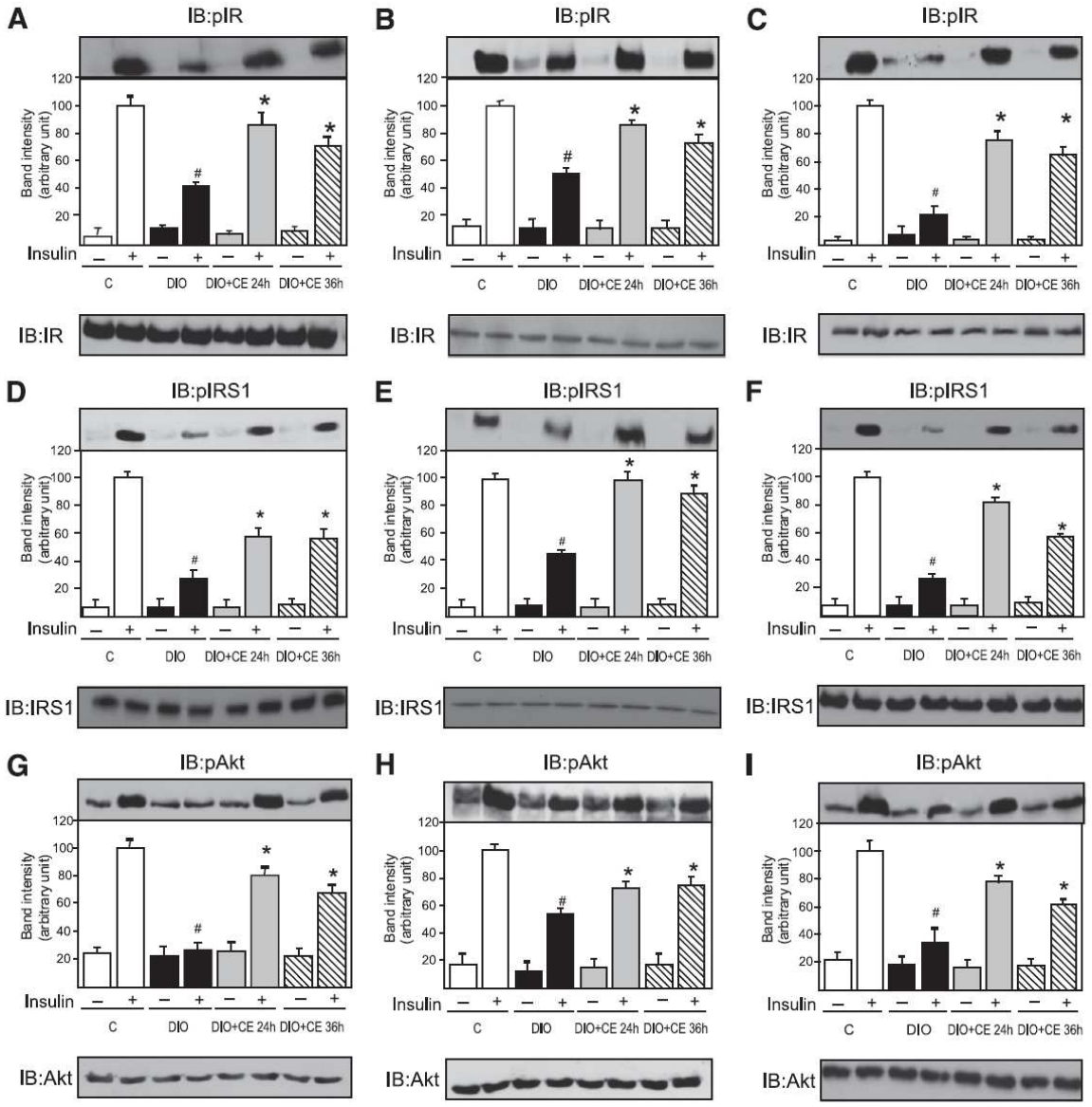


**FIG. 4. Effects of chronic exercise on insulin signaling in rats fed a high-fat diet. Representative blots show tyrosine phosphorylation of IRβ in muscle (*A*), liver (*B*), and adipose (*C*) of control, DIO, DIO+CE24h, and DIO+CE36h rats (*top*). Total protein expression of IRβ (*A–C*, *bottom*). Tyrosine phosphorylation of IRS-1 in muscle (*D*), liver (*E*), and adipose (*F*) of control, DIO, DIO+CE24h, and DIO+CE36h rats (*top*). Total protein expression of IRS1 (*D–F*, *bottom*). Serine phosphorylation of Akt in muscle (*G*), liver (*H*), and adipose (*I*) of control, DIO, DIO+CE24h, and DIO+CE36h rats (*top*). Total protein expression of Akt (*G–I*, *bottom*). Data are presented as means ± SE of 10 rats per group. #$P < 0.05$ vs. control group. *$P < 0.05$ vs. DIO. IB, immunoblot.**

found for ERK1/2 phosphorylation after the acute exercise (Supplementary Fig. 1). Acute exercise was also able to reverse the diet-induced IRS-1 $Ser^{307}$ phosphorylation in muscle, liver, and adipose tissue of both DIO+AE2h and DIO+AE16h rats (Fig. 6*J–L*).

**Acute exercise improves insulin signaling in DIO rats.** Acute exercise, as observed in the chronic group, improved insulin-induced tyrosine phosphorylation of IRβ and IRS-1 in the insulin-sensitive tissues (Fig. 7*A–F*). With regard to Akt, the acute protocol stimulated an impressive increase in serine phosphorylation of this protein in all tissues studied that was similar to that observed in the C group (Fig. 7*G–I*).

**Physical exercise blunted the high levels of cytokine mRNA expression in obese rats.** The tumor necrosis factor (TNF)-α and IL-6 mRNA levels were upregulated in the DIO group and were decreased after both exercise protocols in muscle, liver, and adipose tissue of almost all exercised groups (Supplementary Fig. 1*D–I*).

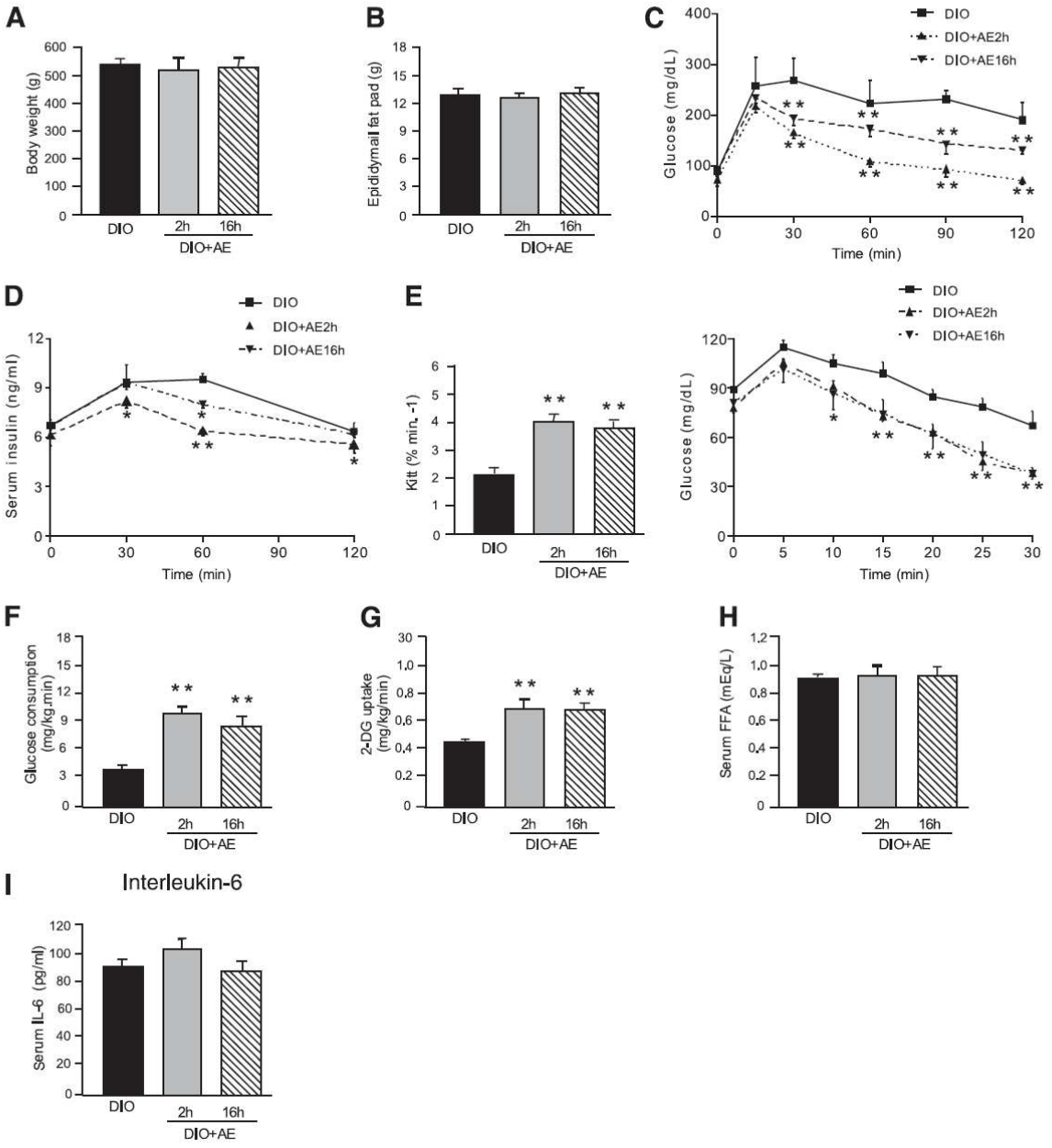


**FIG. 5. Physiologic, metabolic, and insulin tolerance parameters of obese sedentary rats and obese rats submitted to an acute exercise protocol. *A*: Body weight. *B*: Epididymal fat pad weight. *C*: Glucose tolerance test after 20 weeks of a high-fat diet. *D*: Serum insulin levels during the glucose tolerance test after 20 weeks of the diet. *E*: Rate constant for insulin tolerance test and glucose response curve during the insulin tolerance test after 20 weeks of a high-fat diet. *F*: Steady-state glucose infusion rates obtained from averaged rates of 90–120 min of 10% unlabeled glucose infusion during hyperinsulinemic-euglycemic clamp procedures in the DIO sedentary rats and DIO rats submitted to acute exercise. *G*: Glucose transport in gastrocnemius muscle was evaluated by 2-deoxy-D-glucose uptake during the last 45 min of the hyperinsulinemic-euglycemic clamp studies. *H*: Serum FFA concentrations. Data are presented as means ± SEM of 10 rats per group. *$P < 0.05$ vs. DIO. **$P < 0.001$ vs. DIO.**

**Physical exercise reduces serum LPS levels in DIO rats.** Serum LPS levels were significantly higher in obese sedentary rats when compared with control animals. Conversely, in animals of the DIO+AE2h group, acute exercise was able to completely reverse the high levels of LPS, and this was still observed at 16 h after the acute exercise (Fig. 8*A*). Notably, in chronic-exercised animals there was also a reduction

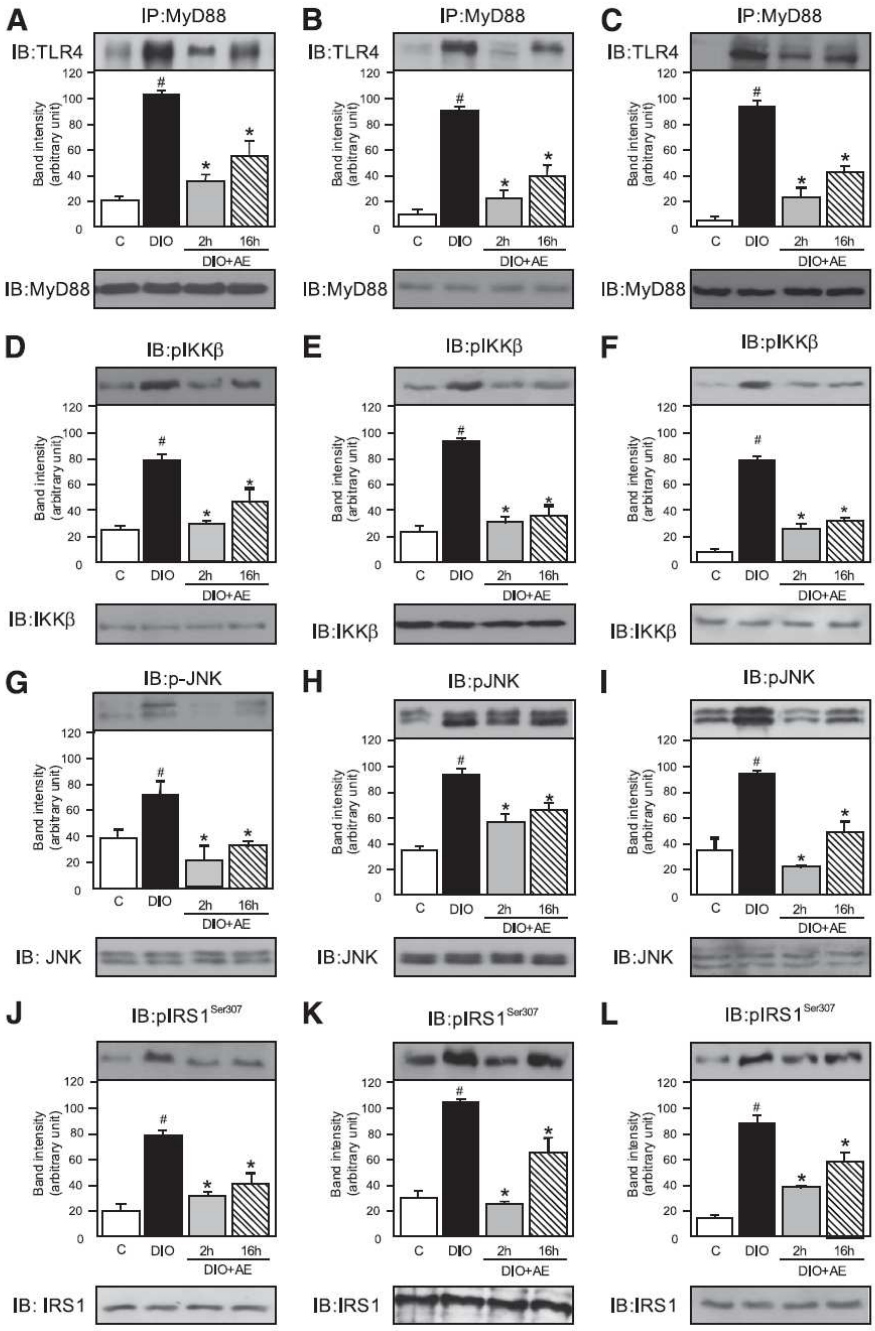


**FIG. 6. Effects of acute exercise on the TLR4 pathway. Representative blots show the TLR4/MyD88 interaction in muscle (*A*), liver (*B*), and adipose (*C*) of control, DIO, DIO+AE2h, and DIO+AE16h rats (*top*). Total protein expression of MyD88 and TLR4 (*A–C*, *bottom*). Phosphorylation of IKKβ in muscle (*D*), liver (*E*), and adipose (*F*) of control, DIO, DIO+AE2h, and DIO+AE16h rats (*top*). Total protein expression of IKKβ (*D–F*, *bottom*). Expression of JNK phosphorylation in muscle (*G*), liver (*H*), and adipose (*I*) of control, DIO, DIO+AE2h, and DIO+AE16h rats (*top*). Total protein expression of JNK (*G–I*, *bottom*). IRS-1 serine 307 phosphorylation in muscle (*J*), liver (*K*), and adipose (*L*) of control, DIO, DIO+AE2h, and DIO+AE16h rats (*top*). Total protein expression of IRS-1 (*J–L*, *bottom*). Data are presented as means ± SE of 10 rats per group. #$P < 0.05$ vs. control group. *$P < 0.05$ vs. DIO. IB, immunoblot; IP, immunoprecipitate.**

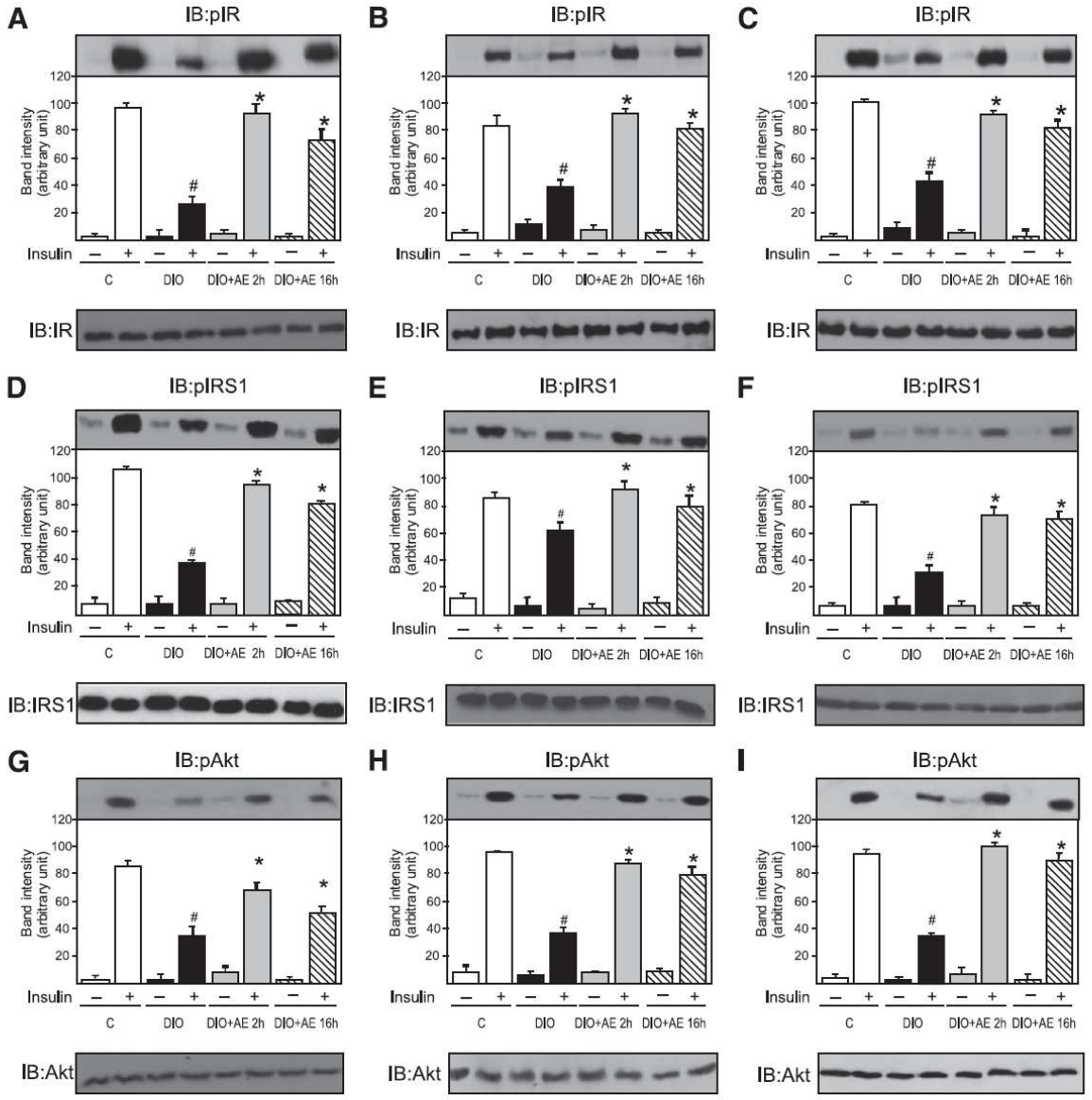


**FIG. 7. Effects of a single session of exercise on insulin signaling in high-fat fed rats. Representative blots show tyrosine phosphorylation of IRβ in muscle (*A*), liver (*B*), and adipose (*C*) of control, DIO, DIOACE2h, and DIO+AE16h rats (*top*). Total protein expression of IRβ (*A–C*, *bottom*). Tyrosine phosphorylation of IRS-1 in muscle (*D*), liver (*E*), and adipose (*F*) of control, DIO, DIO+AE2h, and DIO+AE16h rats (*top*). Total protein expression of IRS-1 (*D–F*, *bottom*). Serine phosphorylation of Akt in muscle (*G*), liver (*H*), and adipose (*I*) of control, DIO, DIO+AE2h, and DIO+AE16h rats (*top*). Total protein expression of Akt (*G–I*, *bottom*). Data are presented as means ± SE of 10 rats per group. #$P < 0.05$ vs. control group. *$P < 0.05$ vs. DIO. IB, immunoblot.**

in serum LPS levels, compared with the DIO group (Fig. 8*A*).

**LPS infusion partially reverses the effects of exercise.** To examine whether the exercised-induced reduction in serum LPS plays an important role in the improvement of inflammatory status and insulin sensitivity, we infused LPS into the peritoneum of obese exercised rats immediately after acute exercise. Exercised animals treated with LPS showed serum levels of LPS similar to the DIO animals and presented significant increases in TLR4/MyD88 interaction, IKKβ, and JNK phosphorylation in the muscle and adipose tissue (DIO+AE2h and DIO+AE16h) (Fig. 8*A–G*), increase in TNF-α mRNA in muscle and adipose tissue, and increase in IL-6 mRNA only in muscle (Supplementary Fig. 2*D–I*). A significant decrease in Akt serine phosphorylation levels was also achieved in LPS-treated animals (Fig. 8*H* and *I*).

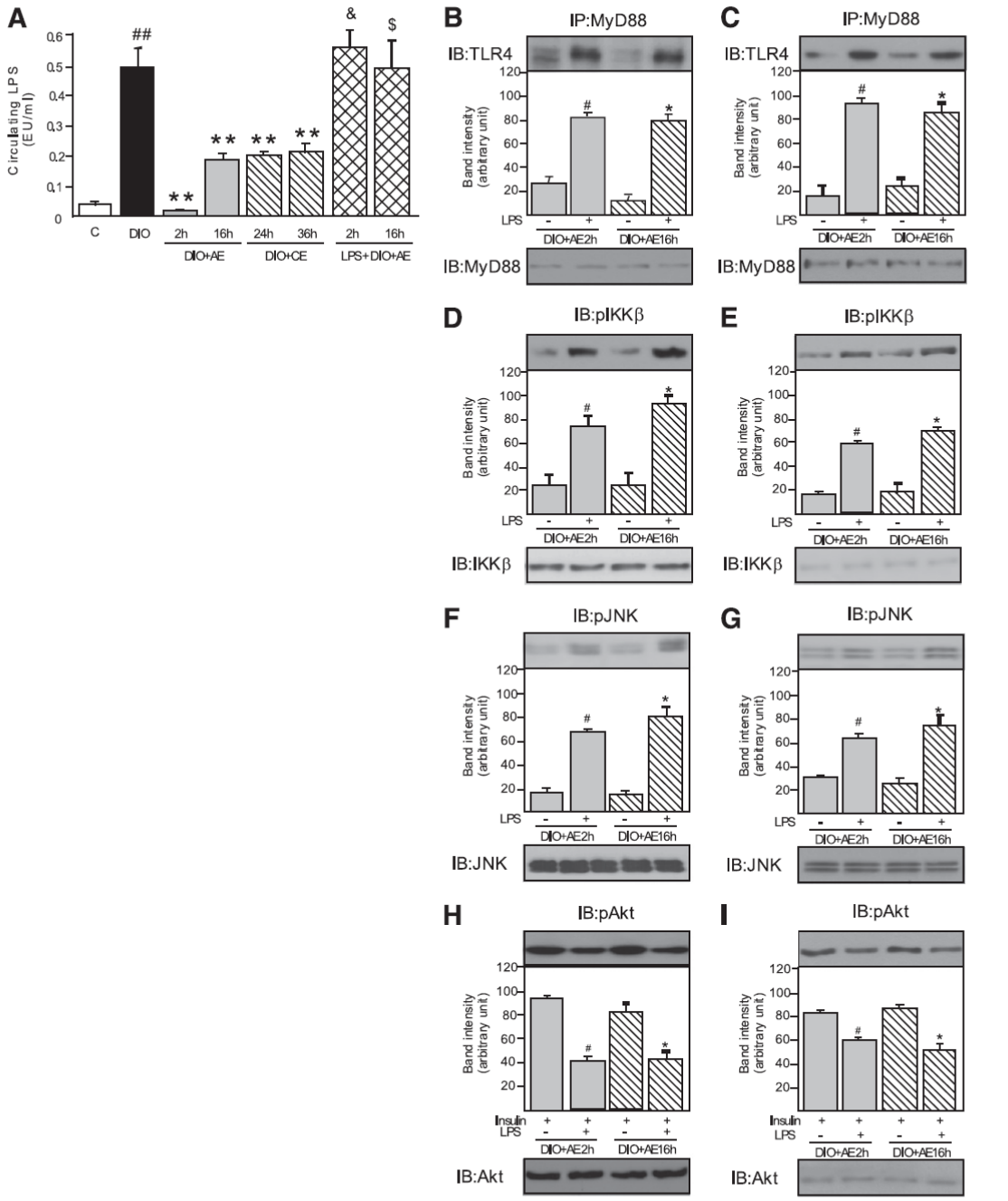


**FIG. 8. Serum LPS concentrations and influence of LPS challenge on the acute exercise effect in obese Wistar rats. *A:* Serum LPS levels in control, DIO, DIO+AE2h, DIO+AE16h, DIO+CE24h, and DIO+CE36h rats, and DIO+AE animals treated with LPS. Representative blots show the TLR4/MyD88 interaction in control, DIO, DIO+AE2h, and DIO+AE16h rats treated with saline or LPS in muscle (*B*) and adipose (*C*). Total protein expression of MyD88 (*B–C*, *bottom*). Phosphorylation of IKKβ in muscle (*D*) and adipose (*E*) of DIO+AE2h and DIO+AE16h rats with saline or LPS (*top*). Total protein expression of IKKβ (*D–E*, *bottom*). Expression of JNK phosphorylation in muscle (*F*) and adipose (*G*) of DIO+AE2h and DIO+AE16h rats treated with saline or LPS (*top*). Total protein expression of JNK (*F–G*, *bottom*). Serine phosphorylation of Akt in muscle (*H*) and adipose (*I*) in control, DIO, DIO+AE2h, and DIO+AE16h rats treated with saline or LPS (*top*). Total protein expression of Akt (*H–I*, *bottom*). Data are presented as means ± SE of 10 rats per group. #$P < 0.05$ vs. control group. ##$P < 0.001$ vs. control group. *$P < 0.05$ vs. DIO. **$P < 0.001$ vs. DIO. &$P < 0.001$ vs. DIO+AE2h. \$$P < 0.001$ vs. DIO+AE16h. IB, immunoblot; IP, immunoprecipitate.**

**Physical exercise does not improve insulin signaling in DIO rats treated with a pharmacologic inhibitor of TLR4 or in C3H/HeJ mice fed with a high-fat diet.** We next investigated whether an inhibitor of TLR4 signaling (TAK-242) could mimic the effect of exercise on the inflammatory pathway and insulin signaling in DIO rats. The administration of TAK-242 for 3 days improved inflammatory status in the muscle and adipose tissue of DIO rats, as measured by the reduction of TLR4/MyD88 interaction and phosphorylation of IKKβ and JNK, in parallel with an improvement in insulin-induced Akt phosphorylation (Supplementary Fig. 3*A*). In DIO+AE2h animals treated with this drug, no additive effect was observed in inflammatory parameters or insulin signaling (Supplementary Fig. 3*A*). In a similar fashion, C3H/HeJ mice fed a high-fat diet did not present insulin resistance, and we did not observe any increase in IKKβ and JNK phosphorylation; furthermore, exercise had no effect on these parameters or on insulin signaling (Supplementary Fig. 3*D–F*).

## DISCUSSION

Our results show that physical exercise, both acute and chronic, induces an important suppression in the TLR4 signaling pathway as evidenced by the reduction in an early step of this pathway, i.e., TLR4/MyD88 interaction, and in main downstream targets, such as IKKβ and JNK phosphorylation. These alterations are associated with a significant improvement in insulin resistance, in glucose uptake in muscle, and in the insulin signaling pathway. In DIO rats, circulating levels of LPS were increased and acute and chronic exercise reduced the circulating levels of this TLR4 ligand. Taken together, these data suggest that exercise is effective in reducing chronic low-grade inflammation, because it downregulates TLR4 ligand and an important pathway of the inflammatory response.

The increase in TLR4 protein levels in the muscle, liver, and adipose tissue of DIO rats was partially reversed after chronic exercise. This reduction in TLR4, after chronic exercise, supports findings of previous studies, in which the deletion of TLR4 in mice has been reported to prevent high-fat diet–induced insulin resistance (9,26). There was also a decrease in TLR4 activation and a parallel improvement in insulin signaling and sensitivity. Accordingly, a recent study has shown that MyD88-deficient mice are protected from high-fat diet–induced weight gain and impairment of peripheral glucose metabolism induced by palmitate (27). In line with this, our study demonstrated that a point mutation or pharmacologic blocking of TLR4 protects DIO rats from inflammation and insulin resistance, but had no additive or synergic effects to exercise in insulin signaling.

In accordance with a reduction in TLR4 activation, our data demonstrate reductions in IKKβ and JNK activities after chronic exercise in obese animals, which culminates in downregulation of both TNF-α and IL-6 mRNA. This finding is in contrast with previous studies that demonstrated an increase in IL-6 mRNA with exercise (28,29). Nevertheless, these previous studies investigated IL-6 mRNA levels during exercise, in control animals, and with high or moderate intensities, whereas our data were obtained in obese rats, at least 2 h after the exercise and with low-intensity. It is also important to mention that these cytokines, possibly through dysregulation of the TNF-α converting enzyme/tissue inhibitor of metalloproteinase 3 proteolytic system, have been shown to play an important role in subclinical inflammation in obesity/type 2 diabetes insulin resistance (30). It is well established that interventions that inhibit or attenuate IKKβ or JNK activity significantly improve peripheral insulin sensitivity (31). In this context, a previous study revealed that endurance training in obese, diabetic subjects suppresses the activation of the IKK/NFκB pathway, as proven by an increased abundance of IκB-α and IκB-β (32). Moreover, Ropelle et al. (18) have demonstrated that a single session of exercise is able to reverse obese-induced JNK activation and consequently promote increased insulin sensitivity. The suppression induced by exercise in these pathways is important because these are overactivated by obesity and can negatively regulate insulin signaling through IRS-1 $Ser^{307}$ phosphorylation (33). Moreover, the activation of the mitogen-activated protein kinase (MAPK) signaling pathway is a mechanism that may also increases IRS-1 $Ser^{307}$ phosphorylation. Our data demonstrate that physical exercise was capable of decreasing ERK1/2 phosphorylation, which may contribute to improving insulin signaling. Previous studies have observed that MAPK activity increases during exhaustive acute exercise (34–36), but the exercise protocol may contribute in explaining these differences; furthermore, the exercise-induced increase in MAPK phosphorylation rapidly decreases on cessation of exercise session and is completely restored to resting levels at 60 min after exercise (37).

Thus, the decrease in TLR4 expression/activation induced by chronic exercise is intriguing and may contribute to explain the amelioration in inflammation status and insulin sensitivity after chronic exercise. These data are in agreement with a previous hypothesis that a physically active lifestyle promotes anti-inflammatory properties (14), as proven in two longitudinal studies showing that regular training may suppress systemic low–grade inflammation (38,39).

Cani et al. (13) recently hypothesized that bacterial LPS, derived from Gram-negative bacteria residing in the gut microbiota, acts as a triggering factor, linking inflammation to high-fat diet–induced diabetes and obesity. They found that high-fat diet feeding resulted in a significant modulation of the dominant bacterial populations within the gut microflora. A reduction in the number of bifidobacteria, *Eubacterium rectal-Clostridium coccoides* group and *Bacteroides*, favoring an increase in the Gram-negative to Gram-positive ratio was observed. This modulation of gut microflora was associated with a significant increase in plasma LPS, fat mass, body weight gain, liver hepatic triglyceride accumulation, insulin resistance, and diabetes. Another study has shown that the treatment of rats with polymyxin B, an antibiotic that specifically targets Gram-negative organisms, reduced LPS expression and hepatic steatosis (40). As expected, an impressive increase in LPS serum level in DIO rats was observed. In contrast, for the first time, we showed a significant reduction in serum LPS levels in chronic-exercised obese rats compared with DIO rats. The reason for this reduction in LPS serum levels in chronic exercised obese rats is not known, but we can speculate that a reduction in gut blood flow during exercise, alterations in intestinal barrier, or reduced LPS absorption by a reduced expression of TLR4 in intestinal cells should be considered (41). On the other hand, the decrease in circulating levels of LPS could also play a role in the reduction of TLR4 mRNA and protein expression induced by chronic exercise, because it is well established that an increase in LPS is associated with

increases in TLR4 expression (42). During sepsis, with very high levels of LPS, the modulation of TLR4 mRNA and protein expression is tissue-specific (43). Our findings show that the modulation of TLR4 induced by chronic exercise was similar in the three tissues investigated and probably has a transcriptional regulation, because our data showed a parallel decrease in TLR4 mRNA and protein expression. On the other hand, acute exercise modulated TLR4 mRNA only in muscle. It may be speculated that exercise and other factors, in addition to LPS levels, may also be involved in the modulation of TLR4 expression or signaling, such as intracellular lipid levels and reactive oxygen species levels (44–46), which are increased in DIO rats and decreased after exercise. In this context, earlier studies showed that reactive oxygen species levels increase during exercise and may participate in mechanisms that increase glucose uptake during activity (36). As such, this point deserves further exploration.

Modest weight loss, achieved by diet and exercise, can enhance insulin sensitivity and even reverse insulin resistance (47,48). In our study, obese trained animals showed a slight reduction in weight, which puts in doubt whether improvement in inflammation and insulin sensitivity are consequences of exercise per se or just weight loss effects. To elucidate this, obese animals were submitted to acute exercise, which had no effect on body weight or epididymal fat pads. Our data showed that acute exercise reduced TLR4 signaling and downstream kinases, such as IKKβ and JNK, and, consequently, also reduced IRS-1 $Ser^{307}$ phosphorylation and improved insulin signaling and sensitivity, as well as induced increased muscle glucose uptake. The main difference between acute and chronic exercise was related to TLR4 protein levels, which did not change after an acute bout of exercise. However, the net effect on TLR4 signaling and insulin resistance was similar. Acute exercise also reduced circulating levels of LPS. This reduction in serum levels of LPS, after an acute bout of exercise, may be related to reduced gut blood flow (49,50). Whether reduced LPS levels may contribute to the reduction in TLR4 signaling and insulin sensitivity is not known, but when we infused a low dose of LPS in obese animals after an acute bout of exercise (to increase circulating levels close to those of obese sedentary animals), the reduction in inflammatory pathways and the improvement in insulin signaling and sensitivity were blunted.

In summary, our results show that physical exercise in DIO rats, both acute and chronic, induces an important suppression in TLR4 signaling pathway in the liver, muscle, and adipose tissue, reduces LPS serum levels, and improves insulin signaling and sensitivity. These data provide considerable progress in our understanding of the molecular events that link physical exercise to an improvement in inflammation and insulin resistance.

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No potential conflicts of interest relevant to this article were reported.

A.G.O. researched data, contributed to discussion, wrote the article, and reviewed and edited the article. B.M.C. researched data and reviewed and edited the article. N.T. researched data and reviewed and edited the article. E.R.R. reviewed and edited the article. J.R.P. researched data. R.A.B. researched data. D.G. researched data. J.B.C.C. contributed to discussion and reviewed and edited the article. M.J.A.S. contributed to discussion, wrote the article, and reviewed and edited the article.

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## ONLINE ONLY APPENDIX

**Effects of exercise on intracellular lipid levels.**

In order to investigate whether exercise induced changes in the intracellular lipid levels, we measured the triglyceride content (TG) in the muscle and liver of all groups studied with a colorimetric kit (Trig/GB; Roche Diagnostics), as described elsewhere (72). The HFD increased TG in the muscle and liver of DIO rats, when compared to the control group and after both acute and chronic exercise the TG content decreased in both tissues (online appendix Fig. S3*A* and *B*).

**Exercise and food intake.**

It is well known that exercise increases energy expenditure and induces weight loss. In contrast, we herein showed that chronic exercise induces only a slight reduction in both body weight and epididymal fat pad. Thus, we hypothesized that chronic exercise results in increased food intake. Food intake was observed to be lower in obese animals after onset of chronic exercise, for 2 days, but thereafter, the food intake rose again and overtook basal levels on the fifth day and remained similar to those of obese sedentary rats (online appendix Fig. S3*C*). Alternatively, acute exercise resulted in lower food intake in the first 12 hours, but after 24 hours, no significant difference was found between exercised and sedentary obese rats (online appendix Fig. S3*D*).

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## Supplemental Table and Figure Legends

**Suplemmental Table 1.** Sequences of primers.

**Figure S1. Effects of DIO and physical exercise on mRNA of TLR4 and cytokines.**TLR4 mRNA expression by real-time PCR in muscle (*A*), liver (*B*), and adipose tissue (*C*) of *Wistar* rats. Muscle (*D* and *G*), liver (*E* and *H*) and adipose tissue (*F* and *I*) mRNA of TNF-α and IL-6 expression by real-time PCR *Wistar* rats. Data are presented as means ± SE of 8 to 12 rats per group, #$P < 0.05$ vs. control group, ##$P < 0.001$ vs. control

group, **$P$ <0.001 vs. DIO, *$P$ <0.05 VS. DIO, &$P$<0.001 vs. DIO+AE 2h and $$P$<0.001 vs. DIO+AE 16h.

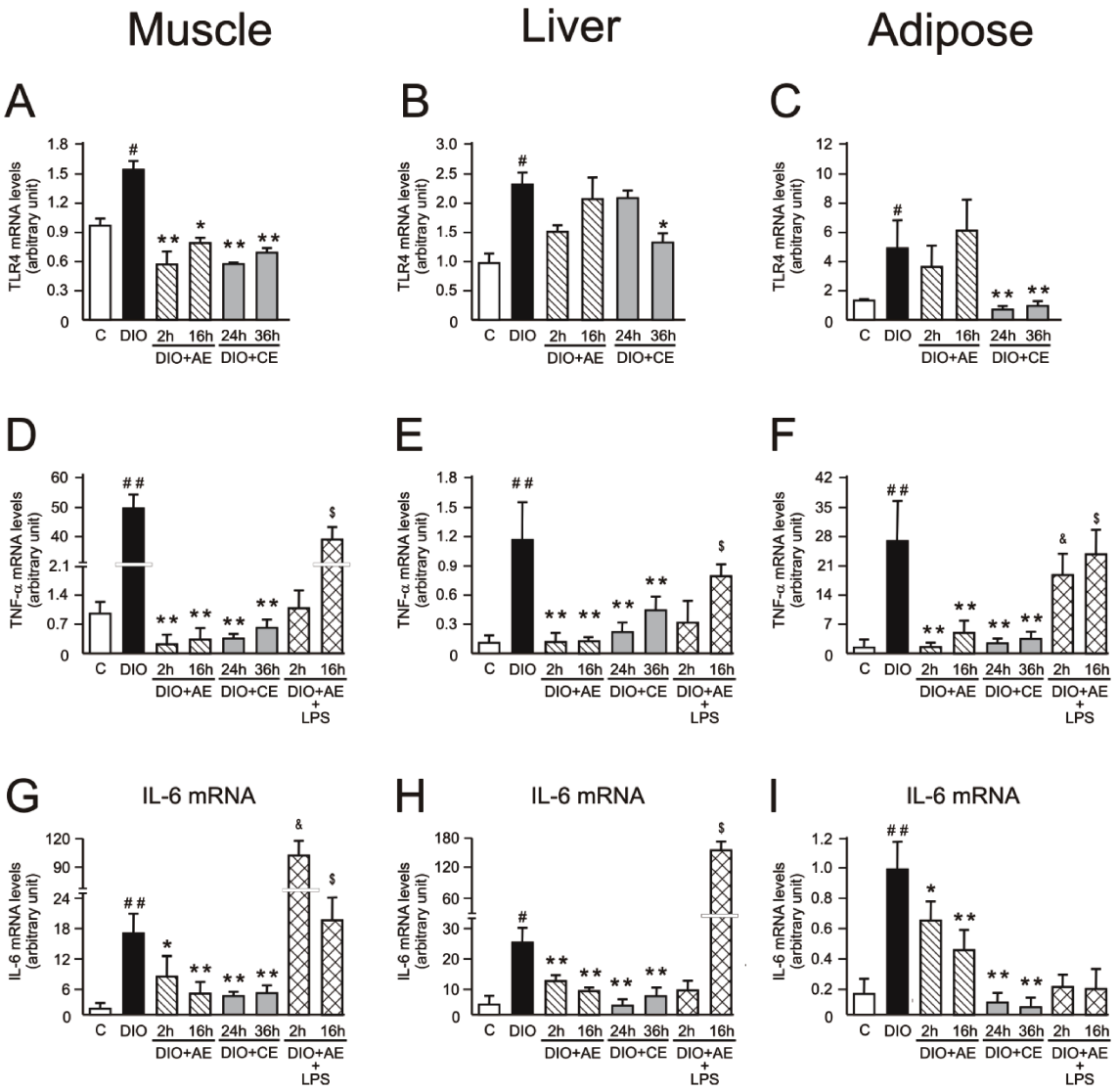
**Figure S2. Physical exercise and pharmacological inhibition of TLR4 in DIO *Wistar* rats, and acute exercise in high-fat fed C3H/HeJ.** Representative blots show the TLR4/MyD88 interaction and phosphorylation and total protein expression of IKKβ, JNK and Akt in DIO rats and DIO+AE2h rats treated with saline or TAK in muscle (*A*). Body weight (*B*), fasting plasma glucose (*C*) and serum LPS levels (*D*) in control groups, DIO groups and DIO+AE 2h groups of mice. Representative blots show the TLR4/MyD88 interaction, IKKβ, JNK and Akt phosphorylation and total protein expression in muscle (*E*) and adipose tissue (*F*) of DIO and DIO+AE 2h groups of mice. Data are presented as means ± SE of eight to ten animals per group. #$P$ < 0.05 vs. control, &$P$<0.001 vs. control, *$P$ < 0.05 vs. DIO and **$P$<0.001 vs. DIO. IB, immunoblot; IP, immunoprecipitate.

**Figure S3. Physiological parameters and effects of exercise on phosphorylation of ERK1/2 in *Wistar* rats.** Intracellular lipid levels in the muscle (*A*) and liver (*B*) of *Wistar* rats. (*C*) 24-hour food intake (kcal) in control rats, DIO rats and DIO+AE rats. (*D*) 4-week food intake (kcal) in controls, DIO rats and DIO+CE rats. Representative blots show the phosphorylation of ERK1/2 and total protein expression of βActin rats in muscle (*E*), and liver (*F*) and adipose (*G*) of *Wistar* rats. Data are presented as means ± SE of eight to ten animals per group. #$P$ < 0.05 vs. control group, &$P$<0.001 vs. control group, *$P$ <0.05 vs. DIO, and **$P$<0.001 vs. DIO.

# Supplemental Table 1

| Gene | Species | Forward primer (5'→3') | Reverse primer (5'→3') |
|---|---|---|---|
| GAPDH | Rat | CCTGCCAAGTATGATGACATCAA | AGCCCAGGATGCCCTTTAGT |
| TLR4 | Rat | CACAACTTCAGTGGCTGGATTTATC | AGCCATGCCATGCCTTGTC |
| IL-6 | Rat | AGGGAGATCTTGGAAATGAGAAA | GTGCATCATCGCTGTTCATACA |
| TNF-α | Rat | TCATCTTCTCAAAACTCGAGTGACA | TGTCTAAGTACTTGGGCAGGTTGA |

Figure S1


Muscle
Liver
Adipose
A
B
C
TLR4 mRNA levels (arbitrary unit)
D
E
F
TNF-α mRNA levels (arbitrary unit)
G
H
I
IL-6 mRNA
IL-6 mRNA levels (arbitrary unit)
C
DIO
2h
16h
24h
36h
DIO+AE
DIO+CE
DIO+AE + LPS

# Figure S2

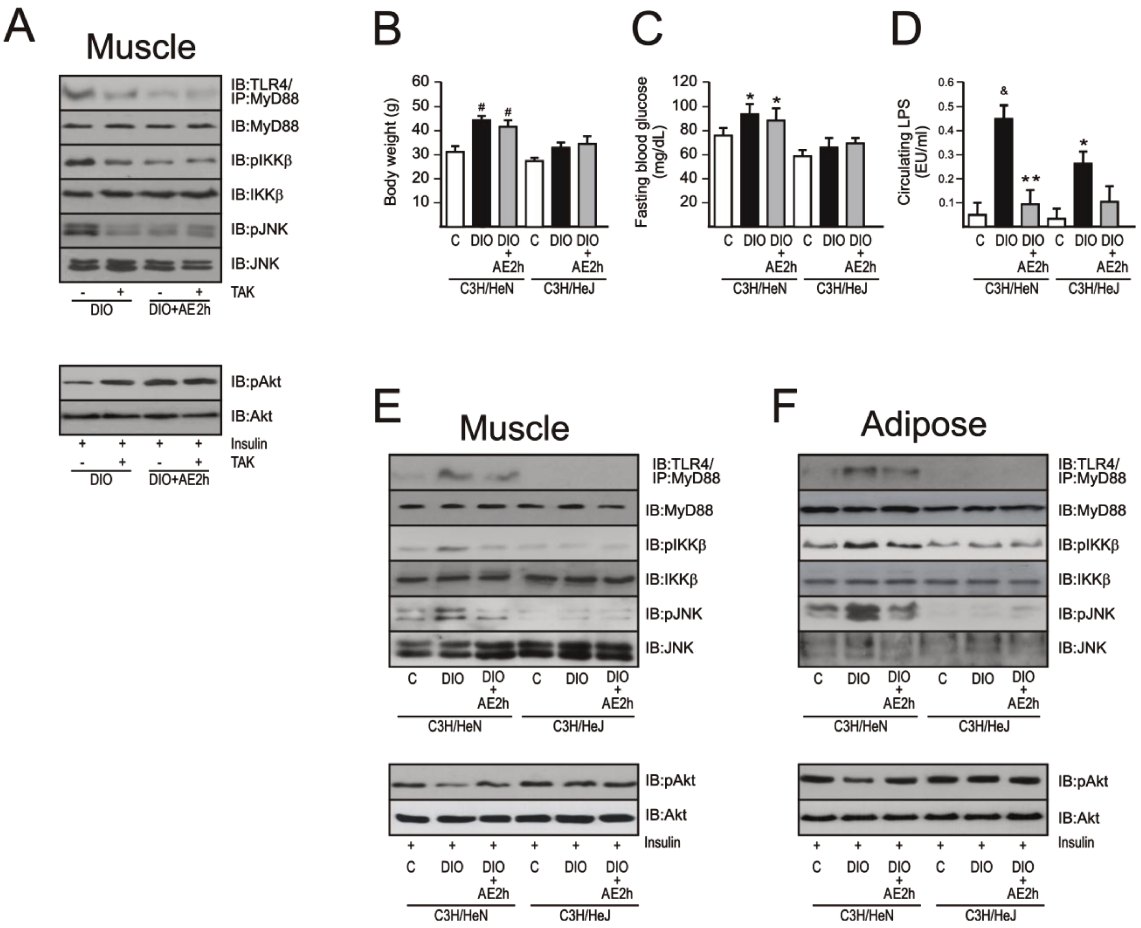
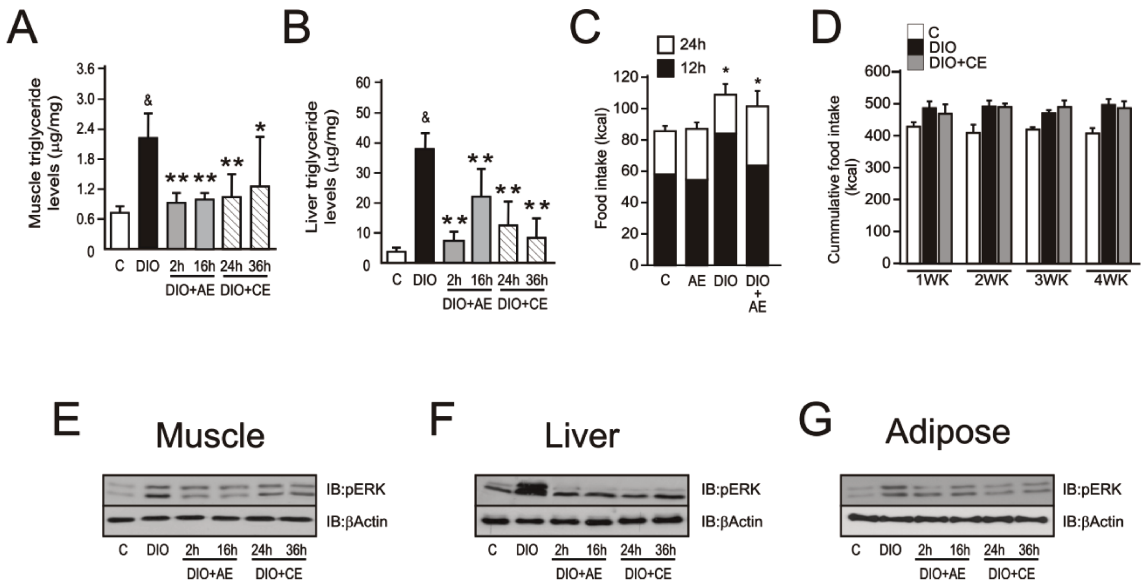

A
Muscle
IB:TLR4/ IP:MyD88
IB:MyD88
IB:pIKKβ
IB:IKKβ
IB:pJNK
IB:JNK
TAK
DIO
DIO+AE2h
IB:pAkt
IB:Akt
Insulin
B
Body weight (g)
C
Fasting blood glucose (mg/dL)
D
Circulating LPS (EU/ml)
C DIO DIO + AE2h
C3H/HeN
C3H/HeJ
E
Muscle
F
Adipose

Figure S3


A
Muscle triglyceride levels (μg/mg)
C DIO 2h 16h 24h 36h
DIO+AE DIO+CE
B
Liver triglyceride levels (μg/mg)
C DIO 2h 16h 24h 36h
DIO+AE DIO+CE
C
24h
12h
Food intake (kcal)
C AE DIO DIO + AE
D
C
DIO
DIO+CE
Cummulative food intake (kcal)
1WK 2WK 3WK 4WK


E
Muscle
IB:pERK
IB:βActin
C DIO 2h 16h 24h 36h
DIO+AE DIO+CE
F
Liver
IB:pERK
IB:βActin
C DIO 2h 16h 24h 36h
DIO+AE DIO+CE
G
Adipose
IB:pERK
IB:βActin
C DIO 2h 16h 24h 36h
DIO+AE DIO+CE

## DISCUSSÃO

Os resultados obtidos no presente trabalho mostraram que o exercício físico promove importante supressão na sinalização do TLR4 como evidenciado através da redução, primeiramente, da interação entre TLR4/MyD88 e em seguida da fosforilação de seus alvos JNK e IKKβ. Observou-se também: melhora na resistência à insulina, na captação de glicose pelo músculo esquelético e na via de sinalização à insulina. Nos animais obesos sedentários os níveis circulantes de LPS ficaram aumentados e o exercício físico foi capaz de reverter esse quadro. Em conjunto, esses dados sugerem que o exercício físico é capaz de reduzir a inflamação crônica observada na obesidade, uma vez que ele promove redução de uma das principais vias de resposta inflamatória.

O aumento dos níveis proteicos de TLR4 no músculo, fígado e tecido adiposo, observados em animais obesos foi revertido, ao menos em parte, pelo exercício crônico. Essa redução no TLR4 reforça os resultados obtidos em estudos anteriores, nos quais a deleção do TLR4 em camundongos foi capaz de prevenir a resistência à insulina induzida pela dieta hiperlipídica (20; 47).

Houve também redução na ativação do TLR4 e ao mesmo tempo melhora na sinalização e sensibilidade à insulina. Nesse sentido, estudo recente demonstrou que os camundongos deficientes da proteína adaptadora MyD88 ficavam protegidos contra o ganho de peso e resistência a insulina induzidos por tratamento com palmitato, ácido graxo com conhecidos efeitos deletérios sobre a sinalização à insulina (73). Na mesma linha, nós demonstramos que tanto a

mutação inativadora quanto o bloqueio farmacológico do TLR4 protegem respectivamente os camundongos e os ratos da inflamação e da resistência à insulina induzidos pela dieta hiperlipídica, e que o exercício físico não foi capaz de promover efeito adicional a esses tratamentos sobre a sinalização à insulina.

Mostrando relação com a diminuição da ativação de TLR4, observamos também reduções nas atividades de IKKβ e JNK, em animais obesos, após o exercício crônico, o que resultou em níveis reduzidos de RNA mensageiro (RNAm) de TNF-α e IL-6. Esses resultados são contrastantes com estudos anteriores que demonstraram aumento do RNAm de IL-6 com o exercício físico (74; 75). Contudo, esses estudos investigaram os níveis de RNAm de IL-6 durante a execução de exercício físico de intensidade elevada em animais magros, enquanto nossos dados foram obtidos em animais obesos, ao menos duas horas depois de uma sessão de exercício de baixa intensidade. É importante mencionar que essas citocinas, possivelmente através de um desequilíbrio do sistema TIMP3/TACE, desempenham papel importante na inflamação sub-clínica observada na obesidade e na resistência à insulina (76).

Já é bem estabelecido que intervenções que inibem ou mesmo atenuam a atividade de IKKβ e JNK melhoram a sensibilidade à insulina (77). Nesse contexto, estudo anterior revelou que o treinamento de endurance suprime a via em IKK/NFκB em obesos diabéticos, como evidenciado pelo aumento deIκB-α e IκB-β (78). Além disso, Ropelle et al (67) demonstraram que uma sessão de exercício físico isolada é capaz de reverter a ativação de JNK induzida pela obesidade e consequentemente promover aumento na sensibilidade à insulina. Sobretudo, a

supressão induzida pelo exercício físico nessa via inflamatória é muito importante, visto que ela fica constantemente ativada em decorrência da obesidade e pode regular negativamente a via de sinalização à insulina através da fosforilação em serina 307 do IRS-1 (18).

Além da JNK, o aumento de outras MAPK podem também promover a fosforilação em serina 307 do IRS-1. Nesse contexto, nossos resultados demonstraram que o exercício físico foi capaz de diminuir a fosforilação de ERK1/2, o que pode ter contribuído para a melhora na sensibilidade à insulina. Por outro lado, estudos mostraram que a atividade das MAPK pode ser aumentada durante o exercício físico agudo extenuante (79-81), contudo, mais uma vez a diferença no protocolo de exercício pode contribuir para explicar essas diferenças, ademais a maior fosforilação de MAPK induzida pelo exercício físico é rapidamente reduzida após o término da sessão e volta aos níveis basais dentro de aproximadamente uma hora (82).

Portanto, a redução da expressão e da ativação do TLR4 induzidos pelo exercício crônico é intrigante e pode contribuir para o entendimento da melhora na inflamação e da sensibilidade após as 8 semanas de treinamento de natação a que os animais obesos foram submetidos. O que parece estar em acordo com a hipótese previa de que o estilo de vida ativo exerce propriedades antiinflamatórias (63), como evidenciado em outros dois estudos longitudinais que mostraram que o treinamento pode suprimir a inflamação sistêmica de baixa intensidade (83; 84).

Recentemente, Cani e colaboradores (53) apresentaram a hipótese de que o LPS, proveniente das bactérias gram-negativas residentes da microbiota

intestinal, age como fator desencadeador da inflamação associada com a resistência à insulina e com a obesidade. Eles observaram que refeições ricas em gordura resultavam em modulação significativa na população bacterial dominante da microflora intestinal. Foi observado redução na quantidade de bífidobacteria, grupo *Eubacterium rectal-Clostridium coccoides*e *Bacteroides*, favorecendo assim o aumento da razão entre gram-positivas e gram-negativas. Essa modulação da flora intestinal foi associada com aumento no LPS plasmático, massa gorda, ganho de peso, acúmulo de triglicerídeos hepático, resistência à insulina e diabetes. Outro estudo demonstrou que o tratamento de ratos com polymixin d, um antibiótico especifico para bactérias gram-negativas, reduziu a expressão de LPS e a esteatose hepática (85). Como era de se esperar, encontramos grande aumento do LPS sérico nos animais do grupo DIO. E em contraste, pela primeira vez, nós observamos nesse estudo uma redução significativa nos níveis de LPS nos animais exercitados cronicamente, quando comparados aos ratos obesos sedentários. A razão para essa diminuição não é conhecida, mas nós especulamos que a redução do fluxo sanguíneo para intestino durante o exercício físico e alterações na barreira intestinal ou diminuição na absorção de LPS induzida pela atenuação da expressão de TLR4 nas células intestinais devam ser consideradas como possíveis explicações para tal resultado (86).

Por outro lado, a redução nos níveis circulantes de LPS pode ainda participar do efeito supressor do exercício crônico sobre a expressão proteica e de RNAm de TLR4, uma vez que já está bem estabelecido que aumentos de LPS promovem elevação da expressão de TLR4(87).Durante a sepse, com altos níveis

de LPS, a modulação proteica e do RNAm de TLR4 é tecido-específica (88). Nossos resultados mostraram que a modulação do TLR4, em decorrência do exercício crônico, foi similar nos três tecidos investigados (muscular, hepático e adiposo) e provavelmente apresente regulação transcricional, pois demonstramos redução no RNAm de TLR4 paralelamente à expressão de proteínas. Especula-se que o exercício físico e outros fatores, além dos níveis de LPS, bem como os lipídios intracelulares e as espécies reativas de oxigênio (89-91), que se encontram aumentadas em ratos DIO e diminuem após o exercício, possam também apresentar algum papel na modulação da expressão e/ou sinalização de TLR4. Ao contrário, foi demonstrado anteriormente que os níveis das espécies reativas de oxigênio podem aumentar durante o exercício e dessa forma participar do aumento da captação de glicose durante a atividade (81). Assim, esse ponto merece maior investigação.

Até mesmo modestas perdas de peso, alcançadas por dieta e exercício físico, podem melhorar a sensibilidade e mesmo reverter a resistência à insulina (92; 93). Nesse contexto, em nosso estudo, os animais obesos treinados apresentaram pequena redução em seu peso corporal, o que gerou duvida se a melhora na inflamação e na sensibilidade à insulina era consequente do exercício por si ou apenas um efeito da perda de peso. Para elucidar essa questão, animais obesos foram submetidos a um protocolo de exercício agudo, que não exerceu qualquer efeito sobre o peso corporal ou sobre o conteúdo de gordura epididimal.

Nossos resultados mostraram que o exercício agudo atenuava a sinalização do TLR4, como evidenciado pela redução da fosforilação de suas quinases alvo

IKKβ e JNK, que resultou em menores níveis de fosforilação em serina 307 do IRS-1 e também na melhora da sinalização e sensibilidade à insulina, bem como maior captação de glicose pelo músculo esquelético. A maior diferença entre os dois tipos de exercício estudados esteve relacionada com a expressão de TLR4, que não sofreu qualquer alteração significativa após o protocolo agudo. Todavia, os efeitos sobre a sinalização do TLR4 e resistência à insulina foram similares entre os dois protocolos utilizados.

Curiosamente, o exercício agudo também foi capaz de reduzir os níveis de LPS circulantes. É possível que essa diminuição do LPS, após uma sessão isolada de exercício físico, esteja relacionada com a redução do fluxo sanguíneo do intestino que ocorre durante o exercício físico(94; 95).Se esse LPS reduzido pode contribuir para a redução da sinalização do TLR4 não se sabe, entretanto quando injetamos uma pequena dose de LPS em animais obesos após a realização do protocolo agudo (no intuito de mimetizar os níveis encontrados nos animais obesos sedentários), a redução na via inflamatória e a melhora na sinalização e sensibilidade à insulina foram atenuadas.

Em síntese, nossos resultados mostraram que o exercício físico (agudo e crônico) em ratos com obesidade induzida por dieta hiperlipídica, promove supressão da sinalização do TLR4 em fígado, músculo e tecido adiposo, reduz os níveis séricos de LPS e melhora a sensibilidade e sinalização à insulina. Esses dados promoveram considerável progresso em nosso entendimento dos eventos moleculares que ligam o exercício físico com a melhora na inflamação e na resistência à insulina.

## CONCLUSÃO

Podemos concluir que o exercício físico é capaz reduzir os níveis circulantes de LPS e promover importante supressão da via de sinalização do TLR4 em músculo, fígado e tecido adiposo; além de melhorar a sinalização e sensibilidade à insulina em animais com obesidade induzida por dieta hiperlipídica.

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