MARIA FERNANDA DE ANDRADE FERNANDES

MODULAÇÃO DA *AMP-ACTIVATED PROTEIN KINASE* (AMPK) EM HIPOTÁLAMO DE RATOS *WISTAR* SUBMETIDOS AO EXERCÍCIO

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Dissertação de Mestrado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas, para obtenção do título de Mestre em Clínica Médica, área de concentração em Ciências Básicas.

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2-DG	2-deoxi-D-glucose
4EBP1	Eukaryotic initiation factor (eif) 4E binding protein
ACC	Acetil Coa Carboxilase
ACTH	Hormônio adrenocorticotrófico
ADO	Adenosina
AgRP	Agouti related peptide
AICAR	5-aminoimidazole-4-carboxamide riboside
AMP	Monofosfato de adenosina
AMPK	AMP-activated protein kinase
ATP	Trifosfato de adenosina
CART	Cocaine-and amphetamine–regulated transcription
CBS	Cystathionine beta synthase
DNA	Ácido desoxirribonucléico
DTT	Ditiotreitol
ECL method	Electrogenerated chemiluminescence method
EDTA	Ácido etilenodiaminotetracético
eif4E	Eukaryotic initiation factor 4e
GβL	G protein β-subunit-like protein
HPLC	High performance liquid chromatography
ICV	Intracerebroventricular
IL-6	Interleucina-6
IMC	Índice de massa corporal

kDa	Quilodalton
KH2PO4	Potassium dihydrogen phosphate
mTOR	Mammalian target of apamycin
Na2HPO4	Disodium hydrogen phosphate,
NaCl	Cloreto de sódio
NPY	Neuropeptídeo Y
OBR	Receptor de leptina
p70S6K1	p70-S6K ribosomal protein S6 kinase
PBST	Phosphate buffered saline Tween
PI(3)K	Fosfatidil inositol-3 quinase
PMSF	Phenyl methylsulphonyl fluoride
POMC	Proopiomelanocortina
PVN	Núcleo paraventricular
RAPTOR	Regulatory associated protein on mTOR
RICTOR	Rapamycin insensitive companion of mTOR
RPM	Rotações por minuto
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
SEM	Standard error of the mean
SNC	Sistema nervoso central
Thr 172	Treonina 172
TORC1	Raptor- mTOR
TORC2	Rictor- mTOR
TRIS	Tri (hidroximetil)-aminometano
α-LA	Ácido alfa lipóico

PÁG.

RESUMO

As proteínas AMPK e mTOR são as principais moduladoras do balanço energético intracelular, e exercem influência decisiva sobre a ação hipotalâmica da leptina. O exercício agudo, através da produção de IL-6, está associado ao aumento da sensibilidade à ação anorexigênica da leptina. Visando investigar a possível influência das vias AMPK e mTOR, neste aumento da sensibilidade hipotalâmica à leptina induzido pelo exercício agudo, ratos Wistar foram submetidos à natação e posteriormente receberam injeção intracerebroventricular de IL-6, ativadores e/ou inibidores da AMPK e mTOR. A IL-6 reduziu a ingestão alimentar dos animais não exercitados; no entanto, o pré-tratamento com ativador da AMPK ou inibidor da mTOR bloqueou esta ação da IL-6. Ativadores da AMPK aumentaram a ingestão alimentar de forma mais significativa nos animais não exercitados. Inibidores da AMPK reduziram a ingestão mais expressivamente nos animais exercitados. A injeção central de leptina reduziu a ingestão de ratos exercitados mais expressivamente do que foi observado nos animais controle. Tanto o pré-tratamento com inibidor da IL-6, como com ativador da AMPK ou inibidor da mTOR, reverteram esta ação da leptina. O exercício também está associado à redução da fosforilação da via da AMPK e à maior fosforilação da via da mTOR, no hipotálamo. A da resposta das vias em questão ao estímulo com leptina provavelmente seja um dos principais determinantes da modulação do set point hipotalâmico pelo exercício agudo.

ABSTRACT

AMP-activated protein kinase (AMPK) and mammalian Target of Rapamycin (mTOR) are key regulators of cellular energy balance and of the effects of leptin on food intake. Acute exercise is associated with increased sensitivity to the effects of leptin on food intake in an IL-6-dependent manner. To determine whether exercise ameliorates the AMPK and mTOR response to leptin in the hypothalamus in an IL-6-dependent manner, rats performed two 3-h exercise bouts, separated by one 45-min rest period. Intracerebroventricular IL-6 infusion reduced food intake and pretreatment with AMPK activators and mTOR inhibitors prevented IL-6-induced anorexia. Activators of AMPK increased food intake in control rats to a greater extent than that observed in exercised ones, whereas inhibitors of AMPK had the opposite effect. Exercise was associated with both reduced phosphorylation of the AMPK/ACC signaling pathway and increased phosphorylation of proteins involved in mTOR signal transduction in the hypothalamus. The regulatory role of IL-6 in mediating the modulation in AMPK and mTOR pathways in the hypothalamus was also investigated. Treatment with leptin reduced food intake in exercised rats that were pretreated with vehicle, although no increase in sensitivity to leptin-induced anorexia after pretreatment with anti-IL6 antibody, AICAR or Rapamycin was detected. Improved responses of AMPK and mTOR to leptin may contribute to the appetite suppressive actions of exercise.

1- INTRODUÇÃO

A obesidade é uma doença crônica e multifatorial, caracterizada como um estado patológico, onde há acúmulo excessivo de tecido adiposo, em função de uma série de alterações crônicas no balanço energético. (1, 2).

No que diz respeito ao estabelecimento de morbidade e mortalidade, a distribuição da gordura corporal é apontada como fator de risco ainda mais importante do que a própria obesidade. O excesso de gordura depositado na região abdominal é o principal responsável pelo estabelecimento da síndrome metabólica; aumentando a associação entre obesidade e doenças cardiovasculares, diabetes tipo 2, câncer, hipertensão, dislipidemias, esteatose hepática não alcoólica, entre outros. (3, 4).

Estima-se que existam 100 milhões de obesos no mundo. Nos Estados Unidos, aproximadamente 21% da população é classificada como obesa. Na região sudeste do Brasil, este índice é de 6,7%. Diante disso, o aumento excessivo do peso corporal decorrente do acúmulo de tecido adiposo, constitui uma das mais relevantes questões de saúde pública da atualidade (5).

Balanço energético

Os fatores envolvidos no balanço energético são: a ingestão calórica e o gasto energético (6). O primeiro, refere-se a todo alimento ingerido que posteriormente, será metabolizado pelo organismo. Já o gasto energético é a somatória do gasto metabólico basal, do efeito térmico da dieta (produção de calor a partir da metabolização dos alimentos) e da atividade física (7).

Em condições fisiológicas, o balanço energético é mantido através do equilíbrio entre a ingestão calórica e o gasto energético. No entanto, quando o desequilíbrio entre estas variáveis persiste por longo período de tempo, o balanço energético pode ser positivo (ingestão calórica maior do que o gasto energético), o que favorece o estabelecimento da obesidade; ou negativo (ingestão calórica menor que o gasto energético), induzindo redução dos estoques energéticos periféricos e, conseqüentemente, redução do peso corporal. (1, 8). Segundo Jéquier, se a ingestão calórica excede em 5% o gasto energético durante longo período de tempo, ocorrerá um aumento aproximado de 5 quilogramas na massa adiposa

daquele indivíduo, o que contribuíra de forma decisiva para o estabelecimento da obesidade (1). Além disso, há estudos evidenciando que o estilo de vida sedentário aumenta a prevalência da obesidade (9), já que foi comprovada a relação inversa entre IMC e atividade física. (10).

A proporção com que a ingestão alimentar e o gasto energético contribuem para a homeostasia do peso corporal varia entre os indivíduos, por serem grandemente influenciadas por componentes genéticos (11). De acordo com as concepções atuais sobre a homeostasia do peso corporal, os indivíduos nascem com sua composição corporal geneticamente determinada. Assim, a relação entre consumo e gasto energético é modulada no sentido de manter e estabilizar os estoques energéticos daquele indivíduo no patamar determinado pelo *set point.* Sempre que esta estabilidade é afetada, uma série de mecanismos fisiológicos atua sobre o balanço energético, visando restabelecer a homeostasia das reservas energéticas (11).

Em 1990, Bouchard e colaboradores desenvolveram um estudo, cujo objetivo principal era avaliar a influência genética no controle do peso corporal. Neste estudo, 12 pares de gêmeos monozigóticos foram submetidos à dieta hipercalórica (1000 Kcal por dia), durante período aproximado de 3 meses. No decorrer deste período, alterações significativas na composição e distribuição da gordura corporal destes indivíduos foram observadas. O aumento do peso corporal entre os pares de gêmeos variou entre 4,3 e 13,3 quilogramas. No entanto, entre os irmãos monozigóticos, não houve variações significativas no aumento do peso corporal, na distribuição do tecido adiposo e na massa adiposa ($p<0,05$). Diante disso, a explicação mais provável para a resistência ao aumento do peso corporal e também para a diferença na distribuição da massa adiposa, observados entre os pares de gêmeos, seria a atuação significativa dos fatores genéticos na determinação do peso e da composição corporal. Estes fatores genéticos são os responsáveis pela determinação do *set point* e, conseqüentemente, pela modulação do gasto energético e da ingestão alimentar. (12).

Entretanto, outros fatores ambientais também contribuem para a regulação do peso corporal, de forma que a intervenção dietética, através do emprego de uma dieta balanceada, é estratégia fundamental em programas de redução do peso corporal. No

entanto, tal estratégia só se mostra realmente efetiva quando associada ao exercício físico (11).

A prática de atividade física influencia decisivamente tanto no tratamento, como na prevenção da obesidade, apesar de corresponde a uma porção relativamente pequena (aproximadamente 15%) do gasto energético total de um indivíduo adulto. (13).

Assim, a prática regular de exercícios sido associada a inúmeros benefícios, como redução da adiposidade, redução da pressão arterial (14), redução da resistência periférica à insulina, controle do diabetes tipo 2. (15) e homeostasia do peso corporal. (16, 17).

O SNC é o principal órgão responsável pelo balanço energético

Atualmente, o hipotálamo é reconhecido como a principal estrutura anatômica do SNC, envolvida no controle da ingestão alimentar. Os núcleos hipotalâmicos arqueado e paraventricular possuem como função integrar as informações periféricas acerca dos estoques energéticos à modulação da ingestão alimentar (18).

Estudos realizados na década de 40 demonstraram que lesões no núcleo hipotalâmico ventromedial de roedores induziam hiperfagia e obesidade; enquanto estímulos no núcleo hipotalâmico lateral induziriam anorexia (19). **(Figura 1)**

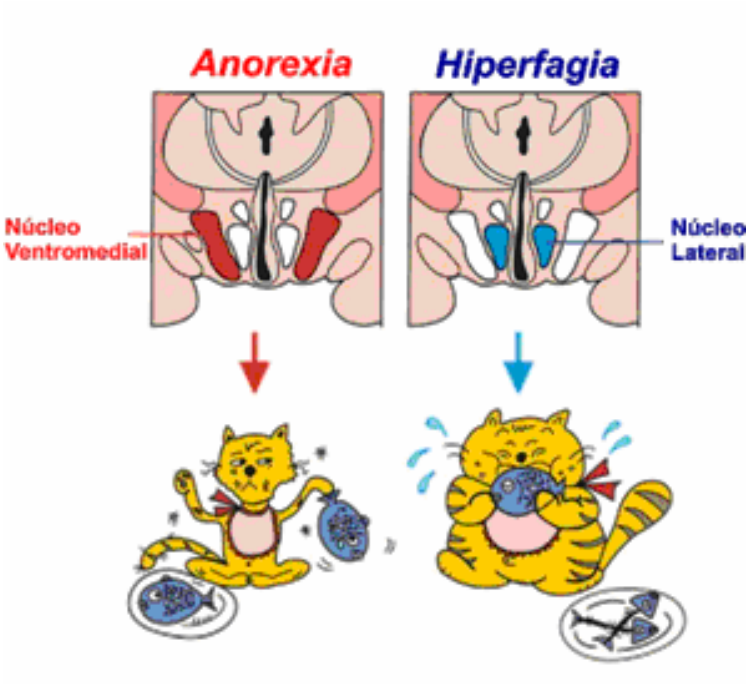

Figura 1- Repercussão de lesões nos núcleos ventromedial e lateral (hipotálamo)

De forma complementar, um estudo subseqüente ressaltou a importância do tecido adiposo e do hipotálamo no controle do peso corporal. Neste estudo, dois ratos (rato I e rato II) foram submetidos à cirurgia de *parabiose* (conexão entre os sistemas circulatórios destes animais). Além disso, o rato I teve o hipotálamo ventromedial lesionado, o que desencadeou um aumento na adiposidade deste animal. No entanto, após parabiose com o rato I (obeso), o animal II, cujo hipotálamo se manteve intacto, reduziu significativamente seu consumo alimentar. Diante disso, os autores concluíram que o rato I, era portador de um "fator de saciedade" circulante, responsável pela redução da ingestão alimentar observada no rato II. Este fator encontrava-se inoperante no rato I, devido à lesão do hipotálamo ventromedial. (20, 21).

Buscando uma melhor compreensão dos mecanismos envolvidos na modulação central da ingestão alimentar, surgiu, na década de 50, a *hipótese lipostática*, segundo a qual, a quantidade de gordura corporal teria correlação negativa com a ingestão alimentar (21).

A despeito destas evidências, durante algum tempo, o tecido adiposo continuou sendo compreendido como um simples "depósito" de armazenamento da gordura corporal. O seu reconhecimento como órgão endócrino dotado de atividade metabólica, ocorreu somente na década de 90, após a identificação de uma série de moléculas sinalizadoras – as adipoquinas - sintetizadas nas células adiposas. (22, 23), incluindo a leptina.

Hormônios e peptídeos que informam ao SNC a quantidade dos estoques energéticos

Leptina

Os camundongos *ob/ob* e *db/db* (modelos animais de síndromes de obesidade geneticamente determinada) apresentam fenótipos idênticos, pesam três vezes mais e sua massa adiposa é cinco vezes maior do que a massa adiposa dos camundongos controle (ainda que submetidos a dieta isocalórica), além de serem diabéticos. Os primeiros estudos informativos sobre os defeitos primários destas linhagens foram obtidos através dos experimentos de parabiose **(Figura 2).** Através da parabiose, os componentes periféricos dos sistemas circulatórios de camundongos de linhagens obesas (*ob/ob* e *db/db*) eram conectados aos de camundongos controle (23). Os resultados demonstraram que:

1) Parabiose entre camundongos obesos (*ob/ob)* e controle: ocorreu normalização do peso corporal dos camundongos *ob/ob;*

2) Parabiose entre camundongos diabéticos (*db/db)* e controle: não ocorreu alteração do peso corporal dos camundongos *db/db;*

3) Parabiose entre camundongos obesos (*ob/ob)* e diabéticos (*db/db)*: ocorreu redução do peso corporal dos camundongos o*b/ob.*

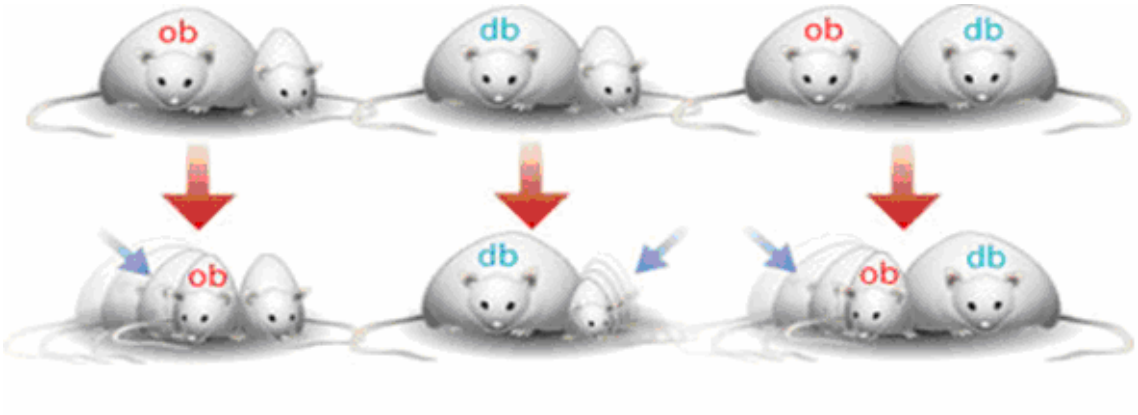

Figura 2- Parabiose entre camundongos ob/ob e db/db

Diante isso, Coleman concluiu que a obesidade desenvolvida pelos camundongos *ob/ob* provavelmente decorria da ausência de um fator sinalizador circulante, que foi reposto após parabiose com os camundongos controle e com a linhagem diabética db/db. Esses resultados também indicaram que a obesidade desenvolvida pelos camundongos *db/db*, provavelmente era decorrente de uma alteração na capacidade de responder a este fator sinalizador, produzido pelo gene *ob*, e que induzia saciedade (23).

As conclusões obtidas por Coleman não foram muito valorizadas na época. Sua confirmação só foi possível após a clonagem e seqüenciamento do produto do gene *ob*: a leptina, por Zhang e colaboradores (24).

A proteína do gene *ob* está presente no plasma de camundongos normais, como um monômero com peso molecular de 16 kDa. Esta proteína não foi detectada em plasma de camundongos *ob/ob*, enquanto foi observada em concentrações elevadas em camundongos *db/db* (24). A administração de leptina em camundongos *ob/ob* reduziu a ingestão alimentar, o peso corporal e também os níveis glicêmicos; além de estimular a atividade simpática do tecido adiposo marrom e, conseqüentemente, aumento o gasto energético. A administração de leptina em camundongos db/db, por sua vez, não induziu qualquer alteração fisiológica. (25).

Os níveis séricos de leptina correlacionam-se de forma positiva com o IMC na

maioria das populações estudadas. A secreção desse hormônio diminui após o jejum prolongado e também frente a estímulos □-adrenérgicos. Por outro lado, tanto a insulina como os glicocorticóides, aumentam a sensibilidade à esta citoquina. A leptina é secretada de forma pulsátil e inversamente relacionada à atividade do eixo ACTH-Cortisol, ou seja, sua secreção apresenta-se reduzida ao amanhecer, sendo estimulada no final da tarde (26).

Seguramente, uma das funções mais claras da leptina é ser uma aferência para o SNC, dentro de uma alça de retroalimentação negativa, responsável por informar ao cérebro sobre os estoques periféricos de energia. Uma vez produzida, a leptina entra na circulação sangüínea e é transportada até os órgãos alvo acoplada a seu receptor (Ob-R). O primeiro estudo a respeito de mutações no gene *ob* foi realizado em 1997 (27) Neste estudo, foram identificados dois primos de origem paquistanesa, homozigóticos para uma mutação no gene *ob*. Esta mutação era responsável pelo fenótipo obeso dos gêmeos (28). Estes indivíduos apresentavam níveis reduzidos de leptina circulante, a despeito da obesidade. Seu peso corporal era considerado normal ao nascimento, porém precocemente se estabeleceu um quadro de obesidade severa em função da hiperfagia característica destes gêmeos. Este estudo ainda destacou o papel fundamental desempenhado pela leptina sobre o sistema imunológico. Todos os indivíduos desta família cujo peso corporal era considerado normal estavam vivos, enquanto sete dos onze indivíduos obesos morreram na infância. Assim como os indivíduos obesos estudados, estes sete indivíduos provavelmente eram leptino-deficientes (29). No entanto, a deficiência de leptina por mutação do gene *ob* é rara na espécie humana (30). A maioria dos indivíduos obesos apresenta níveis elevados de leptina, embora não respondam adequadamente a este fator de sinalização (30, 31) sugerindo que a maior parte dos indivíduos obesos apresenta resistência à leptina. Esta resistência à leptina, por sua vez, pode ser atribuída a uma série de fatores**.** Alguns estudos apontam como principal responsável por este estado, uma deficiência no transporte desta citoquina (32, 33). Entretanto, a etiologia do defeito neste mecanismo de transporte não está bem definida. Podendo ser decorrente de uma saturação ou ainda de algum defeito intrínseco dos transportadores (34). A leptino-resistência também pode ser atribuída a algum defeito na sinalização "pós-receptor de leptina", o que levaria a uma falha na ativação dos mediadores neuroendócrinos envolvidos na regulação do peso corporal (35).

Conforme anteriormente mencionado, o hipotálamo parece ser o principal mediador dos efeitos da leptina sobre o peso corporal e a ingestão alimentar. Mais especificamente, a região ventrobasal do hipotálamo (núcleos arqueado, ventromedial e dorsomedial) possuem ObR em abundância (36). Além disso, a expressão da proteína Fos (um marcador da atividade neuronal) encontra-se aumentada nestas mesmas regiões após administração da leptina (37). De fato, os núcleos: arqueado, ventromedial e dorsomedial são considerados como sítios hipotalâmicos implicados na regulação do comportamento alimentar. Estes núcleos interagem entre si e, assim, enviam aferências para o sistema nervoso autonômico e para regiões corticais, modulando conseqüentemente a ingestão alimentar e o peso corporal (38).

A leptina, após interagir com ObR, atua sobre alguns peptídeos produzidos em neurônios do núcleo arqueado: NPY, AGRP, POMC e CART **(Figura 3)** A leptina suprime a atividade dos neurônios orexigênicos que produzem NPY/AGRP, enquanto exerce ação estimulatória sobre a atividade de neurônios anorexigênicos, responsáveis pela produção da POMC e CART (39).

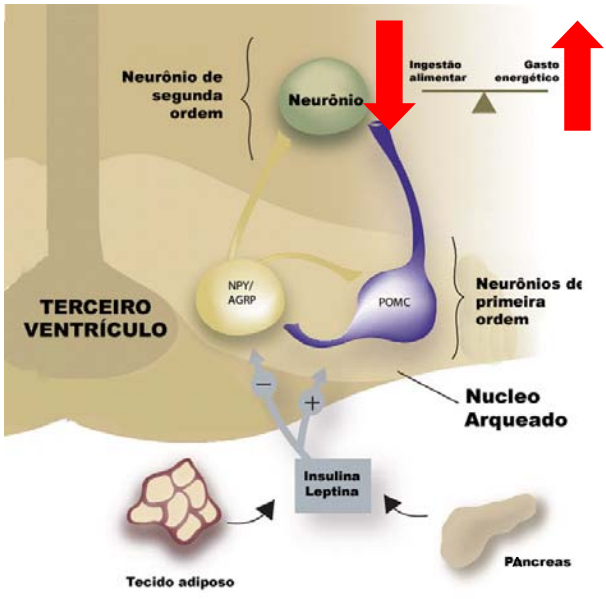

Figura 3- Modulação do Balanço Energético

Além de atuar como "fator de saciedade", a leptina possui diversas funções, como promover a oxidação dos ácidos graxos na musculatura esquelética (40). Minokoshi e colaboradores demonstraram que a mobilização dos estoques de gordura periféricos frente à leptina, assim como a ação anorexigênica deste peptídeo são mecanismos mediados pela enzima AMPK. Atualmente, a AMPK é considerada a principal enzima envolvida no controle da homeostasia energética intracelular (41).

Insulina

Assim como a leptina, a insulina também é considerada um hormônio que sinaliza ao hipotálamo o estoque de tecido adiposo e modula a ingestão alimentar (42-45).A insulina circula em níveis proporcionais ao conteúdo de tecido adiposo e atravessa a barreira hematoencefálica via um sistema de transporte saturável em níveis proporcionais aos plasmáticos (46). Os receptores de insulina são expressos por neurônios envolvidos na ingestão alimentar (47-49).A administração de insulina no sistema nervoso central reduz a ingestão alimentar e diminui o peso corporal, enquanto a deficiência desse hormônio causa hiperfagia (50).

A correlação dos níveis séricos de insulina com o conteúdo de gordura corporal é conseqüência da resistência à insulina induzida pelo aumento da gordura corporal (51). Assim, à medida que o peso corporal aumenta a insulina deve aumentar para compensar a resistência à insulina e manter a homeostasia de glicose (52, 53). A falência da célula β em alcançar este aumento adaptativo causa hiperglicemia, e provavelmente contribui para a associação entre diabetes tipo 2 e obesidade. Acredita-se que o aumento progressivo da secreção de insulina que ocorre durante o desenvolvimento da obesidade atue como uma alça de retroalimentação limitando o acúmulo de tecido adiposo.

Uma situação que merece destaque é o diabetes descompensado, onde a ingestão alimentar aumenta marcadamente (54), mas a quantidade de gordura corporal está baixa, bem como os níveis plasmáticos de leptina (55, 56). Como tanto os níveis séricos de insulina como o de leptina estão baixos nesta situação, a "hiperfagia diabética" poderia ser resultado da diminuição da sinalização da insulina, da leptina ou de ambas. Um estudo recente esclareceu este assunto mostrando que a reposição isolada de leptina (mas não a de

insulina) é capaz de prevenir a "hiperfagia diabética", indicando que a leptina realmente é mais potente que a insulina no controle central da homeostasia energética. Apesar disso, recentemente, foi descrito que a inativação do receptor de insulina apenas no sistema nervoso central de camundongos resulta em aumento da ingestão alimentar e obesidade leve, aumento dos níveis plasmáticos de leptina, aumento da secreção de insulina, bem como resistência à insulina leve, demonstrando a necessidade da integridade da via de sinalização da insulina cerebral para o controle do peso corporal (57).

Adiponectina

A adiponectina é uma citoquina secretada pelos adipócitos, que foi caracterizada em 1995 e 1996 por grupos diferentes (58-60).Sua concentração sérica é inversamente proporcional à massa adiposa, índice de massa corporal, insulinemia e triacilglicerol plasmático (61).

A adiponectina estimula a atividade da AMPK tanto na periferia como no SNC. Assim. nos tecidos periféricos, a ação desta citoquina estimula a oxidação dos ácidos graxos e aumenta a sensibilidade à insulina, enquanto sua ação hipotalâmica (núcleo arqueado) estimula a ingestão alimentar e reduz o metabolismo energético. De acordo com estes dados, é importante ressaltar que camundongos que não possuem adiponectina são resistentes à ativação hipotalâmica da AMPK e, conseqüentemente são hipofágicos, com elevado gasto energético, além de apresentarem resistência à obesidade, quando expostos à dieta hipercalórica (62).

Grelina

A grelina é um hormônio de crescimento, expresso principalmente pelas células da mucosa gástrica, conhecido por sua ação orexigênica: este hormônio estimula os neuropeptídeos NPY e AgRP (núcleo arqueado)(63).

Estudos demonstraram que tanto a administração intraperitoneal (64), como a administração icv (65) de grelina, estimulam a atividade hipotalâmica da AMPK, sugerindo esta quinase como mediadora da ação central da grelina (66).

Além disso, este hormônio desempenha importantes funções nos tecidos periféricos. A grelina inibe a ativação da AMPK tanto no fígado como no tecido adiposo, sugerindo que ação deste hormônio sobre a gliconeogênese e sobre a adiposidade, também depende da enzima AMPK (67, 68).

IL-6

A concentração plasmática de IL-6 aumenta consideravelmente em função do exercício físico (69-72). Além disso, parece que a produção desta citoquina pelo músculo esquelético em contração, constitui a principal fonte de IL-6 durante o exercício físico (73, 74). O estímulo para produção de IL-6 no músculo esquelético são a contração muscular *per se* e ainda, a redução dos estoques de glicogênio (75, 76). Esta IL-6 produzida pelo músculo em contração, em parte é responsável pela produção hepática de glicose, importante para manter a glicemia durante o exercício (72).

Em condições normais, os níveis de IL-6 encontram-se reduzidos no SNC. No entanto, a ocorrência de inflamações, hipóxia ou alguns estados patológicos, induz aumento significativo nos níveis de IL-6. Neste caso, a IL-6 é produzida pelos astrócitos (SNC) (77).

Esta citoquina também pode ser sintetizada e secretada pelos núcleos hipotalâmicos, em situação de stress (78, 79) e durante/após o exercício (80). Nybo e colaboradores observaram um aumento na produção de IL-6 após sessão aguda de exercício (longa duração), em humanos (80). Além disso, há indícios de que ao atuar no SNC, a IL-6 participa do controle da ingestão alimentar, gasto energético e composição corporal. Desta forma, camundongos que não expressam IL-6 tornam-se hiperfágicos e, conseqüentemente, obesos. Este mesmo estudo destaca que a administração de IL-6 (icv) exerce uma ação "anti-obesidade", ao aumentar o consumo de oxigênio em roedores (81). Este aumento no consumo de oxigênio persistiu por 3 horas, após a injeção icv de IL-6 (81). Da mesma forma, o aumento na concentração desta citoquina no sistema nervoso central, em função de uma sessão de exercício prolongado, provavelmente contribua para a manutenção do aumento no consumo de oxigênio, que persiste por algumas horas após finalizado o exercício (82, 83).

Com isso, parece que alguns estudos tendem a sugerir a IL-6 (produzida no SNC) como uma das mediadoras dos efeitos que o exercício físico exerce sobre o balanço energético (84).

Vias de sinalização intracelular

PI(3)K

A sinalização intracelular da insulina em tecidos insulino-sensíveis inicia-se com a ligação do hormônio a um receptor específico de membrana, uma proteína heterotetramérica com atividade quinase, composta por duas subunidades α e duas subunidades β. A ligação da insulina à subunidade α estimula a autofosforilação da região intracelular da subunidade β do receptor. Uma vez ativado, o receptor de insulina fosforila vários substratos protéicos em tirosina incluindo membros da família dos substratos dos receptores de insulina (IRS-1/2/3/4), Shc, Gab-1 e Cbl (**Figura 4**). A fosforilação em tirosina das proteínas IRS cria sítios de reconhecimento para moléculas contendo domínios com homologia à Src 2 (SH2). Dentre estas, destaca-se a PI(3)K. As funções fisiológicas do IRS-1/2 foram recentemente estabelecidas através da produção de camundongos sem os genes que codificam o IRS-1 ou IRS-2 (*knock-out* de IRS-1 e IRS-2). O camundongo que não expressa IRS-1 apresenta resistência à insulina e retardo de crescimento, mas não é diabético (85). Foi demonstrado que o IRS-2 poderia compensar parcialmente a ausência de IRS-1, o que explicaria o fenótipo de resistência à insulina sem hiperglicemia do camundongo *knock-out* de IRS-1. O camundongo que não expressa o IRS-2 foi posteriormente gerado (86) e mostrou um fenótipo diferente do camundongo sem IRS-1: hiperglicemia acentuada devido a diversas anormalidades na ação da insulina nos tecidos periféricos associada à falência da atividade secretória das células β. Esta última alteração é, provavelmente, conseqüência da redução significativa da massa de células β pancreáticas.

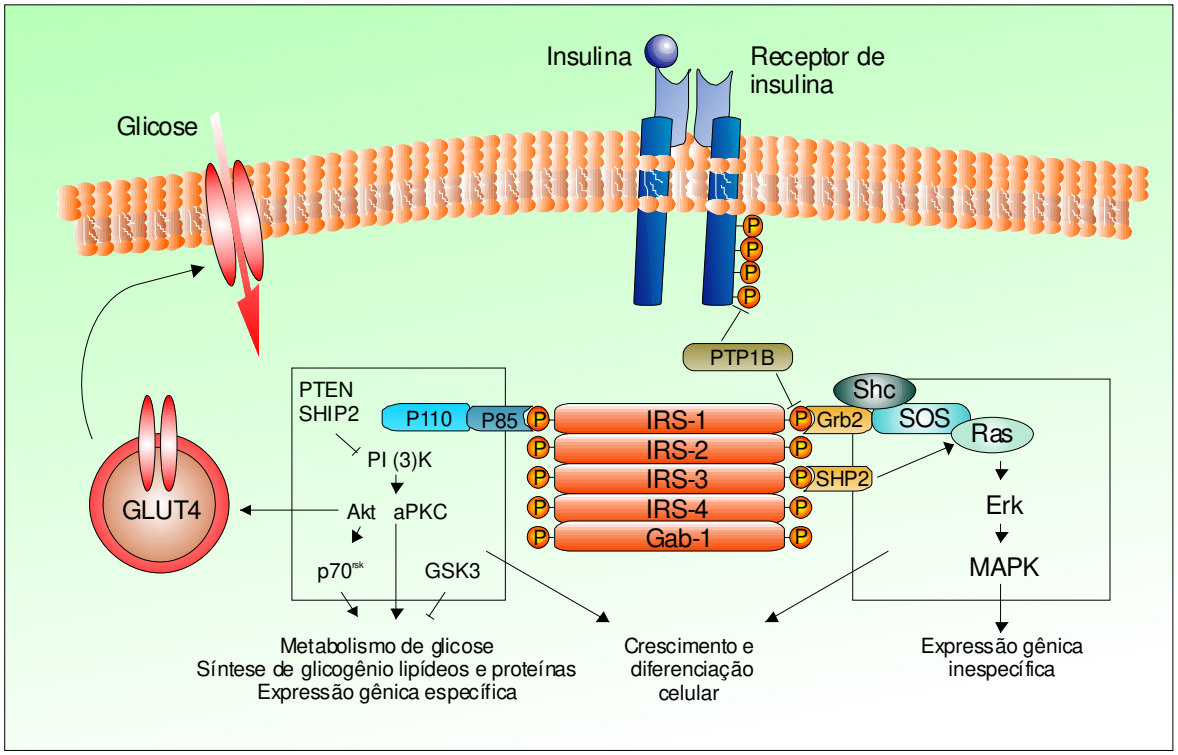

Figura 4- Transmissão do Sinal da Insulina

A fosforilação das proteínas IRSs cria sítios de ligação para a PI(3)K, promovendo a sua ativação. Atualmente, a PI(3)K é a única molécula intracelular inequivocamente considerada essencial para o transporte de glicose (87). As proteínas alvo conhecidas dessa enzima são a Akt e as isoformas atípicas da PKC (□ e λ), porém a função dessas proteínas no transporte de glicose ainda não está bem estabelecida (88-92).

JAK/STAT

A identificação de receptores específicos para leptina em plexo coróide de ratos levou a uma melhor compreensão de como acontece a sinalização da leptina no sistema nervoso central e motivou o desenvolvimento de estudos visando o esclarecimento dos mecanismos envolvidos na gênese da resistência à leptina. O receptor de leptina (OBR) é membro da família gp130 da classe I dos receptores de citocinas (93). É encontrado em muitos tecidos com várias formas de *splicing*, sendo as mais encontradas a forma curta

(OBR_S), expressa em vários tecidos, que apresenta domínios intracelulares truncados, e a forma longa (OBR_L), que apresenta domínios intracelulares longos e é expressa principalmente no hipotálamo (núcleos paraventricular, arqueado, ventromedial e dorsomedial (94-96).

O OBR_S não tem sua função bem definida, mas parece influenciar no transporte da leptina através da barreira hematoencefálica e talvez contribua para a depuração da leptina atuando como uma fonte de receptor solúvel (96)

A homologia do receptor de leptina à classe I dos receptores de citocinas forneceu informações importantes para a descoberta dos possíveis mediadores intracelulares da ação da leptina. Os receptores da classe I das citocinas agem através das famílias das proteínas JAK (*Janus Kinase*) e STAT (*Signal Transducers Activators of Transcription*) (97). Tipicamente, as proteínas JAK estão constitutivamente associadas com seqüências de aminoácidos dos receptores, e adquirem sua atividade tirosina quinase após a ligação do hormônio a seu receptor. Uma vez ativada, a proteína JAK fosforila o receptor induzindo à formação de um sítio de ligação para as proteínas STAT, as quais são ativadas após terem se associado ao receptor e serem fosforiladas pela JAK. As proteínas STAT ativadas são translocadas para o núcleo e estimulam a transcrição.

O OBR_L é capaz de estimular as proteínas STAT em resposta à sua ativação. Dois estudos (97) mostraram que a leptina ativa o STAT3 e o STAT5 em células COS transfectadas com o OBR_L, mas discordaram em relação à atividade do STAT1 e do STAT6. A proteína da família STAT mais importante para a regulação do peso corporal ainda não foi identificada. No entanto é pouco provável que tanto o STAT1 como o STAT6 estejam envolvidos de forma significativa, uma vez que a ausência de expressão desses genes em camundongos *knock-out* não resultou em obesidade (98-101). Embora o OBR_L seja capaz de ativar as proteínas STAT3 e STAT5 em células COS, as proteínas do STAT que são realmente ativadas *in vivo* podem diferir das que são observadas nestas linhagens celulares (102). Apenas a ativação do STAT3 foi detectada no hipotálamo de camundongos, após a administração exógena de leptina (103).

O receptor de leptina é capaz de estimular outras vias de sinalização além da JAK/STAT, tais como a via da proteína quinase ativadora de mitose (MAPK) e a via de fosfatidilinositol 3-quinase (PI(3)K), e é possível que a capacidade do OBR controlar o peso dependa também destas vias de sinalização (102, 104). Além disso, a leptina leva também à fosforilação do SHP2 (105), uma fosfotirosina fosfatase, que diminui o grau de fosforilação da JAK2 e conseqüentemente a ativação do STAT3. Uma outra proteína, SOCS3, quando ativada, diminui a resposta à leptina (106).

AMPK

As pesquisas sobre a AMPK iniciaram em 1994, após a identificação de sua estrutura trimérica (107, 108), e a primeira clonagem do DNA responsável pela codificação da subunidade catalítica da molécula (109).

Atualmente, a AMPK é reconhecida como um complexo heterotrimérico, constituído por uma subunidade catalítica α e pelas subunidades regulatórias β e γ **(Figura 5)**

- A subunidade α possui um domínio serina/treonina quinase em sua região N-terminal, onde o resíduo Thr172, constitui o sítio de fosforilação e ativação da molécula (Hawley *et al.* 1996). A região C-terminal da subunidade α é responsável pela formação de um complexo entre esta subunidade e as subunidades regulatórias β e γ (110)

- A subunidade β apresenta uma região C-terminal, envolvida na formação do complexo entre esta subunidade e as subunidades α e γ; além do domínio N-isoamilase, que atua como sítio de ligação para o glicogênio (111, 112).

- A subunidade γ apresenta em sua estrutura vários domínios CBS (113). Estes domínios CBS se pareiam para formar dois módulos conhecidos por "domínios Bateman" (114). Os "domínios Bateman" são responsáveis pela formação do sítio de ligação para a molécula de AMP ou para a molécula de ATP, embora sua afinidade por este último seja menor do que sua afinidade pelo AMP (115).

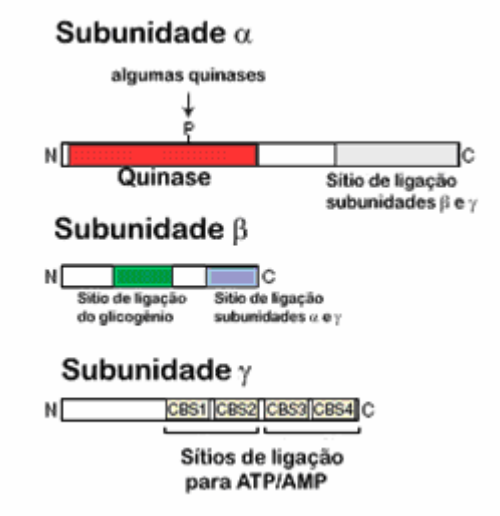

Figura 5- Estrutura Molecular da AMPK

A ativação da AMPK pelo AMP pode ocorrer por três mecanismos (116):

1. Ativação alostérica

2. Fosforilação do resíduo Thr172 por alguma quinase

3. Inibição da desfosforilação do resíduo Thr172 por ataques de proteínas fosfatases

A AMPK atua como um sensor energético intracelular: após sua fosforilação/ativação, esta quinase estimula a atividade de vias catabólicas (que produzem ATP), enquanto silencia a atividade das vias anabólicas (que consomem o ATP), com o objetivo de manter a homeostasia dos estoques energéticos intracelulares (116) **(Figura 6).**

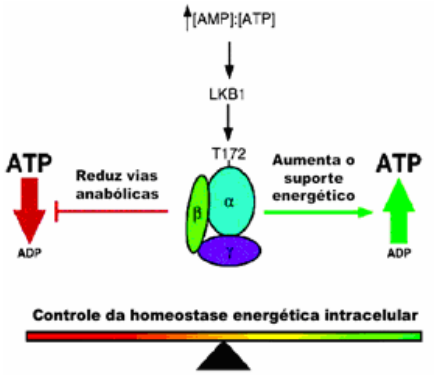

Figura 6- Ativação da AMPK pelo ATP

Embora a elevação na concentração intracelular de AMP, em relação à concentração de ATP, seja o principal mecanismo de ativação da AMPK, esta quinase também pode sofrer modulação de outros mecanismos, como o stress hiperosmótico, que ativa a AMPK independente de alterações na relação ATP/AMP (117), sugerindo a influência de outros mecanismos, ainda não explorados, no controle da via de sinalização da AMPK.

Vários estudos têm evidenciado o importante papel desempenhado pela AMPK no controle da homeostasia energética. Conforme anteriormente mencionado, esta quinase responde à fatores de sinalização como a leptina, por exemplo, e isto ocorre tanto na periferia como no SNC (41) **(Figura 7).** A leptina ativa a AMPK em tecidos periféricos, como a musculatura esquelética, aumentando a oxidação dos ácidos graxos.Por outro lado, a leptina inibe a AMPK no SNC e, com isso, reduz a ingestão alimentar (41). Esta influência exercida pela leptina sobre a atividade hipotalâmica da AMPK foi recentemente demonstrada por Andersson e colaboradores (2004). Segundo os autores, a leptina necessita que a AMPK hipotalâmica seja inibida, para exercer sua função anorexigênica (118).

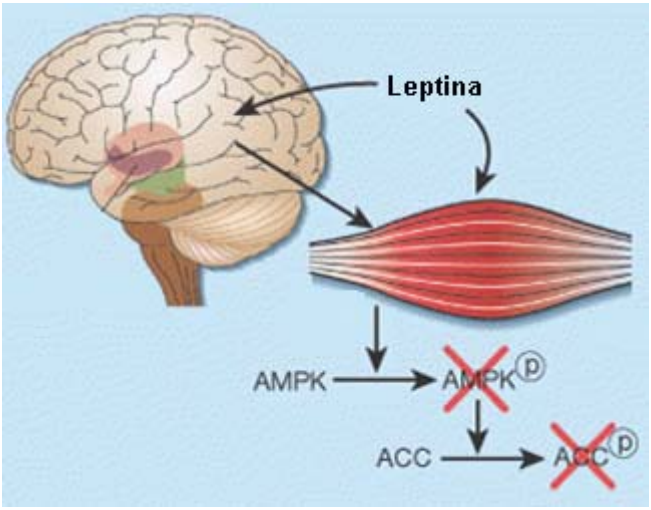

Figura 7- Ação da leptina sobre a AMPK (SNC)

De forma semelhante, foi demonstrado que o ácido α-lipóico, um cofator das desidrogenases mitocondriais, modula a atividade da AMPK de modo similar à leptina: inibe esta quinase no SNC e induz sua ativação no músculo esquelético (119). Além disso, a ativação farmacológica desta quinase no SNC induz hiperfagia (118). De acordo com Minokoshi e colaboradores, o aumento da atividade hipotalâmica da AMPK induz aumento na expressão de NPY, no núcleo arqueado estimulando, portanto, a ingestão alimentar e reduzindo o gasto energético. De forma contrária, a inibição da ação hipotalâmica desta quinase, reduz a expressão de neuropeptídeos orexigênicos e aumentando o gasto energético (41).

Todas estas evidências sugerem a participação efetiva da AMPK nos mecanismos controladores do balanço energético.

mTOR

Outra quinase sensível às variações na disponibilidade energética é a mTOR.

Esta enzima é uma serina-treonina quinase, considerada a principal proteína de uma via sinalizadora responsável principalmente, pelo controle dos mecanismos de proliferação, sobrevivência e crescimento celular. Esta quinase ainda modula a atividade de outras quinases, envolvidas nesta mesma cascata de sinalização, como as enzimas:

p70S6K1, 4EBP1 e eif4E (120). Estudos realizados em mamíferos e *Drosophilas,* sugerem que as proteínas envolvidas na cascata de sinalização da mTOR também atuem sobre os mecanismos de controle da homeostasia energética (121).

A transmissão do sinal pela mTOR ocorre através de dois distintos complexos protéicos: TORC1 e TORC2 **(Figura 8).** O complexo TORC1, é formado pela associação entre as proteínas RAPTOR, GβL e mTOR (122, 123) e, entre outras funções, possui importante função no controle da homeostasia energética, e na atividade das proteínas p70S6K e 4EBP1 (123). O complexo TORC2, descoberto recentemente, é formado pela associação entre as proteínas RICTOR, G□L e mTOR (123).

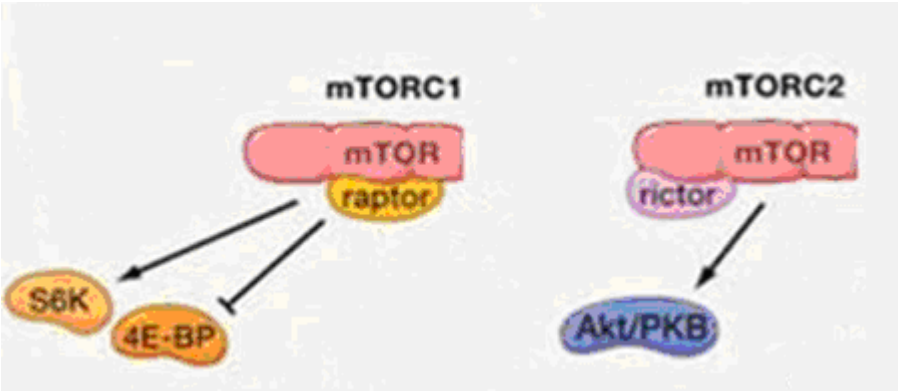

Figura 8- Transmissão do sinal pela mTOR

O complexo TORC1 é modulado por uma variedade de mecanismos (124-126). Sempre que qualquer aumento nos estoques energéticos é detectado, pelas moléculas sinalizadoras, a atividade de algumas vias de sinalização intracelular sofre modulação, visando manter a homeostasia do peso corporal. Dentre estas vias, citamos como exemplo, a via da PI(3)K, e o complexo TORC1 (124-126).

Este complexo também é responsável por estimular a produção de leptina pelos adipócitos. A leptina, cumprindo sua função de molécula sinalizadora, informa o SNC sobre as reservas energéticas periféricas, desencadeando uma série de respostas neurais que culminam com a redução do consumo de alimentos e aumento do gasto energético (127).

Conforme anteriormente citado, sempre que ocorre redução nos estoques energéticos periféricos, a concentração intracelular de AMP aumenta em relação à concentração de ATP, induzindo a ativação da proteína AMPK e, conseqüentemente, inibindo a atividade do complexo TORC1 (128) **(Figura 9).**

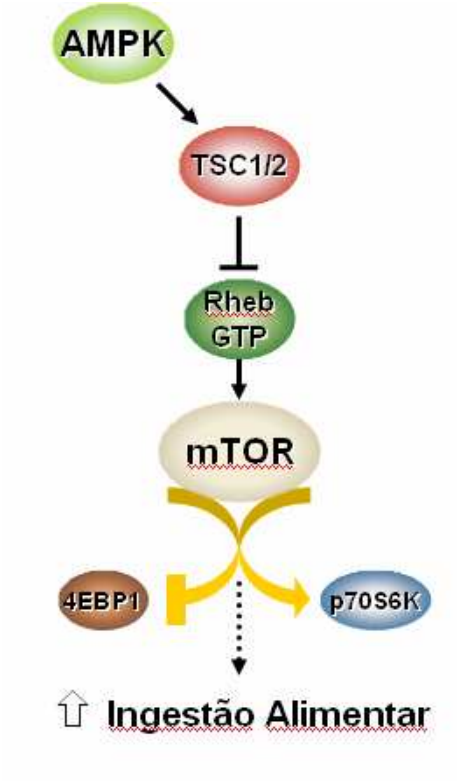

Figura 9- Ação da AMPK sobre a mTOR (SNC)

A rapamicina é uma droga imunossupressora, que modula negativamente a atividade do complexo TORC1. De acordo com Cota e colaboradores, a administração central de rapamicina bloqueia a ação anorexigênica da leptina. Os autores também observaram uma relação direta entre a atividade hipotalâmica da TORC1 e a ação central da leptina, sugerindo a participação deste complexo na sensibilidade hipotalâmica à ação deste hormônio (129).

Assim, diversas vias de sinalização envolvidas no controle do comportamento alimentar convergem no complexo TORC1. As vias envolvidas na modulação da sensibilidade à leptina, como a via da PI(3)K e a via da AMPK, exercem grande influência sobre este complexo e, conseqüentemente, sobre a transmissão do sinal da mTOR (124, 127, 130).

Além disso, o complexo TORC1 e a enzima AMPK, encontram-se localizados na mesma população de neurônios hipotalâmicos. Isto sugere que a ação orexigênica da AMPK, ainda que parcialmente, é mediada pelo complexo TORC1 (130).

Exercício e modulação do set point

Os efeitos decorrentes da prática de exercícios, resultam em aumentos do gasto energético, colaborando para a redução da adiposidade e, conseqüentemente, para a redução do peso corporal. Além disso, existem dados na literatura relacionando a ação do exercício a algumas funções do SNC que, conforme a modulação do balanço energético, também são mediadas por moléculas sinalizadoras (131). Uma vez que o exercício não permite que haja aumento na ingestão alimentar como mecanismo de *feedback,* frente ao aumento do gasto energético, fica claro que, o exercício físico induz produção e liberação de algum fator responsável por inibir este *feedback* (131).

Foi demonstrado recentemente que uma sessão aguda de exercício é capaz de modular o apetite, em ratos *Wistar*. Neste estudo, roedores submetidos ao exercício mostraram-se mais sensíveis à ação anorexigênica dos hormônios insulina e leptina, através da ação da IL-6 (132). Da mesma forma, o exercício mostrou-se eficiente em reduzir o *set point* para o peso corporal em roedores submetidos a dieta hipercalórica (133). Neste mesmo estudo, foi observado que esta modulação do *set point* hipotalâmico não possui qualquer relação com o volume ou o tempo de duração do exercício; e sim com a modulação do apetite. Isto sugere que o exercício monitora o balanço energético não pelo volume de atividade física, mas pelos sinais periféricos moduladores da ingestão alimentar, que são desencadeados pelo exercício (133).

Ainda que os efeitos do exercício em roedores não reproduzam de forma absolutamente fiel os efeitos que este mesmo exercício induziria em humanos, a identificação dos fatores centrais e periféricos induzidos pelo exercício, bem como os mecanismos através dos quais estes fatores modulam a homeostasia energética, apresenta extrema relevância, por representarem a possibilidade de novos alvos para intervenção terapêutica, no tratamento da obesidade.

2- OBJETIVOS

Em estudo recente, foi demonstrado que, em ratos *Wistar,* o exercício agudo modula a sensibilidade hipotalâmica à ação de fatores anorexigênicos, como os hormônios leptina e insulina, através da IL-6 (132). Além disso, vias hipotalâmicas sensíveis ao *status* energético do organismo, como as vias AMPK e mTOR, vêm despontando como importantes coadjuvantes na modulação da sensibilidade à leptina e, conseqüentemente, na modulação da ingestão alimentar (129, 134, 135).

Assim, o presente trabalho tem como principal objetivo, verificar a possível ação moduladora da IL-6, na resposta das vias hipotalâmicas AMPK e mTOR à leptina, após uma sessão aguda de exercício, em ratos *Wistar.*

3- CAPÍTULO 1

EXERCISE MODULATES AMP KINASE ACTIVITY IN THE HYPOTHALAMUS OF WISTAR RATS

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Short running title: Exercise alters AMPK activity in the hypothalamus

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SUMMARY

AMP-activated protein kinase (AMPK) and mammalian Target of Rapamycin (mTOR) are key regulators of cellular energy balance and of the effects of leptin on food intake. Acute exercise is associated with increased sensitivity to the effects of leptin on food intake in an IL-6-dependent manner. To determine whether exercise ameliorates the AMPK and mTOR response to leptin in the hypothalamus in an IL-6-dependent manner, rats performed two 3-h exercise bouts, separated by one 45-min rest period. Intracerebroventricular IL-6 infusion reduced food intake and pretreatment with AMPK activators and mTOR inhibitors prevented IL-6-induced anorexia. Activators of AMPK increased food intake in control rats to a greater extent than that observed in exercised ones, whereas inhibitors of AMPK had the opposite effect. Exercise was associated with both reduced phosphorylation of the AMPK/ACC signaling pathway and increased phosphorylation of proteins involved in mTOR signal transduction in the hypothalamus. Treatment with leptin reduced food intake in exercised rats that were pretreated with vehicle, although no increase in sensitivity to leptin-induced anorexia after pretreatment with anti-IL6 antibody, AICAR or Rapamycin was detected. Thus, improved responses of AMPK and mTOR to leptin may contribute to the appetite suppressive actions of exercise.

INTRODUCTION

Prolonged exercise of medium to high intensity is known to profoundly affect energy balance (1-3). Studies of individuals who have maintained significant weight loss for >1 year have demonstrated that dieters who achieve long-term success are often those who engage in regular and extensive exercise programs (4). Although the energy expenditure aspects of such exercise may contribute to the effects of weight maintenance, it has been suggested that even acute exercise may also contribute to the energy balance by altering food intake (5). Rodents submitted to exercise have increased sensitivity to leptin, conversely animals with diet-induced obesity and most obese humans are resistant to leptin (5-7). Thus, the mechanism for leptin increased sensitivity in exercise is of great interest and understanding this mechanism could lead to new approaches to prevent or treat obesity.

The hypothalamus plays a central role in integrating hormonal (leptin and insulin) and nutritional signals from the periphery and modulating food intake, energy expenditure, and peripheral metabolism (8). Multiple factors regulate food intake, including hormones, fuels and behaviour. AMPK is the downstream component of a kinase cascade that acts as a sensor of cellular energy charge, being activated by rising AMP coupled with falling ATP. Once activated, AMPK phosphorylates acetyl-CoA carboxylase (ACC) and switches on energy-producing pathways at the expense of energy-depleting processes (9). Another target molecule for the control of food intake and energy homeostasis is represented by the phosphoprotein mammalian target of rapamycin, mTOR, in which the PI(3)K/Akt pathway has been suggested to affect the mTOR phosphorylation state and catalytic activity (10). Activated signaling through mTOR phosphorylates the serine/threonine kinase p70S6K and the translational

repressor eukaryotic initiation factor (eIF) 4E binding protein (4EBP1) (11). mTOR signaling is inhibited under conditions of low nutrients, such as glucose and amino acids and low intracellular ATP levels (12). While mTOR was presumed to serve as the direct cellular sensor for ATP levels (13), mounting evidence has implicated AMPK in the regulation of mTOR activity (14-16).

The level of circulating IL-6 increases dramatically in response to exercise (17), with IL-6 being produced by working muscle (18, 19) and adipose tissue (20, 21) and its concentration correlates temporally with increases in AMPK in multiple tissues (22). In addition, AMPK activity is diminished in IL-6 deficient mice at rest and the absolute increases in AMPK activity in these tissues caused by exercise is diminished compared with control mice (23). It also appears that centrally-acting IL-6 plays a role in the regulation of appetite, energy expenditure, and body composition (5, 24). The signaling mechanism of IL-6 in the hypothalamus is, however, not fully understood. In cells, binding IL-6 to the α subunit of its receptor triggers the recruitment of gp130, subsequently leading to the activation of the gp130-associated JAK (25-27). JAK links cytokine receptor to the STAT3 and MAP kinase pathway (25, 26, 28). In addition to JAK/STAT and MAP kinase pathways, IL-6 also activates the PI(3)K/Akt pathway (29).

In this study, we sought to determine whether the improved response of the AMPK and mTOR pathways to leptin could contribute to the increased molecular sensitivity of leptin in rats submitted to exercise in an IL-6-dependent manner. We therefore, examined hypothalamic modulation of AMPK/ACC and mTOR signaling pathways, induced by IL-6, as well as the role of IL-6 in those signaling pathways induced by leptin in rats after acute exercise.

RESULTS

IL-6 decreases hypothalamic AMPK and increases mTOR signaling

To determine whether interleucine-6 (IL-6) modulates hypothalamic AMPK/ACC signaling, we injected IL-6 into the third ventricle of rats and evaluated food intake and AMPK signaling. IL-6 caused a significant reduction in food intake in a dose-dependent manner (Figure 1a). We next investigated whether the microinfusion of IL-6 modulates the hypothalamic ATP concentration. Figure 1b shows that IL-6 (200ng) changed ATP, ADP and AMP concentrations in the hypothalamus of rats, whereas, sixty minutes after IL-6 injection, the ATP content increased by ~88% (Figure 1c) and decreased AMP:ATP ratio by ~54% in Wistar rats (Figure 1d). Consistent with the modulation of the AMP:ATP ratio, we observed reduced hypothalamic AMPK and ACC phosphorylation induced by IL-6 (Figures 1e and f); whilst IL-6 increases p70S6K and 4EBP1 phosphorylation in a dose-dependent manner (Figures 1g and h).

To determine whether the effects of IL-6 on food intake are AMPK-dependent, we first identified a dose of AICAR that did not alter food intake (0.5mM; data not shown) when administered at the onset of the dark cycle. We then evaluated the effect of i.c.v. pretreatment with this dose of AICAR, or its vehicle, on the anorectic response to i.c.v. IL-6 (200ng) and we observed that the anorectic response to i.c.v. IL-6 was reversed by AICAR at the time points studied (figure 1a). These findings indicate that inactivation of neuronal AMPK is necessary for some of the effects of IL-6 on food intake.

IL-6 induction of hypothalamic mTOR does not require changes in PI(3)K signaling

To determine whether interleucine-6 (IL-6) modulates hypothalamic mTOR signaling, we injected IL-6 (200ng) into the third ventricle of rats and evaluated food intake and mTOR signaling. IL-6 caused a significant reduction in food intake (Figure 2a) and induced hypothalamic p70S6K and 4EBP1 phosphorylation (Figures 2b and c). To determine whether the effects of IL-6 on food intake are mTOR-dependent, we first identified a dose of Rapamycin that did not alter food intake (25µg; data not shown) when administered at the onset of the dark cycle. We then evaluated the effect of i.c.v. pretreatment with this dose of Rapamycin or its vehicle, on the anorectic response to i.c.v. IL-6 and we observed that IL-6 reduction of food intake was reversed by Rapamycin.

We next examined whether PI(3)K signaling is required for the IL-6-dependent reduction of food intake, by IL-6 administration in LY294002 pretreated (i.c.v.) animals. Pretreatment with LY294002 at a dose that did not alter food intake (50µM; data not shown) had no effect on anorectic response to i.c.v. IL-6 (Figure 2d). Consistent with these data, we observed that a single IL-6 i.c.v. injection did not change Akt phosphorylation status in the hypothalamus (Figure 2e). These findings indicates that activation of neuronal mTOR is necessary for some of the effects of IL-6 on food intake and that these effects of IL-6 do not require any change in PI(3)K signaling.

Physiological parameters measured in basal conditions after exercise protocol

The plasma glucose level was lower in the exercised group compared to the control group (3.6±0.8 *vs* 4.6±0.5 mmol/L; n=5; $p<0.05$) and the insulin levels were also lower (88±12 *vs* 193±17 pmol/L, n=5; $p<0.05$). Exercise did not, however, reduce plasma leptin (2.6±0.5 *vs* 2.3±0.7 ng/ml). Insulinemia and leptinemia were not altered by third ventricle microinjection of leptin (data not shown).

Exercise partially reverses the effects of AICAR on food intake through modulation of the AMPK-mTOR signaling pathway in the hypothalamus.

To test the role of a single session of exercise on AICAR-increased food intake, AICAR (2mM) or its vehicle were administered (i.c.v.) to control and exercised animals. 12-hour total food intake was measured after exercise. In exercised rats, AICAR (2mM) did not cause any acute change in food intake but, in the control group, AICAR (2mM) increased food intake by 32% (Figure 3a), suggesting that AICAR is not effective in exercised rats. Comparing AICAR-treated groups (control *vs*. exercise), exercised animals showed a 33% reduction in 12-hour total food intake (Figure 3a).

Next, AMPK, ACC, p70S6K and 4EBP1 phosphorylation levels were assessed in the hypothalamus of all rats. Western blotting of hypothalamic whole-extracts was performed using anti-phospho-AMPK, anti- phospho-ACC, anti-phospho-p70S6K and anti-phospho-4EBP1 antibodies. Consistent with food intake, AICAR increased AMPK threonine and ACC serine phosphorylation levels in the hypothalami of control rats, whilst in exercised animals, AICAR did not change AMPK/ACC phosphorylation status (Figures 3b and c). Comparing AICAR treated groups (control *vs*. exercise), exercised animals showed reductions in AMPK threonine and ACC serine phosphorylation of 52% and 31%, respectively. AICAR also reduced p70S6K and 4EBP1 threonine phosphorylation levels in the hypothalamus of control and exercised rats. However, comparing AICAR treated groups (control *vs*. exercise), exercised animals showed increases in p70S6K and 4EBP1 threonine phosphorylation of 230% and 310%, respectively (Figures 3d and e). Similar results were observed after intraperitoneal treatment with 2-DG, another pharmacological activator of AMPK (Figure 4).

Intracerebroventricular α-LA reduces food intake by modulating AMPK-mTOR hypothalamic phosphorylation levels to a greater extent in exercised animals

The effects of α-LA (3μg) intracerebroventricular administration, or its vehicle, on food intake control were studied by measuring the 12-hour total food intake after an acute exercise bout. In exercised rats, α-LA (3μg) reduced food intake by 86% while control group showed a reduction of 58%. Comparing α-LA treated groups (control *vs*. exercise), exercised animals showed a 64% reduction in 12-hour total food intake (Figure 5a).

To determine the effects of exercise on the AMPK-mTOR signaling pathway, α-LA was i.c.v. administered and AMPK, ACC, p70S6K and 4EBP1 phosphorylation levels were assessed in the hypothalamus of all rats. Western blotting of hypothalamic whole-extracts was performed using anti-phospho-AMPK, anti-phospho-ACC, anti-phospho-p70S6K and anti-phospho-4EBP1 antibodies. α-LA reduced AMPK and ACC phosphorylation levels, in the hypothalami of control and exercised rats. Comparing α-LA treated groups (control *vs.* exercise), in exercised animals α-LA reduced both AMPK threonine phosphorylation and ACC serine phosphorylation of 39% and 57%, respectively (Figures 5b and c).

α-LA also induced p70S6K and 4EBP1 threonine phosphorylation in the hypothalami of control and exercised rats. Comparing α-LA treated groups (control *vs.* exercise), in exercised animals, α-LA increased p70S6K and 4EBP1 threonine phosphorylation of 19% and 11%, respectively (Figures 5d and e).

Intracerebroventricular leptin reduces food intake by modulating AMPK-mTOR hypothalamic phosphorylation levels to a greater extent in exercised animals.

The effects of leptin (10^{-6}M) i.c.v. administration or its vehicle on food intake control were studied by measuring the 12-hour total food intake after an acute exercise bout. In exercised rats, leptin (10^{-6}M) reduced food intake by 43%, when compared with exercised plus vehicle treated group, while the control group showed a reduction of 25%, when compared with vehicle treated group. Comparing leptin-treated groups (control *vs*. exercise), exercised animals showed a 31% reduction in 12-hour total food intake (Figure 6a).

To determine the effects of exercise on AMPK-mTOR signaling pathway, leptin was i.c.v. administered and AMPK, ACC, p70S6K and 4EBP1 phosphorylation levels were assessed in the hypothalamus of all rats. Western blotting of hypothalamic whole-extracts was performed using anti-phospho-AMPK, anti-phospho-ACC, anti-phospho-p70S6K and anti-phospho-4EBP1 antibodies. Leptin reduced AMPK and ACC phosphorylation levels in the hypothalami of control and exercised rats. Comparing leptin-treated groups (control *vs*. exercise), in exercised animals, leptin reduced both AMPK threonine phosphorylation and ACC serine phosphorylation of 57% and 45%, respectively (Figures 6b and c). Leptin also induced p70S6K and 4EBP1 threonine phosphorylation in the hypothalamus of control and exercised rats. Comparing leptin treated groups (control *vs*. exercise), in exercised animals, leptin increased both p70S6K and 4EBP1 threonine phosphorylation of 30% and 40% respectively (Figures 6d and e).

Role of IL-6 in anorectic response to leptin.

IL-6 expression was detected in control animals; however, a 420% increase was observed in the exercised group (Figure 7a). We tested whether the inhibitory effects of leptin on food intake depends on IL-6, by i.c.v. infusion of anti–IL-6 into exercised rats. Treatment with leptin (10^{-6}M) markedly reduced 12-h food intake in exercised rats

pretreated with vehicle, although pretreatment with anti–IL-6 antibody blocked exercise-induced leptin sensitivity in a concentration-dependent manner (Figure 7b).

Both AMPK and ACC phosphorylation levels, reduced by exercise, were reversed by anti–IL-6 (Figure 7c and d). We also observed that the increased phosphorylations of p70S6K and 4EBP1, induced by exercise, were also reversed by anti–IL-6 (Figures 7e and f).

Blocking effects of AICAR and Rapamycin on leptin-induced anorexia.

We tested whether the i.c.v. administration of AICAR or Rapamycin, 60 minutes before the administration of leptin (10^{-6}M), prevents the anorexigenic effects of leptin. Leptin (10^{-6}M) treatment markedly reduced 12-h food intake in both control and exercised groups, although leptin was much more effective in exercised rats. AICAR (0.5mM) or Rapamycin (25μg), at doses that do not alter ingestion (data not shown), completely blocked the suppression of food intake induced by an i.c.v. injection of leptin (10^{-6}M) (Figure 8a). The i.c.v. administration of leptin (10^{-6}M) to exercised rats pretreated with vehicle reduced AMPK and ACC phosphorylation in the hypothalamus by 63% and 60% respectively, compared with the control group. Comparing exercised animals, i.c.v. administration of leptin (10^{-6}M) to rats pretreated with AICAR increased both AMPK and ACC phosphorylation levels in the hypothalamus. Exercised animals pretreated with Rapamycin also demonstrated increases in hypothalamic AMPK and ACC phosphorylation (Figures 8b and c).

The i.c.v. administration of leptin (10^{-6}M) to exercised rats pretreated with vehicle induced p70S6K and 4EBP1 phosphorylation in the hypothalamus of 60% and 70%, respectively, compared with the control group. Comparing exercised animals,

i.c.v. administration of leptin (10^{-6}M) to rats pretreated with AICAR reduced both p70S6K and 4EBP1 phosphorylation levels in the hypothalamus. Exercised animals pretreated with Rapamycin also reduced hypothalamic p70S6K and 4EBP1 phosphorylation (Figures 8d and e).

DISCUSSION

The molecular mechanisms by which exercise controls food intake are unclear. Our data indicate that IL-6 signaling through AMPK and mTOR reduces food intake in a dose-dependent manner. Leptin, as well as α-LA infusion, reduced food intake in exercised rats to a greater extent than that observed in control animals. Conversely, AICAR and 2-DG increased food intake in exercised rats to a lower extent than that observed in control animals. Exercise was associated with a modulation in the activity of the AMPK/mTOR pathway in the hypothalamus. In addition, we investigated the regulatory role of IL-6 in mediating the increase in leptin sensitivity in the hypothalamus. Treatment with leptin markedly reduced food intake, AMPK activity and increased mTOR activity in exercised rats that were pretreated with vehicle, although no increase in sensitivity to leptin-induced anorexia and modulation of AMPK/mTOR pathway were detected after pretreatment with anti-IL-6 antibody. Taken together, these results suggest that IL-6 is a major component of the effects of exercise on the control of food intake.

A number of recent studies have shown that AMPK plays a key role in regulating both energy intake and expenditure (30, 31). In peripheral tissues, such as skeletal muscle, activation of AMPK switches on energy producing pathways and switches off energy consuming pathways. In the hypothalamus, activation of AMPK leads to increased feeding, thereby increasing energy intake. Conversely, inhibition of AMPK in the hypothalamus reduces food intake. These dual functions of AMPK suggest that it may act to coordinate energy expenditure with energy intake. There is already some evidence that this may be the case in one situation. Leptin activates AMPK in skeletal muscle, leading to increased fatty acid oxidation (32), whilst

inhibiting AMPK in the hypothalamus leads to decreased food intake (33, 34). Furthermore, inhibition of hypothalamic AMPK is necessary for the anorexigenic effects of leptin (34). The aim of this study was to investigate whether IL-6 could affect AMPK activity in the hypothalamus, thereby providing a potential mechanism for the coordination of energy expenditure and energy intake during, or following exercise. We detected changes in the hypothalamic AMPK activity in rats subjected to a single bout of exercise; furthermore, IL-6 markedly decreased phospho-AMPK abundance (an index of activity) in the hypothalamus. In accordance with the reduction in $AMPK^{thr172}$ phosphorylation, we observed that, after IL-6 administration, hypothalamic AMP:ATP ratio was decreased.

The mTOR, an evolutionary conserved serine-threonine kinase, central to integrating similar signals to control food intake, has now emerged as a detector of hormonal and nutritional signals in the hypothalamus (10). In this study, we investigated whether IL-6 activates mTOR. IL-6 increased mTOR activity; moreover inhibition of central mTOR reversed the anorectic effect of IL-6. In addition, the anorexigenic effect of IL-6 was absent in AICAR- and Rapamycin-pretreated rats, however, pretreatment with LY294002 - a PI(3)K inhibitor - had no effect on IL-6 induced anorexia, indicating that, in the hypothalamus, the effect of IL-6 is independent of the PI(3)K pathway. Signaling through gp130 commonly results in activation of PI(3)K, and IL-6 can activate PI(3)K (35) and its downstream target Akt (36-39), but it should be noted that this effect has not been observed in all studies (40), suggesting a tissue-dependent effect.

Next, we investigated whether the increased sensitivity of the leptin action on food intake induced by exercise, could be due to the modulation of AMPK activity. As

previously shown (41), exercise, per se, does not alter AMPK activity in the hypothalamus; however, we observed that the normal inhibition of AMPK phosphorylation and activity in the hypothalamus, induced by leptin administration, was improved in exercised rats.

In addition, we did not observe any normal stimulation of AMPK activity by AICAR in the hypothalamus of exercised rats, indicating that AMPK pathway is disrupted. This observation agrees with data from aging studies in which acute stimulation with AICAR was blunted in skeletal muscle of old rats (42). Furthermore, the use of another activator of AMPK (2-DG) in exercising rats resulted in a reduced activation of AMPK when compared to the control animals. In contrast, the pharmacological inhibition of AMPK by α-LA results in a greater inhibition of AMPK activity, compared to control animals.

The mechanism by which exercise inhibits AMPK-induced food intake in the hypothalamus is not clear. Several lines of evidence point to a possible link between inhibited AMPK-induced food intake in the hypothalamus and IL-6 signaling through the AMPK/mTOR pathway. Firstly, we found that the leptin-inhibited food intake enhanced by exercise was blunted by anti-IL-6 antibody. Secondly, exercise induced increased sensitivity of leptin-inhibited AMPK signaling was reverted by AICAR. Finally, exercise induced increased sensitivity of leptin stimulated mTOR signaling was reverted by rapamicyn.

Our data are in accordance with earlier studies demonstrating that IL-6 treatment enhances energy expenditure in both rodents and humans (24, 43-45). In exercising rats, hypothalamic leptin and insulin sensitivity are increased in an IL-6-dependent manner (5). It has been previously shown that IL-6 treatment stimulates energy expenditure at

the level of the brain in rodents (24, 44, 46), and it might be assumed that endogenous IL-6 also acts on the brain during exercise. The IL-6 exerting this effect during exercise could be produced by the brain itself, which has been shown to have increased IL-6 production during exercise (47). Alternatively, the large quantities of endocrine IL-6 produced from working skeletal muscle (47) might reach appropriate sites in the brain (17, 48, 49).

Increased sensitivity of leptin action in the hypothalamus, through modulation of the AMPK-mediated pathway by exercise, could be pathophysiologically important in the prevention of obesity. Recent studies have shown that modulation of leptin signaling through the AMPK pathway could be involved in the development of obesity (50). Taken together these data may indicate that the anti-obesity actions, induced by leptin, could be increased due to the partial inhibition of the AMPK pathway provoked by exercise. If the mechanism used by IL-6 to reduce food intake is AMPK-dependent, as our results suggest, the defective activation of AMPK in the hypothalamic neurons induced by exercise may increase the ability of leptin to reduce food intake.

In conclusion, exercise alters the AMPK/mTOR signaling pathway in the hypothalamus and increases the response to leptin administration. This increased dynamic responsiveness of the AMPK/mTOR pathway to leptin could provide information regarding the molecular mechanism underlying the biological sensitivity to leptin in exercise. Furthermore, these findings provide support to the hypothesis that AMPK and mTOR interact in the hypothalamus to regulate feeding in exercised rats, in an IL-6-dependent manner.

METHODS

Antibodies and Chemicals

Reagents for SDS-polyacrylamide gel electrophoresis and immunoblotting were from Bio-Rad (Richmond, CA, USA). Tris[hydroxymethyl]amino-methane (Tris), aprotinin, ATP, dithiothreitol, phenylmethylsulfonyl fluoride, Triton X-100, Tween 20, glycerol, and bovine serum albumin (fraction V) were from Sigma Aldrich (St. Louis, MO, USA). Protein A-Sepharose 6 MB and ^{125}I-protein A and Nitrocellulose paper (Hybond ECL, 0.45 μm) from Amersham Pharmacia Biotech United Kingdom Ltd. (Buckinghamshire, United Kingdom). Ketamin was from Parke-Davis (São Paulo, SP, Brazil) and diazepam and thiopethal were from Cristália (Itapira, SP, Brazil). Anti-phospho-[Ser^{79}] ACC (rabbit polyclonal, #07-184) was from Upstate Biotechnology (Charlottesville, VA, USA). anti-ACC (goat polyclonal, sc-26816) and anti-IL-6 antibody (rabbit polyclonal, sc-7920) were from Santa Cruz Biotechnology, Inc. Anti-phospho-[Thr^{172}] AMPKα (rabbit polyclonal, #2531), anti-AMPKα (rabbit polyclonal, #2532), anti-phospho-[Thr389] p70S6K (rabbit polyclonal, #9205), anti-p70S6K (rabbit polyclonal, #9202), anti-phospho- [Thr70]4EBP1 (rabbit polyclonal, #9455), anti-4EBP1 (rabbit polyclonal, #9452), anti-phospho- [Ser 473]Akt (rabbit polyclonal, #9271), and anti-Akt (rabbit polyclonal, #9272) were from Cell Signalling Technology (Beverly, MA, USA). Leptin, LY294002, and Interleukin-6 were from Calbiochem (San Diego, CA, USA); 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), 2-Deoxy-D-glucose and α-lipoic acid were from Sigma Chemical Co. (St. Louis, MO). Rapamycin was from LC Laboratories (Woburn, MA, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Experimental Animals and Intracerebroventricular (i.c.v.) Cannulation

Male Wistar rats (8 weeks old/250–300 g) obtained from the University of Campinas Animal Breeding Center were used in the experiments. The investigation was approved by the ethics committee and followed the University guidelines for the use of animals in experimental studies and conforms to the *Guide for the Care and Use of Laboratory Animals,* published by the US National Institutes of Health (NIH publication no. 85-23 revised 1996). The animals were maintained on 12h:12h artificial light–dark cycles and housed in individual cages. After the acclimatizing period (3 days), the animals were stereotaxically instrumented under intraperitoneal injection of a mix of ketamin (10mg) and diazepam (0.07mg) (0.2ml/100g body weight) with a chronic 26-gauge stainless steel indwelling guide cannula, aseptically placed into the third ventricle (0.5 mm posterior and 8.5 mm ventral to bregma), as previously described (51). After a 1-wk recovery period, cannula placement was confirmed by a positive drinking response after administration of Angiotensin II (40 ng/2 μL); animals that did not drink 5 mL of water within 15 minutes after treatment were not included in the experiment.

Exercise Protocol

Rats were acclimated to swimming for 2 days (10 min per day). On the day of the experiment, animals swam in groups of four, in plastic barrels of 45cm in diameter, filled to a depth of ≈50 cm. Water temperature was maintained at 34-35°C. They performed two 3-h exercise bouts, separated by one 45-min rest period. After the last exercise bout, some rats were injected into the cannula and food intake was determined over the next 4 and/or 12 h; the other rats were injected into the cannula and then anesthetized with intraperitoneal injection of sodium thiopethal (5mg/100g body weight) and hypothalamus was removed.

Treatments

For acute treatments, rats were deprived of food for 6 h with free access to water and i.p. injected (200µl bolus injection) with either vehicle or 2-DG (500mg/kg) or i.c.v. injected (3 µl bolus injection) with either vehicle, IL-6 (100ng or 200ng), AICAR (0.5 or 2.0mM), Rapamycin (25µg), α-LA (3µg), leptin (10^{-6}M), LY294002 (50µM) or anti-IL-6 antibody (12.5; 50 or 25ng). Similar studies were carried out in rats that were initially pre-treated with i.c.v. microinjection of vehicle, AICAR, Rapamycin, anti-IL-6 antibody or LY294002, and after 60 min with i.c.v. microinjection of IL-6 or leptin. Thereafter, standard chow was given and food intake was determined by measuring the difference between the weight of chow given and the weight of chow at the end of 4 and/or 12-h periods. All acute treatments were performed at 5:00 and 6:00 p.m.

Western Blot Analysis

After exercise and i.c.v. treatments, rats were anaesthetized with intraperitoneal injection of a mix of ketamin (10mg) and diazepam (0.07mg) (0.2ml/100g body weight), and used as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The rats were killed, and hypothalamus was quickly removed, minced coarsely and homogenized immediately in a freshly prepared ice-cold buffer (1% Triton X-100, 100mmol/l Tris pH 7,4, 100mmol/l sodium pyrophosphate, 100mmol/l sodium fluoride, 10mmol/l EDTA, 10mmol/l sodium vanadate, 2mmol/l phenyl methylsulphonyl fluoride and 0,0,1mg/ml aprotinin) suitable for preserving phosphorylation states of enzymes and Western blot was performed, as previously described (52). Insoluble material was removed by centrifugation (50000g) for 25 minutes at 4^{o}C. For phospho-AMPK, AMPKα2, phospho-ACC, ACC, phospho-p70S6K, p70S6K, phospho-4EBP1, 4EBP1, phospho-Akt and Akt detection, total extracts of hypothalamus were prepared and 0.25mg total protein were separed by SDS-PAGE. After SDS-PAGE (15%

resolving gels for p-4EBP1 and 4EBP1; 12% resolving gels for p-AMPK, AMPKα2; phospho-p70S6K, p70S6K, phospho-Akt and Akt; 6,5% resolving gels for ACC and phospho-ACC), proteins were transferred from gel to nitrocellulose membrane. Membranes were blocked in 5% nonfat dried milk in PBST (139mM NaCl, 2,7mM KH_2PO_4, 9,9mM Na_2HPO_4, and 0,1% Tween 20) for 2 hours and then incubated overnight with specific antibodies. After incubation with the relative second antibody, immune complexes were detected using the ECL method. Results were visualized by autoradiography using preflashed Kodak XAR film (Eastman Kodak, Rochester, N.Y., USA) with Cronex Lightning Plus intensifying screens at –80°C for 12–48 h. (Mass., USA). Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image software - *Scion Corporation*, Frederick, Md., USA).

Chromatography

Chromatographic analyses were carried out on a Waters Alliance equipment series 2695 (Milford, MA, USA) equipped with a quaternary pump, an autosampler, a degasser, and a Waters 2475 fluorescence detector model. The fluorescence of derivatized compounds (ATP, ADP, AMP, and ADO) was monitored with excitation and emission wavelengths set at 280 and 420 nm, respectively. Chromatographic separations of the compounds were achieved at room temperature, using a reversed-phase Cosmosil 5C18-MS column (150X4.6 mm i.d.; 5 µm particle size) with a Cosmosil guard column (5C18-MS 10X4.6 mm) purchased from Phenomenex (Torrance, CA, USA). The mobile phase composition was 50 mmol/L KH_2PO_4, 25 mmol/L citric acid (pH 4.5), and methanol (90:10, v/v), which was prepared immediately before use and filtered through a 0.45 µm filter (Millipore, Milford, MA, USA). The column was equilibrated and eluted under isocratic conditions using a flow rate of 1.0 ml/min. The chromatographic run time for

each analysis was 20 min. Aliquots of 25 µl were injected into the HPLC system. System control, data acquisition, and processing were performed with a PC-Pentium IV Processor personal computer from Dell, operated with Microsoft Windows XP version 2003 and Waters Empower 2002 chromatography software. A validation chromatographic run included a set of calibration samples assayed in duplicate and quality control samples at four levels in triplicate. The standard calibration curves for known amounts of ATP, ranging from 0.025 to 10.0 µmol/L, were linear (R>0.999) and could be described by the linear regression equation: $y = 0.4992*x - 0.0463$ (n=4, P<0.0001, r=0.9997), in which y is the ATP concentration in micromoles and x is the chromatogram peak area.

Statistical Analysis

All numeric results are expressed as the means ±SEM of the indicated number of experiments. The results of blots are presented as direct comparisons of bands or spots in autoradiographs and quantified by optical densitometry (Scion Image). Statistical analysis was performed by employing the ANOVA test with *Bonferroni post test*. Significance was established at the p<0.05 level.

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FIGURE LEGENDS:

Figure 1. Effects of IL-6 on food intake, hypothalamic AMPK/mTOR activity and ATP content. (a) Effect of i.c.v. administration of IL-6 on food intake; pretreatment with AICAR blocks IL-6-induced anorexia (*n* = 12-15 animals per group). (b, c, d) Typical chromatographic run (b) depicting the ATP, ADP, and AMP fractions in control (black line) and in i.c.v. IL-6 treated animals (red line), as mean ATP content (e) and as AMP:ATP ratio (c, d). (e, f, g, h) Representative Western blots of four independent experiments showing hypothalamic lysates from Wistar rats. Phospho-AMPK^{thr172}, threonine-phosphorylated AMPK (e); phospho-ACC^{ser79}, serine phosphorylated ACC (f); phospho-p70S6Kinase^{thr389}, threonine phosphorylated p70S6Kinase (g); phospho-4EBP1^{thr70}, threonine phosphorylated 4EBP1 (h). Data are the means ± SEM. *$p< 0.05$, *vs.* control group; **$p< 0.01$, *vs.* control group; # $p< 0.05$, *vs.* other groups

Figure 2. Effects of IL-6 on food intake and hypothalamic PI(3)K/mTOR activity. (a) Pretreatment with Rapamycin blocks IL-6-induced anorexia (*n* = 10-12 animals per group). (b, c) Representative Western blots of four independent experiments showing hypothalamic lysates from Wistar rats. Phospho-p70S6Kinase^{thr389}, threonine phosphorylated p70S6Kinase (b); phospho-4EBP1^{thr70}, threonine phosphorylated 4EBP1 (c). (d) Pretreatment with LY294002 had no effect on anorectic response to IL-6 (*n* = 10-12). (e) Representative western blot of four independent experiments showing hypothalamic lysates from Wistar rats. Phospho-Akt^{ser473}, serine phosphorylated Akt. Data are the means ± SEM. **$p< 0.01$, *vs.* control group; # $p< 0.05$, *vs.* other groups.

Figure 3. AICAR effects on 12-h cumulative food intake and AMPK/mTOR signaling, in the hypothalami of control and exercised rats. (a) AICAR (2mM) was administered in control and exercised rats. Animals were immediately exposed to food for a 12-hour period (*n* = 12-15 animals per group). (b, c, d, e) Representative Western blots of five independent experiments showing hypothalamic lysates from Wistar rats. Phospho-AMPK^{thr172}, threonine-phosphorylated AMPK (b); phospho-ACC^{ser79}, serine phosphorylated ACC (c); phospho-p70S6Kinase^{thr389}, threonine phosphorylated p70S6Kinase (d); phospho-4EBP1^{thr70}, threonine phosphorylated 4EBP1 (e). Data are the means ±SEM. * p<0.05, ** p<0.0, *vs.* control group; # p<0.05, ## p<0.001 *vs.* AICAR-stimulated control group.

Figure 4. 2-DG effects on 12-h cumulative food intake and AMPK/mTOR signaling, in the hypothalami of control and exercised rats. (a) 2-DG (500mg/Kg) was administered in control and exercised rats. Animals were immediately exposed to food for a 12-hour period (*n* = 12-15 animals per group). (b, c, d, e) Representative Western blots of five independent experiments showing hypothalamic lysates from Wistar rats. Phospho-AMPK^{thr172}, threonine-phosphorylated AMPK (b); phospho-ACC^{ser79}, serine phosphorylated ACC (c); phospho-p70S6Kinase^{thr389}, threonine phosphorylated p70S6Kinase (d); phospho-4EBP1^{thr70}, threonine phosphorylated 4EBP1 (e). Data are the means ± SEM. * p<0.05, ** p<0.01, *vs.* control group; # p<0.05, # # p<0.01 *vs.* 2-DG-stimulated control group.

Figure 5. α-LA effects on 12-h cumulative food intake and AMPK/mTOR signaling, in the hypothalami of control and exercised rats. (a) α-LA (3μg) was administered in control and exercised rats. Animals were immediately exposed to food

for a 12-hour period (*n* = 8-10 animals per group). (b, c, d, e) Representative Western blots of four independent experiments showing hypothalamic lysates from Wistar rats. Phospho-AMPK^{thr172}, threonine-phosphorylated AMPK (b); phospho-ACC^{ser79}, serine phosphorylated ACC (c); phospho-p70S6Kinase^{thr389}, threonine phosphorylated p70S6Kinase (d); phospho-4EBP1^{thr70}, threonine phosphorylated 4EBP1 (e). Data are the means ± SEM. * $p<0.05$, ** $p<0.0$, *vs.* control group; # $p<0.05$, ## $p<0.01$ *vs.* α-LA-stimulated control group.

Figure 6. Leptin effects on 12-h cumulative food intake and AMPK/mTOR signaling, in the hypothalami of control and exercised rats. (a) Leptin (10^{-6} M) was administered in control and exercised rats. Animals were immediately exposed to food for a 12-hour period (*n* = 12-15 animals per group). (b, c, d, e) Representative Western blots of five independent experiments showing hypothalamic lysates from Wistar rats. Phospho-AMPK^{thr172}, threonine-phosphorylated AMPK (b); phospho-ACC^{ser79}, serine phosphorylated ACC (c); phospho-p70S6Kinase^{thr389}, threonine phosphorylated p70S6Kinase (d); phospho-4EBP1^{thr70}, threonine phosphorylated 4EBP1 (e). Data are the means ± SEM. * $p<0.05$, ** $p<0.0$, *vs.* control group; # $p<0.05$, ## $p<0.01$ *vs.* leptin-stimulated control group.

Figure 7. Blockade of leptin induced inhibition of food intake by anti–IL-6. Hypothalami from rats were prepared as described in *Research Design and Methods*. (a) Tissue extracts from control and exercised rats were immunoblotted with anti–IL-6 antibody. (b) Leptin was injected intracerebroventricularly in control rats, exercised rats and exercised rats pretreated with anti–IL-6 at the doses indicated, and the animals were immediately exposed to food for a 12-hour period (*n* = 10-12 animals per group). (c, d,

e, f) Representative Western blots of four independent experiments showing hypothalamic lysates from Wistar rats. Phospho-AMPK^{thr172}, threonine-phosphorylated AMPK (c); phospho-ACC^{ser79}, serine phosphorylated ACC (d); phospho-p70S6Kinase^{thr389}, threonine phosphorylated p70S6Kinase (e); phospho-4EBP1^{thr70}, threonine phosphorylated 4EBP1 (f). Data are the means ± SEM. #$p< 0.05$, vs. control, *$p< 0.05$, *vs.* exercise.

Figure 8. Blocking effects of AICAR and Rapamycin on leptin-induced anorexia. (a) Effect of i.c.v. administration of leptin on exercised rats; pretreatment with AICAR or Rapamycin (n = 8-10 animals per group). (b, c, d, e) Representative Western blots of five independent experiments showing hypothalamic lysates from Wistar rats. Phospho-AMPK^{thr172}, threonine-phosphorylated AMPK (b); phospho-ACC^{ser79}, serine phosphorylated ACC (c); phospho-p70S6Kinase^{thr389}, threonine phosphorylated p70S6Kinase (d); phospho-4EBP1^{thr70}, threonine phosphorylated 4EBP1 (e). Data are the means ± SEM. *$p< 0.05$, *vs.* other groups.

Fig. 1

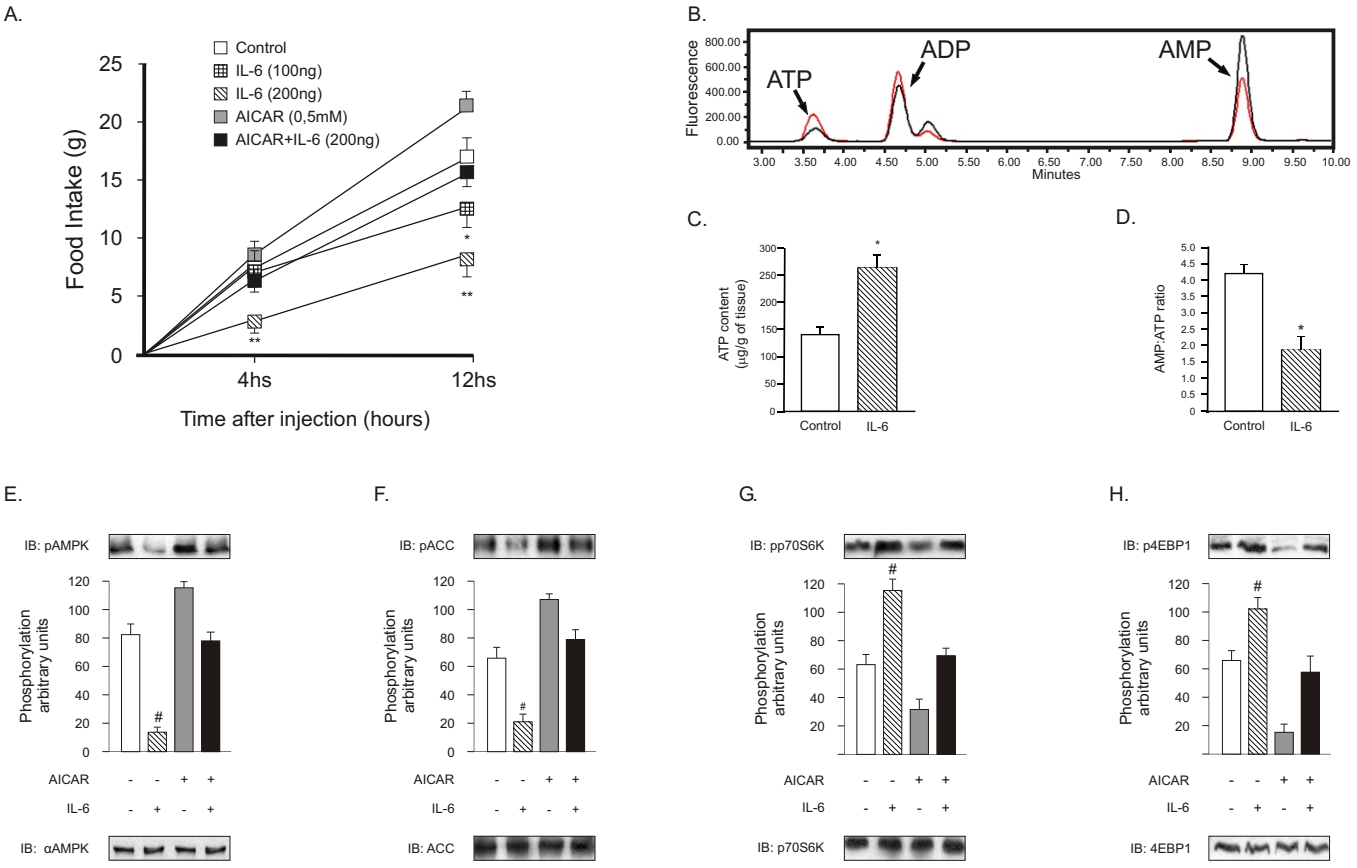

A.
Control
IL-6 (100ng)
IL-6 (200ng)
AICAR (0,5mM)
AICAR+IL-6 (200ng)
Food Intake (g)
Time after injection (hours)
4hs
12hs
B.
ATP
ADP
AMP
Fluorescence
Minutes
C.
ATP content (µg/g of tissue)
Control
IL-6
D.
AMP:ATP ratio
Control
IL-6
E.
IB: pAMPK
Phosphorylation arbitrary units
AICAR
IL-6
IB: αAMPK
F.
IB: pACC
Phosphorylation arbitrary units
AICAR
IL-6
IB: ACC
G.
IB: pp70S6K
Phosphorylation arbitrary units
AICAR
IL-6
IB: p70S6K
H.
IB: p4EBP1
Phosphorylation arbitrary units
AICAR
IL-6
IB: 4EBP1

Fig. 2

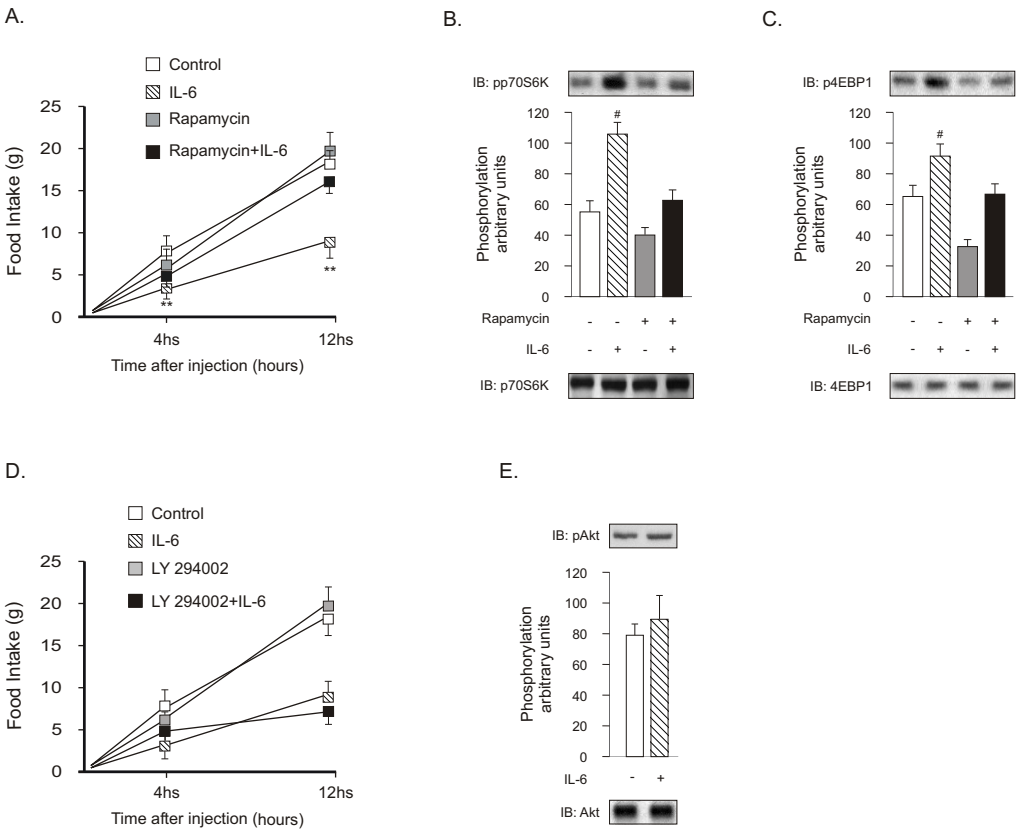
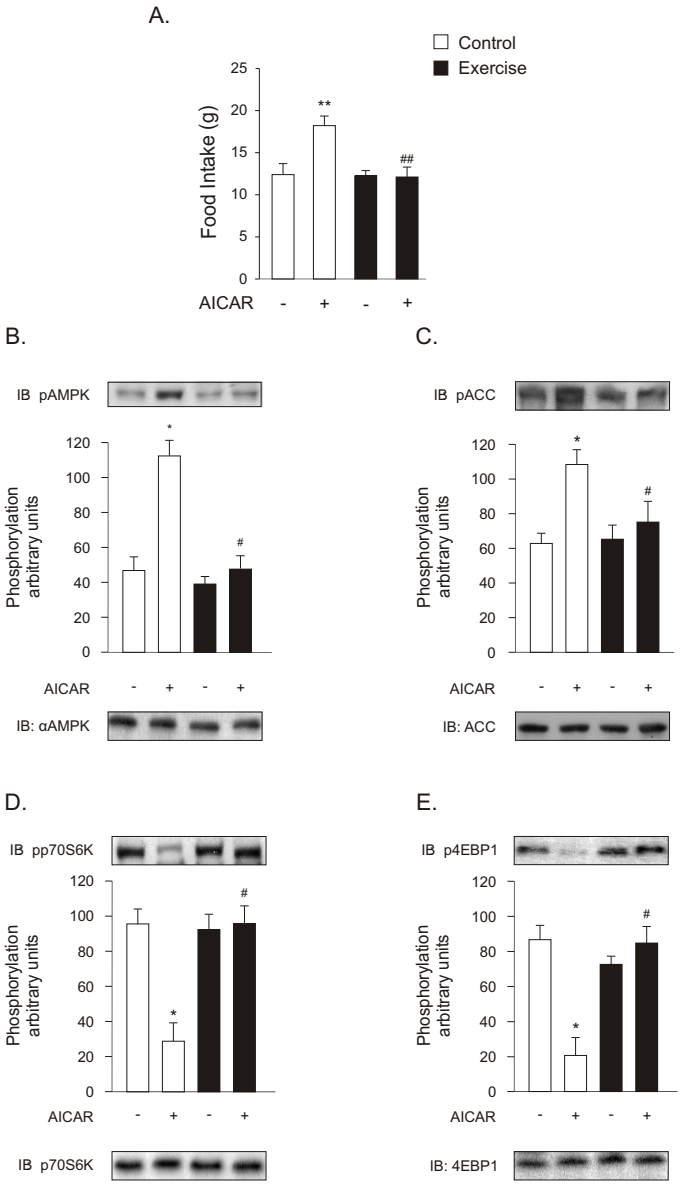

A.
Control
IL-6
Rapamycin
Rapamycin+IL-6
Food Intake (g)
25
20
15
10
5
0
4hs
12hs
Time after injection (hours)
**
**
B.
IB: pp70S6K
Phosphorylation arbitrary units
120
100
80
60
40
20
0
#
Rapamycin - - + +
IL-6 - + - +
IB: p70S6K
C.
IB: p4EBP1
Phosphorylation arbitrary units
120
100
80
60
40
20
0
#
Rapamycin - - + +
IL-6 - + - +
IB: 4EBP1
D.
Control
IL-6
LY 294002
LY 294002+IL-6
Food Intake (g)
25
20
15
10
5
0
4hs
12hs
Time after injection (hours)
E.
IB: pAkt
Phosphorylation arbitrary units
120
100
80
60
40
20
0
IL-6 - +
IB: Akt

Fig. 3


A.
Control
Exercise
Food Intake (g)
25
20
15
10
5
0
**
##
AICAR - + - +
B.
IB pAMPK
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
AICAR - + - +
IB: αAMPK
C.
IB pACC
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
AICAR - + - +
IB: ACC
D.
IB pp70S6K
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
AICAR - + - +
IB p70S6K
E.
IB p4EBP1
Phosphorylation arbitrary units
120
100
80
60
40
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0
*
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AICAR - + - +
IB: 4EBP1

Fig. 4

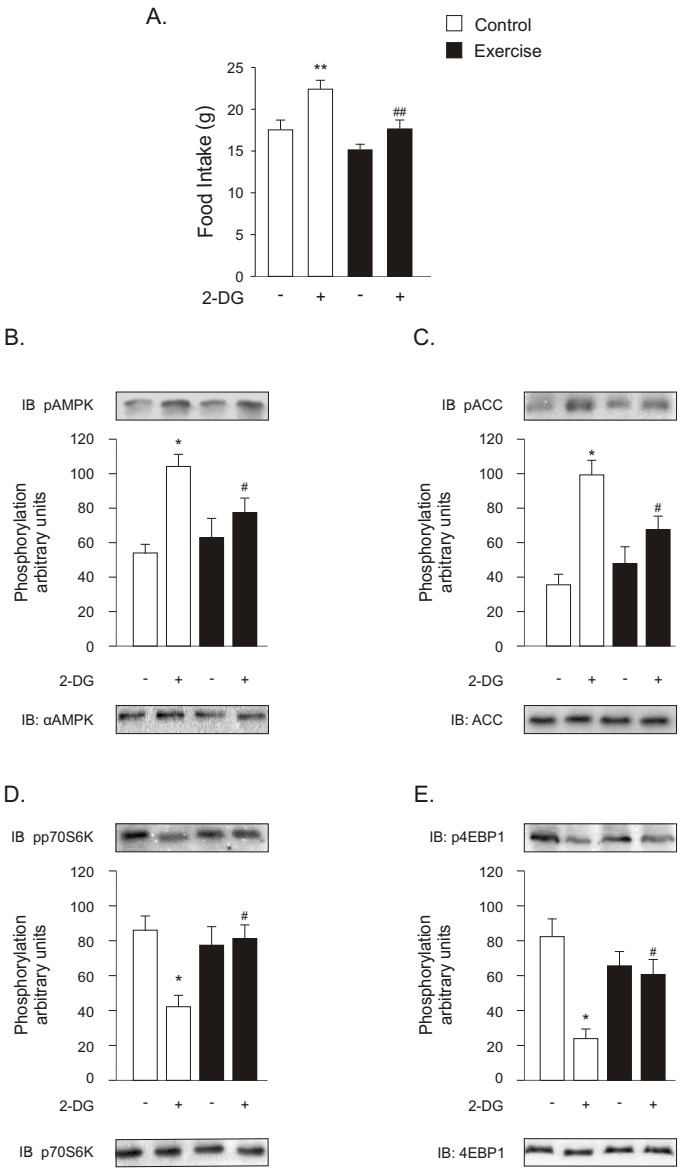
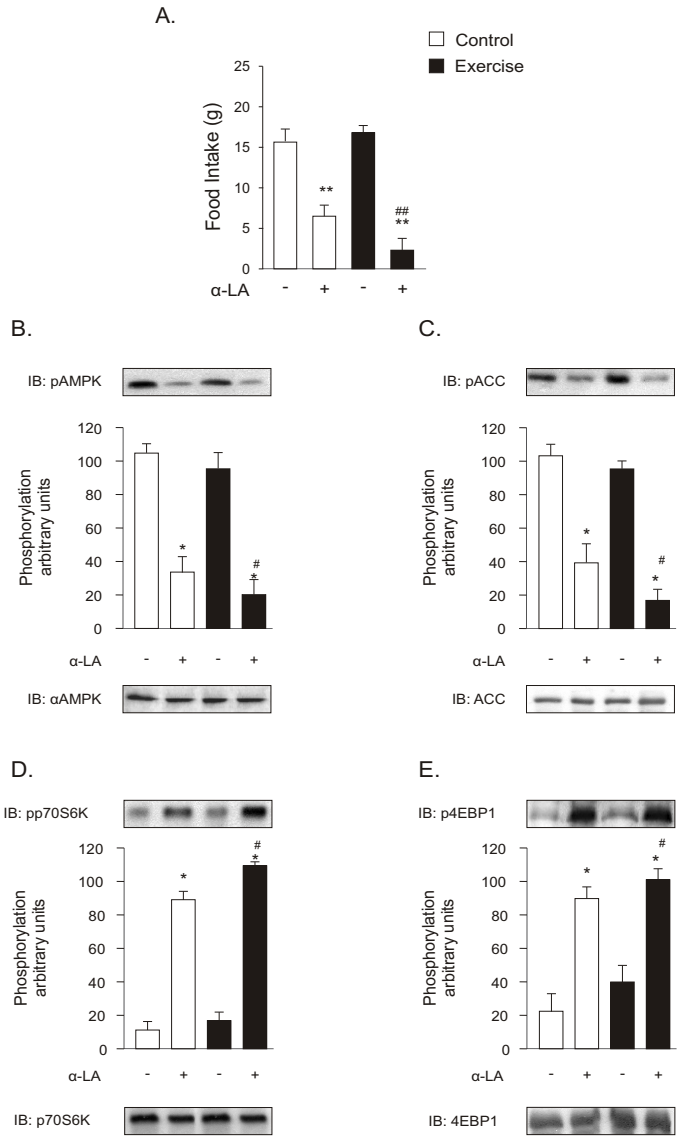

A.
Control
Exercise
Food Intake (g)
25
20
15
10
5
0
**
##
2-DG
-
+
-
+
B.
IB pAMPK
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
2-DG
-
+
-
+
IB: αAMPK
C.
IB pACC
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
2-DG
-
+
-
+
IB: ACC
D.
IB pp70S6K
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
2-DG
-
+
-
+
IB p70S6K
E.
IB: p4EBP1
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
2-DG
-
+
-
+
IB: 4EBP1

Fig. 5


A.
Control
Exercise
Food Intake (g)
25
20
15
10
5
0
**
##
**
α-LA
-
+
-
+
B.
IB: pAMPK
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
*
α-LA
-
+
-
+
IB: αAMPK
C.
IB: pACC
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
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*
α-LA
-
+
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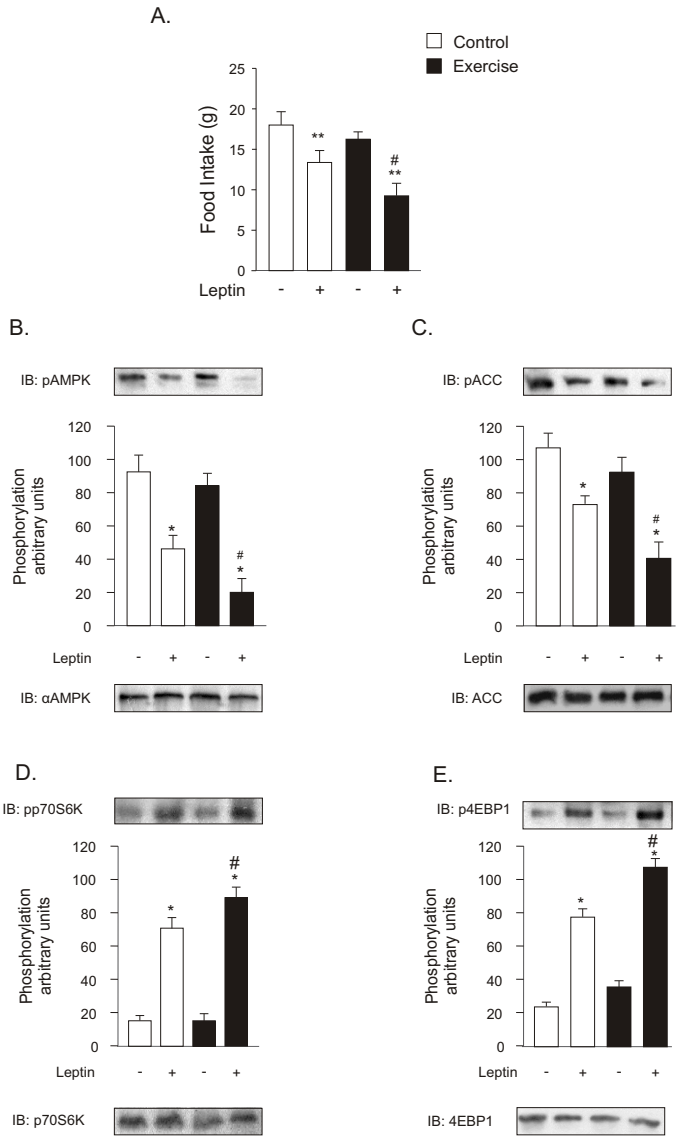
IB: ACC
D.
IB: pp70S6K
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
*
α-LA
-
+
-
+
IB: p70S6K
E.
IB: p4EBP1
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
*
α-LA
-
+
-
+
IB: 4EBP1

Fig. 6


A.
Control
Exercise
Food Intake (g)
25
20
15
10
5
0
**
#
**
Leptin - + - +
B.
IB: pAMPK
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
*
Leptin - + - +
IB: αAMPK
C.
IB: pACC
Phosphorylation arbitrary units
120
100
80
60
40
20
0
*
#
*
Leptin - + - +
IB: ACC
D.
IB: pp70S6K
Phosphorylation arbitrary units
120
100
80
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40
20
0
*
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*
Leptin - + - +
IB: p70S6K
E.
IB: p4EBP1
Phosphorylation arbitrary units
120
100
80
60
40
20
0
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#
*
Leptin - + - +
IB: 4EBP1

Fig. 7

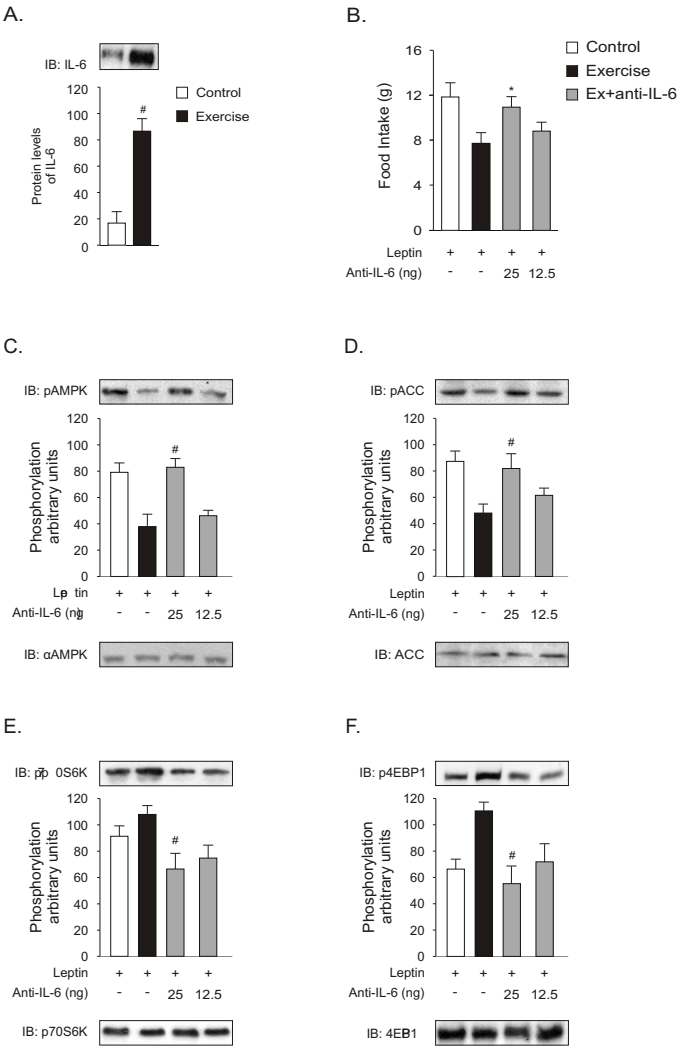
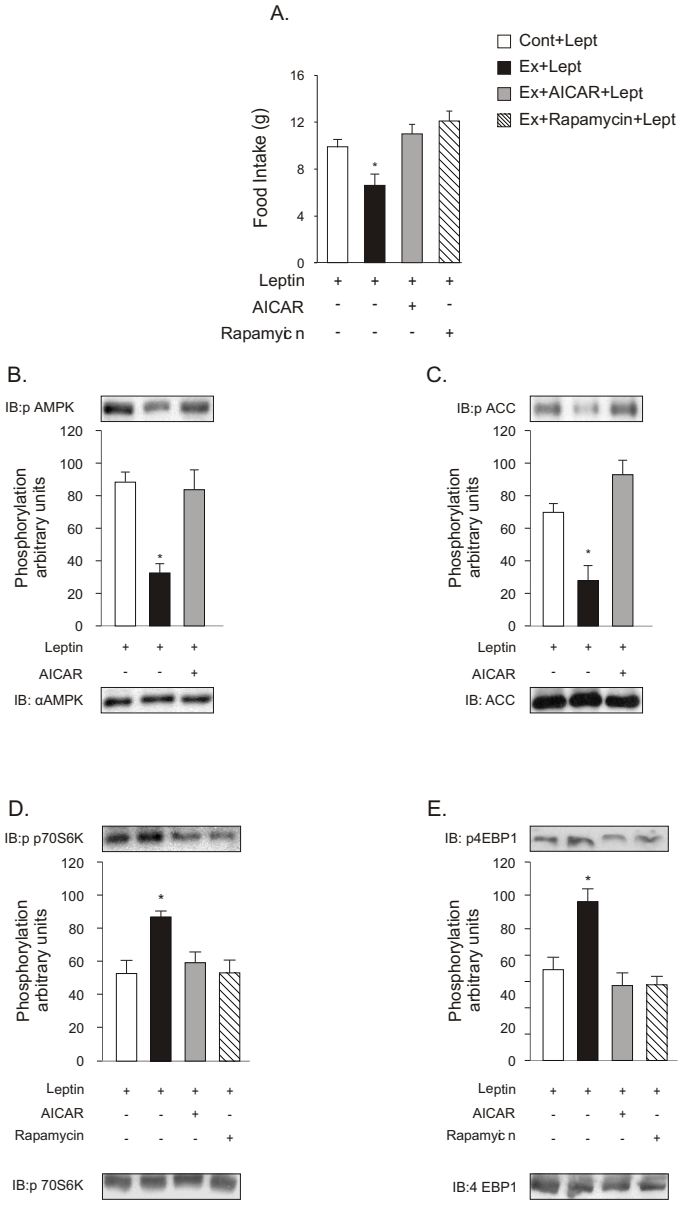

A.
IB: IL-6
Protein levels of IL-6
Control
Exercise
B.
Food Intake (g)
Control
Exercise
Ex+anti-IL-6
Leptin
Anti-IL-6 (ng)
25
12.5
C.
IB: pAMPK
Phosphorylation arbitrary units
IB: αAMPK
D.
IB: pACC
IB: ACC
E.
IB: p70S6K
IB: p4EBP1
F.
IB: 4EBP1

Fig. 8


A.
Cont+Lept
Ex+Lept
Ex+AICAR+Lept
Ex+Rapamycin+Lept
Food Intake (g)
16
12
8
4
0
Leptin + + + +
AICAR - - + -
Rapamycin - - - +
B.
IB:p AMPK
Phosphorylation arbitrary units
120
100
80
60
40
20
0
Leptin + + +
AICAR - - +
IB: αAMPK
C.
IB:p ACC
Phosphorylation arbitrary units
120
100
80
60
40
20
0
Leptin + + +
AICAR - - +
IB: ACC
D.
IB:p p70S6K
Phosphorylation arbitrary units
120
100
80
60
40
20
0
Leptin + + + +
AICAR - - + -
Rapamycin - - - +
IB:p 70S6K
E.
IB: p4EBP1
Phosphorylation arbitrary units
120
100
80
60
40
20
0
Leptin + + + +
AICAR - - + -
Rapamycin - - - +
IB:4 EBP1

4- CONCLUSÕES

A modulação das vias hipotalâmicas AMPK / mTOR pelo exercício agudo, favorece a sinalização da leptina neste tecido e, com isso, constitui um mecanismo fisiológico de grande relevância no que diz respeito à prevenção e tratamento da obesidade.

Estudos recentes têm demonstrado a existência de uma relação entre modulação da ação anorexigênica da leptina pela AMPK e desenvolvimento da obesidade (Martin, Alquier *et al.*, 2006). Assim, a ação "anti-obesidade" exercida pela leptina, provavelmente seja decorrente da inibição parcial da sinalização hipotalâmica da AMPK observada após o exercício agudo.

Já que, como a leptina, a IL -6 reduz a ingestão alimentar modulando a atividade da AMPK no SNC, conforme nossos resultados sugerem. Fatores que acarretam prejuízos à sinalização da AMPK, como o exercício agudo, favorecem a transmissão do sinal pela leptina e, conseqüentemente, a redução do apetite.

Concluindo, o exercício altera a resposta das vias de sinalização AMPK / mTOR no SNC e assim, aumenta a sensibilidade do hipotálamo à leptina. A capacidade das vias AMPK / mTOR em responder à leptina, representa um importante mecanismo molecular, envolvido na modulação do apetite pelo exercício agudo. Além disso, nossos resultados dão suporte à hipótese que, através da IL-6 produzida durante / após o exercício, as vias AMPK e mTOR interagem no hipotálamo para modular o apetite.

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6- APÊNDICES

MATERIAIS E MÉTODOS

Anticorpos e Reagentes

Reagentes utilizados na confecção de gel SDS-poliacrilamida para eletroforese e *immunoblotting* foram adquiridos pela *Bio-Rad* (Richmond, CA, USA). *Tris[hydroxymethyl]amino-methane* (Tris), aprotinina, ATP, *dithiothreitol* (DTT), *phenylmethylsulfonyl fluoride* (PMSF), Triton X-100, Tween 20, glicerol, and *bovine serum albumin* (albumina - fração V) foram obtidos pela *Sigma Aldrich* (St. Louis, MO, USA). ^{125}*I-protein A* (Iodo radioativo) e membranas de nitrocelulose (Hybond ECL, 0.45 μm) foram obtidos pela *Amersham Pharmacia Biotech United Kingdom Ltd.* (Buckinghamshire, United Kingdom). *Ketamin* (Cloridrato de Cetamina) foi fornecida pela *Parke-Davis* (São Paulo, SP, Brazil); *Diazepan* e *Thiopethal* foram obtidos pela *Cristália* (Itapira, SP, Brazil).

Anti-p-[Ser^{79}] ACC (*rabbit polyclonal,* #07-184) obtido pela *Upstate Biotechnology* (Charlottesville, VA, USA). Anti-ACC (*goat polyclonal*, sc-26816) e anti-IL-6 (*rabbit polyclonal*, sc-7920) foram obtidos pela *Santa Cruz Biotechnology Inc.* Anti-p-[Thr^{172}] AMPKα (*rabbit polyclonal*, #2531), anti-AMPKα (*rabbit polyclonal*, #2532), anti-p-[Thr389] p70S6K (*rabbit polyclonal*, #9205), anti-p70S6K (*rabbit polyclonal*, #9202), anti-p-[Thr70]4EBP1 (*rabbit polyclonal*, #9455), anti-4EBP1 (*rabbit polyclonal*, #9452), anti-p- [Ser 473]Akt (*rabbit polyclonal*, #9271), e anti-Akt (*rabbit polyclonal*, #9272) foram obtidos pela *Cell Signalling Technology* (Beverly, MA, USA). Leptina, *LY294002*, e Interleucina-6 foram obtidos pela *Calbiochem* (San Diego, CA, USA); *5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside* (AICAR), 2-Deoxi-D-glicose e Ácido α-lipóico foram obtidos pela *Sigma Chemical Co.* (St. Louis, MO). Rapamicina foi fornecida por *LC Laboratories* (Woburn, MA, USA). Os reagentes de rotina utilizados foram obtidos pela *Sigma Chemical Co.* (St. Louis, MO).

Animais e Cirurgia de Canulação

Foram utilizados ratos (*Rattus norvergicus*) machos, da linhagem Wistar entre quatro e dez semanas de idade, provenientes do Biotério Central da UNICAMP (CEMIB), os quais foram alimentados com ração comercial para roedores (Nuvilab CR-1) da Nuvital,

oferecida *ad libitum*, assim como água. Os animais foram mantidos em gaiolas coletivas sob condições padronizadas de iluminação (ciclo claro/escuro de 12 horas) e temperatura de 22 ± 2° C.

Para canulação do terceiro ventrículo hipotalâmico, os animais (250 - 300g) foram previamente anestesiados por via intraperitonial com uma mistura de 1:1 (0,8 ml) de cloridrato de cetamina (50 mg/ml) e diazepan (5,0 mg/ml). Depois de testados os reflexos corneano e pedioso os animais foram posicionados no aparelho de estereotaxia para implantação de uma cânula no terceiro ventrículo do hipotálamo, seguindo as coordenadas do Atlas Paxinos-Watson: 0.5 mm ântero-posterior; 8.5 mm profundidade, em relação ao bregma (Carvalheira, Siloto *et al.*, 2001).

Após o período de uma semana de recuperação da cirurgia estereotáxica, os animais foram submetidos a um teste de resposta de ingestão hídrica subseqüente ao tratamento com angiotensina II (2,0 µl de solução 10^{-6} M) para avaliação da adequação da posição da cânula. Ratos com resposta positiva à angiotensina II foram selecionados.

Protocolo de Exercício

Os ratos passaram por um período (2 dias) de adaptação ao meio líquido. No dia do experimento, estes animais foram divididos em grupos (4 animais por grupo) e submetidos a sessão aguda de natação, dividida em dois períodos de 3hs, separados por 45 minutos de descanso. A natação foi realizada em baldes plásticos de 45cm de diâmetro e profundidade de ≈50 cm. A temperatura da água foi mantida entre 34-35°C.

Imediatamente após as últimas 3hs de natação, alguns ratos receberam infusão icv de drogas.

Tratamentos

- Injeções intraperitoneais – ip - (200µl):

2-DG: 500mg/kg (controle recebeu salina)

- Injeções intracerebroventriculares – icv - (3µl):

IL-6: 100ng ou 200ng (controle recebeu salina)

AICAR (0.5 ou 2.0mM) (controle recebeu salina)

Rapamicina (25µg) (controle recebeu salina)

Ácido α-Lipóico (3µg) (controle recebeu salina)

Leptina (10^{-6}M) (controle recebeu salina)

LY294002 (50µM) (controle recebeu salina)

anti-IL-6 (12.5; 50 ou 25ng) (controle recebeu salina)

Avaliação da Ingestão Alimentar

Os animais foram colocados individualmente em gaiolas metabólicas e aclimatados ao ambiente por 2 dias. No terceiro dia, estes animais foram divididos nos grupos "controle" e "exercício" e, mantidos em jejum por 6 horas (duração do exercício). Imediatamente após a última sessão de natação, os ratos foram submetidos aos diferentes tratamentos por via icv, e a ingestão alimentar foi verificada após 4hs e/ou 12hs. A avaliação do consumo alimentar se deu por aferição da diferença de peso entre o alimento oferecido e o alimento restante na gaiola.

Dissecção do hipotálamo

Os animais foram anestesiados por meio da administração intraperitonial de tiopental sódico (0,6 ml; 15 mg/kg) e a perda dos reflexos pedioso e corneano foi utilizada como controle da anestesia. Após os diferentes tratamentos, o crânio foi aberto, o hipotálamo retirado e em seguida homogeneizado em aproximadamente 10 volumes de tampão de solubilização (1% Triton X-100; 100 mM Tris pH 7,4; contendo 100 mM de pirofosfato de sódio; 100 mM de fluoreto de sódio; 10 mM de vanadato de sódio; 2 mM PMSF e 0,1 mg de aprotinina/mL) a 4° em "Politron PTA 20S generator" (Brinkmann Instruments mode PT 10/35) com velocidade máxima por 30 segundos. O homogeneizado

foi então centrifugado a 11.000 rpm por 30 minutos para remoção de material insolúvel. No sobrenadante foi determinada a concentração de proteínas utilizando-se o método de Bradford (Bradford, 1976).

Western Blot

Após rápida fervura (5 minutos) as amostras foram aplicadas em gel de poliacrilamida para separação por eletroforese (SDS-PAGE). As proteínas separadas por SDS-PAGE foram transferidas para membrana de nitrocelulose em aparelho de transferência da BIO-RAD durante 120 min a 80 Volts, em gelo, e banhadas com tampão de transferência. As membranas de nitrocelulose foram incubadas por 12 a 14 horas com anticorpo específico. A ligação do anticorpo a proteínas não específicas foi minimizada pela pré-incubação das membranas de nitrocelulose com tampão de bloqueio (5% de leite em pó desnatado; 10 mmol/l de Tris, 150 mmol/l de NaCl, 0,02% de Tween 20) por 1,5 hora. A detecção do complexo antígeno-anticorpo fixo à membrana de nitrocelulose foi obtida por meio de tratamento com ^{125}I-proteína A em 10 ml de tampão de bloqueio por 2 horas em temperatura ambiente e exposição a filmes de RX Kodak a 80°C

Cromatografia

As análises cromatográficas foram realizadas no equipamento *Waters Alliance equipment series 2695* (Milford, MA, USA).

A fluorescência emitida pelos compostos (ATP, ADP, AMP e adenosina) foi monitorada através dos comprimentos de onda de excitação e emissão (280 e 420nm, respectivamente). A separação cromatográfica destes compostos aconteceu em temperature ambiente, através do uso de uma coluna de fase-reversa *Cosmosil 5C18-MS column* (150X4.6 mm i.d.; tamanho da partícula: 5 µm), com um *Cosmosil guard column* (5C18-MS 10X4.6 mm), obtido pela *Phenomenex* (Torrance, CA, USA). A fase móvel foi composta por 50 mmol/L KH_2PO_4, 25 mmol/L ácido cítrico (pH 4.5), e metanol (90:10, v/v), que foi preparado imediatamente antes do uso e filtrado em filtro 0.45 µm (*Millipore*, Milford, MA, USA). A coluna foi equilibrada e eluída em condições isocráticas,

usando fluxo de 1.0 ml/min. O tempo de corrida cromatográfica de cada análise foi de 20 minutos. Alíquotas de 25 µl foram adicionadas ao sistema HPLC. Controle do sistema, aquisição e processamento dos dados foram realizados em computador *PC-Pentium IV Processor (Dell)* , sistema operacional *Microsoft Windows XP* (versão 2003). O software de cromatografia utilizado foi *Waters Empower 2002*.

Para validação dos dados obtidos, foi realizada uma corrida cromatográfica, com amostras de calibração em 4 níveis (foram realizadas triplicatas). As curvas padrão de calibração para o ATP variaram entre 0,025 e 10,0 µmol/L, onde linearidade (R>0.999), podendo ser descrita como a seguinte equação de regressão linear:

$y = 0.4992*x - 0.0463$ (n=4, P<0.0001, r=0.9997), onde y corresponde à concentração de ATP, expressa em micromoles; e x corresponde à área do pico cromatográfico.

Análise Estatística

Os resultados foram expressos como média ± erro padrão da média. Quando comparados dois grupos, foi utilizado o teste *t* de *Student* para dados não pareados. Quando necessário, utilizou-se análise de variância (ANOVA *Bonferroni post test*). Foi adotado o nível de significância p<0.05.

OUTROS TRABALHOS DESENVOLVIDOS DURANTE O MESTRADO

Original Article

Exercise Improves Insulin and Leptin Sensitivity in Hypothalamus of Wistar Rats

Marcelo B.S. Flores, Maria Fernanda A. Fernandes, Eduardo R. Ropelle, Marcel C. Faria, Mirian Ueno, Lício A. Velloso, Mario J.A. Saad, and José B.C. Carvalheira

Prolonged exercise of medium to high intensity is known to promote a substantial effect on the energy balance of rats. In male rats, moderately to severely intense programs lead to a reduction in food intake. However, the exact causes for the appetite-suppressive effects of exercise are not known. Here, we show that intracerebroventricular insulin or leptin infusion reduced food intake in exercised rats to a greater extent than that observed in control animals. Exercise was associated with a markedly increased phosphorylation/activity of several proteins involved in leptin and insulin signal transduction in the hypothalamus. The regulatory role of interleukin (IL)-6 in mediating the increase in leptin and insulin sensitivity in hypothalamus was also investigated. Treatment with insulin or leptin markedly reduced food intake in exercised rats that were pretreated with vehicle, although no increase in sensitivity to leptin- and insulin-induced anorexia after pretreatment with anti–IL-6 antibody was detected. The current study provides direct measurements of leptin and insulin signaling in the hypothalamus and documents increased sensitivity to these hormones in the hypothalamus of exercised rats in an IL-6–dependent manner. These findings provide support for the hypothesis that the appetite-suppressive actions of exercise may be mediated by the hypothalamus. ***Diabetes*** **55:2554–2561, 2006**

Prolonged exercise of medium to high intensity is known to profoundly affect energy balance (1–3). Studies of individuals who have maintained significant weight loss for >1 year have demonstrated that dieters who achieve long-term success are often those who engage in regular and extensive exercise programs (4). Although the energy expenditure aspects of such exercise may contribute to the effects of weight maintenance, it has been suggested that even acute exercise may also contribute to the energy balance by altering appetite and reducing food intake. However, the mechanisms underlying the effects of exercise on food intake have not yet been identified.

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IL, interleukin; IRS, insulin receptor substrate; JAK, janus kinase; PI, phosphatidylinositol; STAT, signal transducer and activator of transcription.

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The circulating peptide leptin is secreted predominantly by white adipose tissue and provides feedback information on the extent of the body's fat stores to hypothalamic leptin receptors (ObRs) that coordinate food intake and body weight homeostasis (5,6). Wild-type ObRs possess a number of signaling capabilities; these include activation of the janus kinase–signal transducer and activator of transcription (JAK-STAT) (7–11) and mitogen-activated protein kinase pathways and stimulation of tyrosine phosphorylation of insulin receptor substrate (IRS)-1, IRS-2, and phosphatidylinositol (PI) 3-kinase (10–13).

Insulin acts at the same hypothalamic areas as leptin to suppress feeding (6,14). The insulin receptor is a protein tyrosine kinase that is activated by insulin binding, undergoing rapid autophosphorylation and phosphorylating intracellular protein substrates, including IRS-1 and -2 (15,16). After tyrosine phosphorylation, the IRSs act as docking proteins for several SH2 (Src homology 2) domain–containing proteins, including PI 3-kinase, Grb2 (growth factor receptor-bound protein-2), SHP2 (src-homology phosphatase-2), Nck, and Fyn (17–21). PI 3-kinase activates two kinases: phosphoinositide-dependent protein kinase 1, which phosphorylates Akt on threonine 308, and a putative phosphoinositide-dependent protein kinase 2, which phosphorylates Akt on serine 473, leading to an increase in Akt kinase activity (22).

The level of circulating interleukin (IL)-6 increases dramatically in response to exercise (23), with IL-6 being produced by working muscle (24,25) and adipose tissue (26–28). IL-6 seems to have several important roles in metabolism, including induction of lipolysis (26,29) and enhancement of insulin sensitivity when injected into IL-6–deficient mice (30). Furthermore, it appears that centrally acting IL-6 plays a role in the regulation of appetite, energy expenditure, and body composition (30). Intracellular interactions between different signaling systems may enhance or counterregulate hormone actions. Thus, it is possible that the effects of acute exercise on central insulin and leptin sensitivity may be dependent on IL-6. The status of leptin and insulin signaling in hypothalamus has not previously been assessed in rats after acute exercise. We therefore examined hypothalamic JAK-STAT and IRS-1/2–PI 3-kinase signaling pathways as well as the role of IL-6 in insulin and leptin signaling in rats after acute exercise.

RESEARCH DESIGN AND METHODS

The reagents for SDS-PAGE and immunoblotting were from Bio-Rad. Tris, aprotinin, ATP, dithiothreitol, phenylmethylsulfonyl fluoride, Triton X-100, Tween 20, glycerol, and BSA (fraction V) were from Sigma (St. Louis, MO). Protein A-Sepharose 6MB, ^{125}I-protein A, and nitrocellulose paper (Hybond

ECL, 0.45 μm) were from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). Sodium amobarbital (Amytal) and human recombinant insulin (Humulin R) were from Eli Lilly (Indianapolis, IN). Leptin was from Calbiochem (San Diego, CA). Ketamine hydrochloride was from Cristália (Itapira, Brazil). Antibodies to insulin receptor, IRS-1, IRS-2, Akt, JAK2, ObR, SOCS3 (suppressor of cytokine signaling 3), PTP1b (protein-tyrosine phosphatase 1b), STAT3, and IL-6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Akt phosphoserine 473–specific and phosphothreonine 308–specific antibodies and the STAT3 phosphotyrosine 705–specific antibody were from New England Biolabs (Beverly, MA), and the antibody to the p85 subunit of PI 3-kinase was from Upstate Biotechnology (Lake Placid, NY). Routine reagents were purchased from Sigma, unless otherwise specified.

Animals and surgical procedure. Male Wistar rats (200–250 g) from the University of Campinas Central Animal Breeding Center were used in the experiments. All experiments involving animals were in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and were approved by the ethics committee at the University of Campinas. Rats were maintained on a 12-h light/dark cycle and were provided free access to water and standard rodent chow before the exercise; they were randomly assigned to one of two groups: those exercised for 6 h or control rats. After an overnight fast, the rats were anesthetized with ketamine hydrochloride plus diazepan and positioned on a Stoelting stereotaxic apparatus. At 10 days before the exercise protocol, the catheter was implanted into the third ventricle as previously described (10). After a 1-week recovery period, catheter placement was confirmed by a positive drinking response after administration of angiotensin II (40 ng per 2 μl), and animals that did not drink 5 ml of water within 15 min after treatment were not included in the experiment.

Exercise protocol. Rats were acclimated to swimming for 10 min per day for 2 days. The swimming protocol was performed as previously described (31). The rats swam in groups of three in plastic barrels 45 cm in diameter that were filled to a depth of 50 cm, and the water temperature was maintained at 34–35°C. Animals performed two 3-h exercise bouts separated by one 45-min rest period.

Treatments and measurement of food intake. After the last bout of exercise, animals were injected (2-μl bolus injection i.c.v.) with either vehicle, insulin (Eli Lilly), or leptin (rat leptin from National Institutes of Health) at the doses indicated. Thereafter, standard chow was given, and food intake was determined by measuring the difference between the weight of chow given and the weight of chow at the end of a 12-h period. Similar studies were carried out after the last bout of exercise in rats that were initially intracerebroventricularly injected with anti–IL-6 (rabbit anti–IL-6 at the doses indicated; Santa Cruz Biotechnology) or vehicle and then, after 30 min, with insulin or leptin. In preliminary experiments, we determined plasma glucose levels in animals that received intracerebroventricular insulin infusion. Plasma glucose was not altered by third ventricle insulin or saline microinjection.

Western blot analysis. Immediately after the last exercise bout, animals were treated with vehicle, insulin, or leptin, according to the protocols described in the preceding section, and then they were decapitated, and the hypothalami were removed at the time points indicated. The hypothalami were minced coarsely and homogenized immediately in solubilization buffer containing 100 mmol/l Tris (pH 7.6), 1% Triton X-100, 150 mmol/l NaCl, 0.1 mg aprotinin, 35 mg/ml phenylmethylsulfonyl fluoride, 10 mmol/l Na_3VO_4, 100 mmol/l NaF, 10 mmol/l $Na_4P_2O_7$, and 4 mmol/l EDTA, using a polytron PTA 20S generator (Model PT 10/35; Brinkmann Instruments, Westbury, NY) operated at maximum speed for 30 s and clarified by centrifugation. Equal amounts of protein were used for immunoprecipitation followed by Western blot analysis with the indicated antibodies and ^{125}I-protein A. ^{125}I-protein A bound to anti-peptide antibodies was detected by autoradiography, using preflashed Kodak XAR film (Eastman Kodak, Rochester, NY) with Cronex Lightning Plus intensifying screens (DuPont, Wilmington, DE) at −80°C for 12–48 h. Band intensities were quantitated by optical densitometry (Scion Image software; ScionCorp, Frederick, MD) of the developed autoradiographs.

PI 3-kinase assay. Aliquots of supernatants containing equal amounts of protein were incubated overnight at 4°C, using antibodies against IRS-1 or -2, and the immunocomplexes were precipitated with a 50% solution of protein A-Sepharose 6MB. In vitro PI 3-kinase assays were performed as previously described (17). The ^{32}P-labeled 3-P-PI was quantitated using Scion Image software.

Statistical analysis. Where appropriate, the results are expressed as the means ± SE accompanied by the indicated number of rats used in experiments. Comparisons among groups were performed using parametric two-way ANOVA, where F ratios were significant; further comparisons were performed using the Bonferroni test.

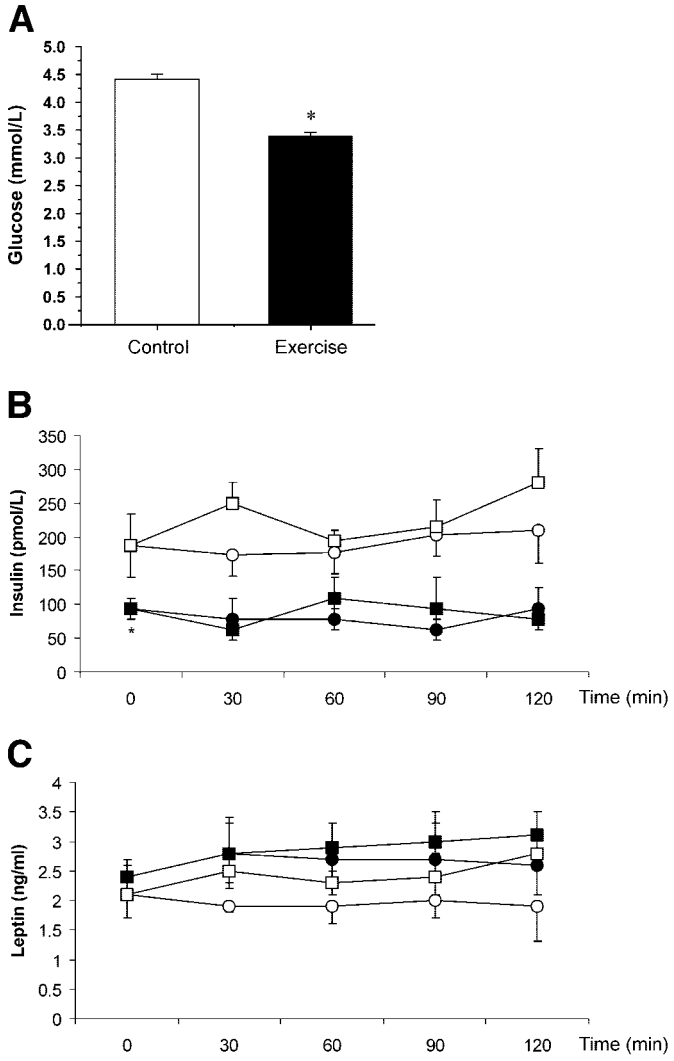

FIG. 1. Physiological characteristics of control and exercised rats. Effects of exercise on plasma glucose concentration (mmol/l) (*A*), plasma insulin concentration (pmol/l) (*B*), and plasma leptin concentration (ng/ml) (*C*). Data are the means ± SE, $n = 6$ animals per group. *$P < 0.01$ vs. control. □, control: insulin i.c.v.; ○, control: leptin i.c.v.; ■, exercise: insulin i.c.v.; ●, exercise: leptin i.c.v.

RESULTS

Physiological parameters measured in basal conditions after 6 h of exercise. The plasma glucose level was lower in the exercised group compared with the control group (3.39 vs. 4.41 mmol/l, respectively) (Fig. 1*A*), and the insulin levels were also lower (93.6 vs. 187.2 pmol/l) (Fig. 1*B*). Exercise did not, however, reduce plasma leptin (2.1 vs. 2.4 ng/ml) (Fig. 1*C*). As shown in Figs. 1*B* and *C*, insulinemia and leptinemia were not altered by third ventricle microinjection of insulin or leptin.

Intracerebroventricular leptin reduces food intake and activates the hypothalamic JAK-STAT pathway in exercised rats to a greater extent than in control animals. The effect of leptin (5, 10, and 20 μg), or its vehicle, on the control of food intake was studied by measuring the total food intake for 12 h after a session of exercise and a single intracerebroventricular injection of leptin or its vehicle. Leptin induced reductions in the 12-h food intake in both exercised and control rats in a concentration-dependent manner. In the exercised rats, leptin (5, 10, and 20 μg) reduced food intake by 38.5, 44.6, and

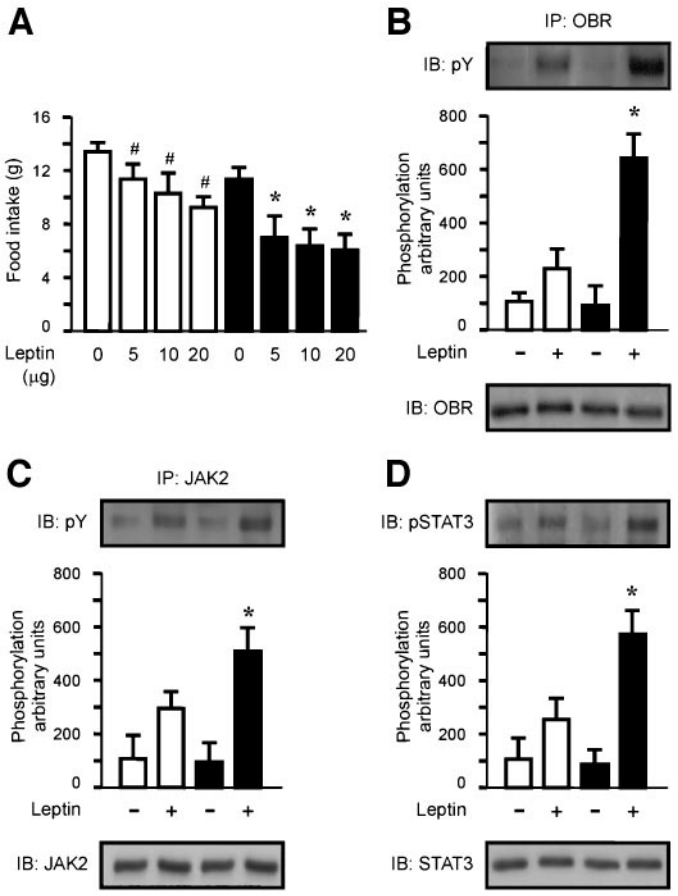

FIG. 2. Leptin inhibition of the 12-h cumulative food intake and leptin signaling in the hypothalamus of control and exercised rats. Vehicle (−) or leptin (+) was injected intracerebroventricularly after a 6-h session of exercise, and rats were immediately exposed to food for 12 h. Data are the means ± SE of 8–14 animals per group (*A*). At 15 min after the infusion, tissue extracts were immunoprecipitated (IP) with anti-ObR and anti-JAK2 and immunoblotted (IB) with anti-phosphotyrosine antibody (pY) (*B* and *C*, *upper panels*). Whole-tissue extracts were immunoblotted with pSTAT3 antibody (*D*, *upper panel*). Stripped membranes were reblotted with anti-ObR, anti-JAK2, and anti-STAT3 antibodies (*B–D*, *lower panels*). The results of scanning densitometry were expressed as arbitrary units. Columns and bars represent the means ± SE, $n = 8$ animals per group. *$P < 0.05$, leptin control vs. leptin exercise; #$P < 0.05$, leptin control vs. control. □, control; ■, exercise.

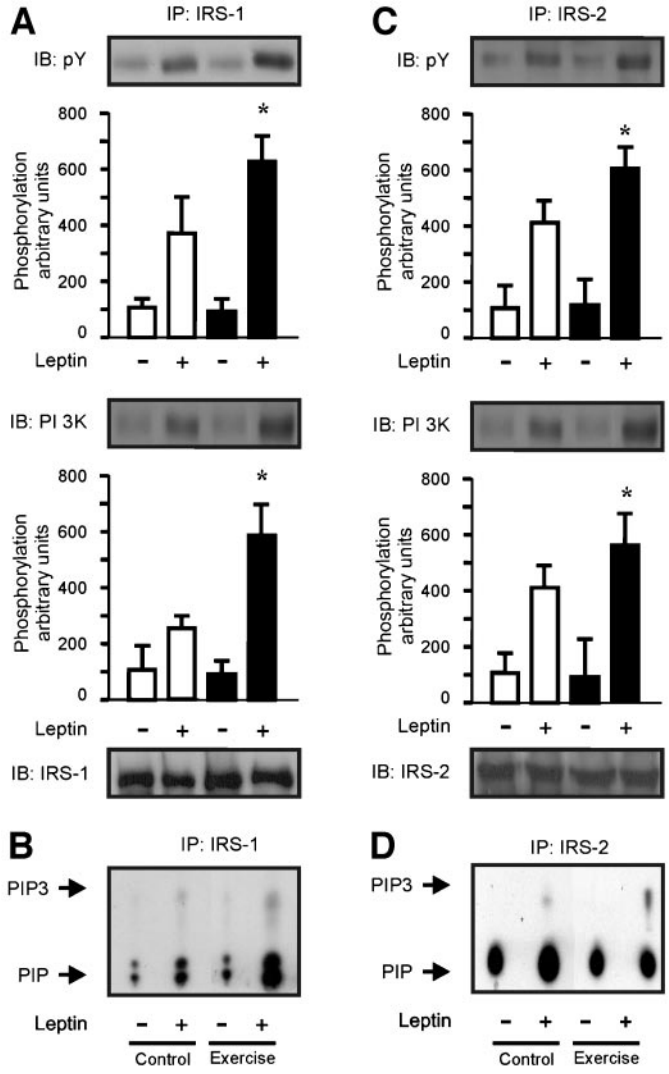

FIG. 3. Leptin signaling in the hypothalamus of control and exercised rats. Hypothalamus extracts from rats injected with vehicle (−) or leptin (+) were prepared as described in RESEARCH DESIGN AND METHODS. At 5 min after the infusion tissue, extracts were immunoprecipitated (IP) with anti–IRS-1 and anti–IRS-2 antibodies and immunoblotted (IB) with anti-phosphotyrosine (pY) (*A* and *C*, *upper panels*), anti–PI 3-kinase (*A* and *C*, *middle panels*), anti–IRS-1, and anti–IRS-2 antibodies (*A* and *C*, *lower panels*). PI 3-kinase assays were performed as described. Fluorographs show the silica thin-layer chromatography plates of IRS-1– or IRS-2–associated PI 3-kinase activity (*B* and *D*). The results of scanning densitometry were expressed as arbitrary units. Columns and bars represent the means ± SE, $n = 6$ animals per group. *$P < 0.05$, leptin control vs. leptin exercise. □, control; ■, exercise. PIP, the migration position of PI 3–phosphate.

46.4%, respectively, whereas in the control group, these doses induced reductions of 15.7, 23.2, and 31.2%, indicating that leptin was much more effective in exercised rats (Fig. 2*A*).

To determine the effects of exercise on the early steps of the leptin signaling pathway, a dose of 10 μg was administered and the ObR and JAK2 tyrosine phosphorylation was assessed in the hypothalamus of exercised and control rats. Immunoprecipitation and Western blotting of hypothalamic extracts were performed using anti-ObR, anti-JAK2, and anti-phosphotyrosine antibodies. Leptin induced increases in ObR and JAK2 tyrosine phosphorylation levels in hypothalami from both control and exercised rats. In the exercised animals, leptin increased ObR and JAK2 tyrosine phosphorylation by 6.0- and 4.7-fold, respectively, compared with 2.1- and 2.7-fold increases in the hypothalami from control rats, representing increases in ObR tyrosine phosphorylation of 4.7- and 2.2-fold, respectively (Fig. 2*B* and *C*, *upper panels*). The same membranes used to detect tyrosine phosphorylation of ObR and JAK2 were reblotted with ObR and JAK2 antibodies, and, as expected, there were no changes in ObR and JAK2 protein expression (Fig. 2*B* and *C*, *lower panels*). Hypothalamic extracts from exercised and control rats that were stimulated with leptin (10 μg) were lysed and the proteins separated by SDS-PAGE gel and blotted with pSTAT3 antibodies. In the hypothalami from exercised animals, leptin increased STAT3 tyrosine phosphorylation by 5.3-fold compared with 2.4-fold increases in the hypothalami from control rats, representing 3.2-fold increases in STAT3 tyrosine phosphorylation (Fig. 2*D*, *upper panel*). No changes were observed in STAT3 protein expression (Fig. 2*D*, *lower panel*).

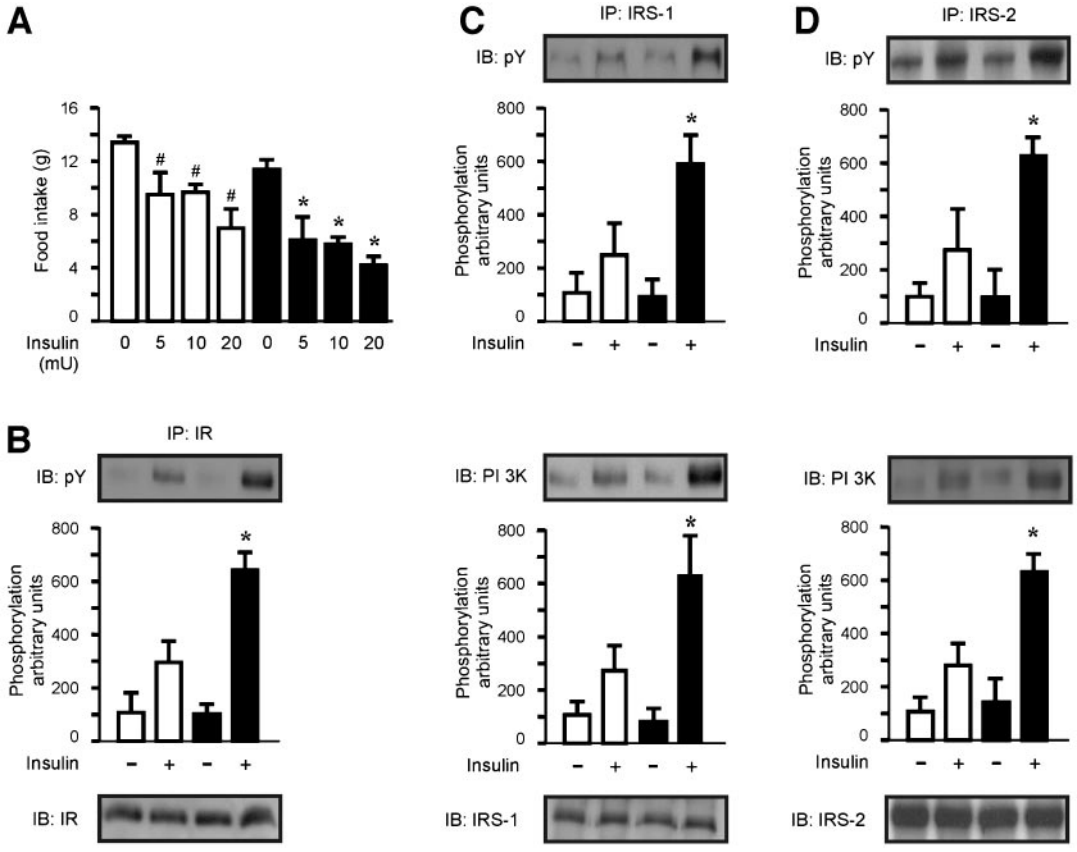

FIG. 4. Insulin inhibition of 12-h cumulative food intake and insulin signaling in the hypothalami of control and exercised rats. Vehicle or insulin was injected intracerebroventricularly after a session of 6-h exercise, and rats were immediately exposed to food for 12 h. Data are the means ± SE of 8–14 animals per group (*A*). At 15 min after the infusion, tissue extracts were immunoprecipitated (IP) with anti–insulin receptor antibody and immunoblotted (IB) with anti-phosphotyrosine antibody (pY) (*B*, *upper panel*) and anti–insulin receptor antibody (*B*, *lower panel*). Tissue extracts were also immunoprecipitated with anti–IRS-1 and anti–IRS-2 antibodies and immunoblotted with anti-phosphotyrosine (*C* and *D*, *upper panels*), anti–PI 3-kinase (*C* and *D*, *middle panels*), or anti–IRS-1 or anti–IRS-2 antibodies (*C* and *D*, *lower panels*). The results of scanning densitometry were expressed as arbitrary units. Columns and bars represent the means ± SE, $n = 6$ animals per group. *$P < 0.05$, insulin control vs. insulin exercise; #$P < 0.05$, insulin control vs. control. □, control; ■, exercise.

Intracerebroventricular leptin activates the hypothalamic IRSs–PI 3-kinase pathway in exercised rats to a greater extent than in control animals. Immunoprecipitation and Western blotting of hypothalamic extracts were performed using anti–IRS-1, anti–IRS-2, and anti-phosphotyrosine antibodies. Leptin (10 μg) induced increases in IRS-1/2 tyrosine phosphorylation levels in hypothalami from both control and exercised rats. In the exercised animals, leptin increased IRS-1 and -2 tyrosine phosphorylation by 5.8- and 5.6-fold, respectively, compared with 3.4- and 3.8-fold increases in the hypothalami from control rats, representing increases in IRS-1 and -2 tyrosine phosphorylation of 2.0- and 1.6-fold, respectively (Fig. 3*A* and *C*, *upper panels*).

The same membranes used to detect tyrosine phosphorylation of IRS-1 and -2 were reblotted with antibodies against the p85 subunit of PI 3-kinase. The PI 3-kinase association with IRS-1 and -2 paralleled the changes in the phosphorylation of these proteins (Fig. 3*A* and *C*, *middle panels*). There were no changes in IRS-1 and -2 protein expressions (Fig. 3*A* and *C*, *lower panels*). To determine whether there was PI 3-kinase activity in IRS-1 and -2 immunoprecipitates, hypothalami were prepared and immunoprecipitated with anti–IRS-1 or anti–IRS-2 antibodies from both control and exercised rats. After treatment with leptin, there was an increase in PI 3-kinase activity associated with IRS-1 and -2. In the exercised animals, leptin increased PI 3-kinase activity associated with IRS-1 and -2 by 5.5- and 5.2-fold, respectively, compared with 2.4- and 3.8-fold increases in the hypothalami from control rats, representing increases in PI 3-kinase activity associated with IRS-1 and -2 of 2.0- and 1.6- fold, respectively (Fig. 3*B* and *D*).

Intracerebroventricular insulin reduces food intake and activates the hypothalamic IRS-1/2–PI 3-kinase pathway in exercised rats to a greater extent than in control animals. The effect of insulin, or its vehicle, on the control of food intake was studied by measuring total food intake for 12 h after a session of exercise and a single intracerebroventricular injection of insulin or its vehicle. Insulin induced reductions in 12-h food intake in both exercised and control rats in a concentration-dependent manner. In the exercised animals, insulin (5, 10, and 20 mU) reduced food intake by 46.4, 49.1, and 63.3%, respectively, whereas in the control group, these doses induced reductions of 29.2, 27.7, and 47.9%, indicating that insulin was much more effective in exercised rats (Fig. 4*A*).

To determine the effects of exercise on the early steps of the insulin signaling pathway, insulin receptor, IRS-1, and IRS-2 tyrosine phosphorylation was assessed in the hypothalamus of trained and control rats. Immunoprecipitation and Western blotting of hypothalamic extracts were performed using anti–insulin receptor, anti–IRS-1, anti–IRS-2, and anti-phosphotyrosine antibodies. Insulin (10 mU) in-

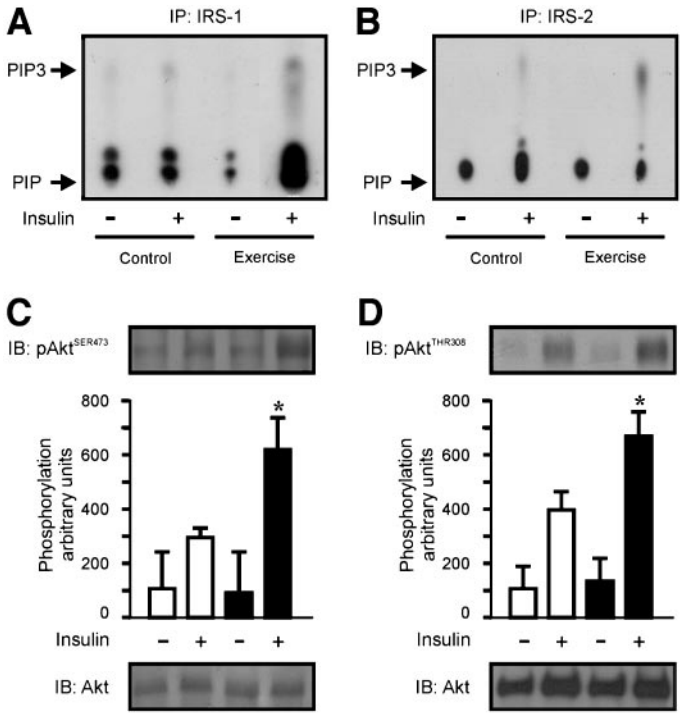

FIG. 5. Insulin signaling in the hypothalami of control and exercised rats. Hypothalami from rats injected with vehicle (−) or insulin (+) were prepared 15 min after the infusion, as described in RESEARCH DESIGN AND METHODS. Fluorographs show the silica thin-layer chromatography plates of IRS-1– or IRS-2–associated PI 3-kinase activity. At 15 min after the infusion, whole-tissue extracts were immunoblotted (IB) with anti–pAkt serine 473 (*C*, *upper panel*) or anti–pAkt threonine 308 (*D*, *upper panel*) and with anti-Akt antibodies (*C* and *D*, *lower panels*). The results of scanning densitometry were expressed as arbitrary units. Columns and bars represent the means ± SE, $n = 6$ animals per group. *$P < 0.05$, insulin control vs. insulin exercise. □, control; ■, exercise. PIP, the migration position of PI 3–phosphate (*A* and *B*).

duced increases in insulin receptor, IRS-1, and IRS-2 tyrosine phosphorylation levels in the hypothalamus of both control and exercised rats. In the exercised animals, insulin increased insulin receptor, IRS-1, and IRS-2 tyrosine phosphorylation by 6.0-, 5.5-, and 5.8-fold, respectively, compared with 2.7-, 2.3-, and 2.5-fold increases in the hypothalami from control rats, representing increases in insulin receptor and IRS-1/2 tyrosine phosphorylation of 3.0-, 3.6-, and 3.2-fold, respectively (Fig. 4*B*, *C*, and *D*, *upper panels*). The same membranes used to detect tyrosine phosphorylation of IRS-1/2 were reblotted with antibodies against the p85 subunit of PI 3-kinase. The PI 3-kinase association to IRS-1 and -2 paralleled the changes in phosphorylation of these proteins (Fig. 4*C* and *D*, *middle panels*).

To determine whether there was PI 3-kinase activity in the IRS-1/2 immunoprecipitates, hypothalami from control and exercised rats treated with insulin (10 mU), after a session of exercise, were prepared and immunoprecipitated with anti–IRS-1 or anti–IRS-2 antibodies. After treatment with insulin, there was an increase in PI 3-kinase activity associated with IRS-1 and -2. In the exercised animals, insulin increased PI 3-kinase activity associated with IRS-1 and -2 by 5.8- and 6.0-fold, respectively, compared with 2.5- and 2.6-fold increases in the hypothalami from control rats, representing increases in PI 3-kinase activity associated with IRS-1 and -2 of 2.0- and 1.6-fold, respectively (Fig. 5*A* and *B*).

Hypothalamic extracts from exercised and control rats that were stimulated with insulin (10 mU) were lysed and the proteins separated by SDS-PAGE on gel and blotted with pAkt antibodies. In the hypothalami from exercised animals, insulin increased Akt serine 473 and Akt threonine 308 phosphorylation by 5.7- and 6.0-fold, respectively, compared with 2.8- and 3.7-fold increases in the hypothalami from control rats, representing increases in Akt serine phosphorylation of 2.7-fold (Fig. 5*C*, *upper panel*). No changes were observed in Akt protein expression (Fig. 5*C*, *lower panel*).

Role of IL-6 in anorectic response to leptin and insulin. IL-6 expression was detected in control animals; however, a 2.8-fold increase was observed in exercised animals (Fig. 6*A*). We tested whether the inhibitory effects of leptin and insulin on food intake depend on IL-6 by intracerebroventricular infusion of anti–IL-6 into exercised rats. Treatment with leptin or insulin markedly reduced 12-h food intake in exercised rats pretreated with vehicle, although pretreatment with anti–IL-6 blocked exercise-induced leptin (Fig. 6*B*) and insulin (Fig. 6*C*) sensitivity in a concentration-dependent manner, respectively (Fig. 6*B* and *C*). Consistent with the increase in leptin sensitivity, JAK2 (Fig. 6*D*) and STAT3 (Fig. 6*E*) phosphorylation were induced by exercise and reversed by anti–IL-6 in accordance with the control of food intake. Insulin induced a significant increase in insulin receptor (Fig. 6*F*), Akt serine 473 (Fig. 6*G*), and threonine (Fig. 6*H*) phosphorylation in the hypothalamus of exercised rats pretreated with vehicle. In animals pretreated with anti–IL-6, the effect of exercise on insulin signaling was reversed in a concentration-dependent manner.

DISCUSSION

Exercise training has multiple effects on metabolism and gene expression (31). However, little is known about the mechanisms by which exercise leads to reduced appetite. Here, we provide evidence for a molecular mechanism to account for increased insulin and leptin action in hypothalamus after exercise. Intracerebroventricular insulin or leptin infusion in doses that did not alter insulinemia or leptinemia reduced food intake in exercised rats to a greater extent than that observed in control animals. Exercise was associated with a marked increase in the phosphorylation/activity of several proteins involved in leptin and insulin signal transduction in hypothalamus. In addition, we investigated the regulatory role of IL-6 in mediating the increase in leptin and insulin sensitivity in hypothalamus. Treatment with insulin or leptin markedly reduced food intake in exercised rats that were pretreated with vehicle, although no increase in sensitivity to leptin- and insulin-induced anorexia was detected after pretreatment with anti–IL-6 antibody. Increased leptin and insulin action in the brain may thus contribute to the modulation of energy homeostasis in exercised rats.

Despite a recent publication showing that the 12-week wheel exercise protocol reduced the expression of ObRb mRNA in the arcuate nucleus (32), our data demonstrate that after a session of exercise, there were no changes in the expression of hypothalamic proteins involved in leptin and insulin signal transduction. However, the phosphorylation status of these proteins was deeply modified. Exercise led to an increase in leptin- and insulin-stimulated ObR/JAK2 and insulin receptor tyrosine phosphorylation, respectively. The next step in leptin and insulin signaling may involve the tyrosine phosphorylation of IRS-1 and -2.

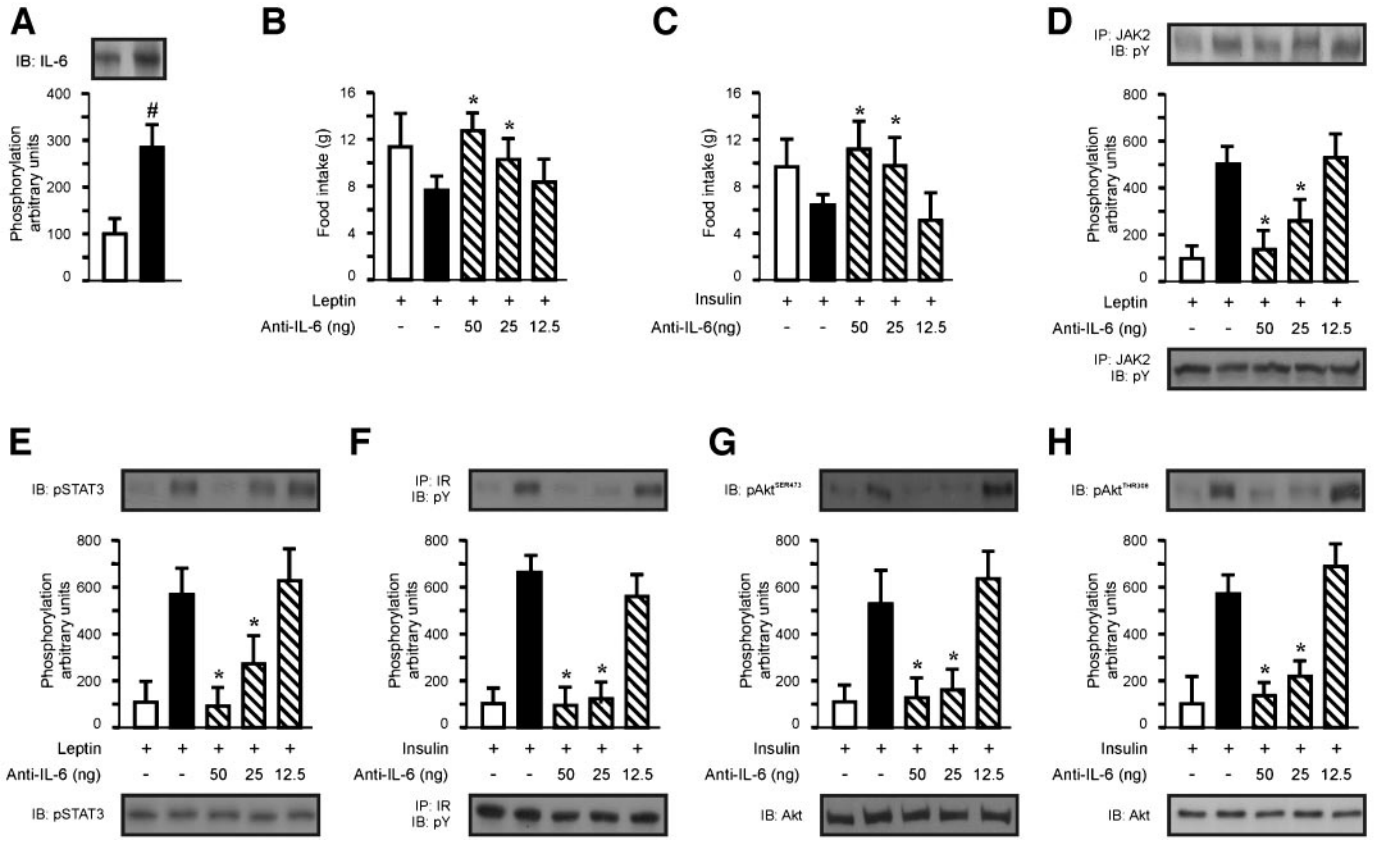

FIG. 6. Blockade of leptin and insulin-induced inhibition of food intake by anti–IL-6. Hypothalami from rats were prepared as described in RESEARCH DESIGN AND METHODS. Tissue extracts from control and exercised rats were immunoblotted with anti–IL-6 antibody (*A*). Leptin and insulin were injected intracerebroventricularly in control rats, exercised rats, and exercised rats pretreated with anti–IL-6 at the doses indicated, and the animals were immediately exposed to food for 12 h. Data are the means ± SE of 8–14 animals per group (*B* and *C*). Tissue extracts from control rats, exercised rats, and exercised rats pretreated with anti–IL-6 were treated with leptin and immunoprecipitated (IP) with anti-JAK2 antibody and immunoblotted (IB) with anti-phosphotyrosine antibody (*D*, *upper panel*) and with anti-JAK2 (*D*, *lower panel*). Whole-tissue extracts were immunoblotted with anti–phospho STAT3 antibody (*E*, *lower panel*) and with anti-STAT3 (*E*, *lower panel*). Hypothalamus tissue extracts from control, exercised, and exercised rats pretreated with anti–IL-6 were treated with insulin and immunoprecipitated with anti–insulin receptor antibody and immunoblotted with anti-phosphotyrosine antibody (*F*, *upper panel*) and with anti–insulin receptor (*F*, *lower panel*). Whole-tissue extracts were immunoblotted with anti–phosphoserine 473 and anti–threonine 308 Akt antibody (*G* and *H*, *lower panels*) and with anti-Akt (*G* and *H*, *lower panels*). The results of scanning densitometry are expressed as arbitrary units. Columns and bars represent the means ± SE, $n = 8$ animals per group. #$P < 0.05$, exercise vs. control; *$P < 0.05$, exercise + anti–IL-6 vs. exercise. □, control; ■, exercise; ▨, exercise + anti–IL-6.

As shown above, the amounts of IRS-1 and -2 were unchanged in the hypothalamus of exercised rats. In contrast, the phosphorylation of IRS-1 and -2 after stimulation with leptin or insulin increased in those rats compared with control animals. Because IRS-1 and -2 are the main molecules linking leptin and insulin signaling to PI 3-kinase activity, we examined the leptin- and insulin-induced association of IRS-1 and -2 with the p85 subunit of PI 3-kinase and found it to be increased in the hypothalamus of exercised rats. After IRS-1– or IRS-2–PI 3-kinase association, PI 3-kinase is activated and may in turn activate Akt, a serine kinase with pleiotropic actions in several tissues (33). The activation of Akt-1/protein kinase B is accompanied by an increase in its serine and threonine phosphorylation (22). Thus, the increase in the association between IRS-1 or IRS-2 and PI 3-kinase, and the increase in PI 3-kinase activity after leptin or insulin infusion in the hypothalamus of exercised rats, may play a role in the increased responsiveness to leptin and insulin in these animals.

Selective impairment of leptin and insulin signaling through the PI 3-kinase pathway in the hypothalamus could be pathophysiologically important in the development of obesity. Recent studies have shown that activation of the PI 3-kinase pathway could be involved in the anorexigenic effect of insulin or leptin (14,34,35). Our findings, in a model of exercise training, are relevant because insulin-induced tyrosine phosphorylation of IRSs and PI 3-kinase activity are reduced in the hypothalamus of different animal models of obesity (14,36,37). Thus, exercise training may be one therapeutic strategy to restore impaired leptin and insulin signal transduction in the hypothalamus of obese individuals.

In addition to the increased insulin and leptin sensitivity observed in the PI 3-kinase pathway, our data also provide evidence that there is an increase in leptin sensitivity in the JAK2/STAT3 pathway. Leptin activation of STAT3 requires the leptin receptor, which associates with and activates JAK2 in a ligand-dependent manner (8,9,12,38). One potential mediator of increased STAT3 activation in the hypothalamus of exercised rats is expression of SOCS-3, a suppressor of cytokine signaling. Forced expression of SOCS-3 in mammalian cells antagonizes leptin signaling, probably by binding and antagonizing JAK activity (39). Using Western blotting, we examined the expression of SOCS-3 in hypothalami of exercised rats. No significant differences were found between the two groups (data not shown). In addition, we found no significant

difference in hypothalamic protein-tyrosine phosphatase 1b expression and its association with JAK2 and ObR between the two groups (data not shown). Thus, a molecular basis for the observed increase in leptin's ability to activate STAT3 signaling after 1 day of exercise remains to be determined.

Perhaps the most striking finding was the reversal of exercise-induced increased hypothalamic insulin and leptin sensitivity by blocking the action of IL-6 action. These data are in accordance with earlier studies demonstrating that IL-6 treatment enhances energy expenditure in both rodents and humans (30,40–42). It has been previously shown that IL-6 treatment stimulates energy expenditure at the level of the brain in rodents (30,41,43), and it might be assumed that endogenous IL-6 also acts on the brain during exercise. The IL-6 exerting this effect during exercise could be produced by the brain itself, which has been shown to have increased IL-6 production during exercise (44). Alternatively, the large quantities of endocrine IL-6 produced from working skeletal muscle (45) might reach appropriate sites in the brain (23,46,47).

Numerous biological responses of different cell types are induced by IL-6, which activates STAT3 and Ras–extracellular signal-regulated kinase-1/2 via JAKS, and the balance of activation of both pathways is considered to direct the cell fate in response to IL-6 (48). The cross talk of signals mediated by a cytokine and growth factor has been previously reported in the case of the phosphorylation of tyrosine kinase receptors by the growth hormone–activated JAK2 (49). This suggests that the IL-6–induced activation of JAK2 is involved in the activation of insulin and leptin receptor–mediated signals in rat hypothalamus. Conversely, it has been reported that the PI 3-kinase and Akt pathways may be activated via gp130 (the glycoprotein of 130 kDa) recruitment of adaptor molecules to create binding sites to the SH2 domain of the p85 subunit of PI 3-kinase (50).

In the current study, using an in vivo approach, we saw a synergistic effect of IL-6 on the insulin-stimulated tyrosine phosphorylation of IRS-1 and on the serine phosphorylation of protein kinase B/Akt in rat hypothalamus. These results are clearly different from the findings of Senn et al. (51) in HepG2 cells and may indicate that the induction of SOCS-3 by IL-6 either follows a different time course in hepatocytes or that the major effect of IL-6 is exerted through other mechanisms, such as the transcriptional regulation identified in the current work. However, the recent finding (52) that a high IL-6 infusion for 2 h in rats did not reduce the insulin effect during a euglycemic clamp clearly supports the theory that any acute inhibitory effects of IL-6, mediated through a transient activation of SOCS-3, are of less importance for whole-body insulin sensitivity. Similar results have recently been reported in humans (53). Because IL-6 has been shown both to interfere with insulin-signaling pathways in the liver and adipocytes in an inhibitory manner and to reduce insulin-stimulated glycogen synthesis in hepatocytes, the cross talk of IL-6 with insulin-signaling pathways appears to be tissue specific.

The current study provides direct measurements of leptin and insulin signaling in the hypothalamus, and it documents increased sensitivity to these hormones in the hypothalamus of exercised rats in an IL-6–dependent manner. These findings provide support for the hypothesis that exercise could have appetite-suppressive actions mediated by the hypothalamus.

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Reversal of diet-induced insulin resistance with a single bout of exercise in the rat: the role of PTP1B and IRS-1 serine phosphorylation

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Reversal of diet-induced insulin resistance with a single bout of exercise in the rat: the role of PTP1B and IRS-1 serine phosphorylation

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Lifestyle interventions including exercise programmes are cornerstones in the prevention of obesity-related diabetes. In this study, we demonstrate that a single bout of exercise inhibits high-fat diet-induced insulin resistance. Diet-induced obesity (DIO) increased the expression and activity of the protein tyrosine phosphatase 1B (PTP1B) and attenuated insulin signalling in gastrocnemius muscle of rats, a phenomenon which was reversed by a single session of exercise. In addition, DIO was observed to lead to serine phosphorylation of insulin receptor substrate 1 (IRS-1), which was also reversed by exercise in muscle in parallel with a reduction in c-Jun N-terminal kinase (JNK) activity. Thus, acute exercise increased the insulin sensitivity during high-fat feeding in obese rats. Overall, these results provide new insights into the mechanism by which exercise restores insulin sensitivity.

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Insulin resistance of skeletal muscle glucose transport is a key defect in the development of impaired glucose tolerance and type 2 diabetes. It is well established that chronic exercise can have beneficial effects on insulin action in insulin-resistant states (Henriksen, 2002). It is important to note that improvements in glucose tolerance can be observed in people with mild type 2 diabetes mellitus after acute exercise (Azevedo *et al.* 1995; Kennedy *et al.* 1999). The molecular mechanism for enhanced glucose uptake with chronic exercise may be partly related to increased expression and activity of key proteins known to regulate glucose metabolism in skeletal muscle (Hjeltnes *et al.* 1998; Chibalin *et al.* 2000; Zierath, 2002).

The action of insulin is mediated by receptor binding at the surface of insulin-sensitive tissue (Czech & Corvera, 1999). The insulin receptor (IR) is a protein with endogenous tyrosine kinase activity that, following activation by insulin, undergoes rapid auto-phophorylation and subsequently phophorylates intracelular protein substrates, such as insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) (Cheatham & Kahn, 1995). Phosphorylation of IRS-1 and IRS-2 tyrosine residues induces activation of phosphatidylinositol 3-kinase (PI3-K) by binding the p85 subunit and activating the catalytic p110 subunit (White & Kahn, 1994). Activation of a serine/threonine kinase Akt occurs downstream from PI3-K. Once phosphorylated, Akt contributes to various biological processes including regulation of glucose uptake (Virkamaki *et al.* 1999).

Dephosphorylation of IR and IRS-1 or serine phosphorylation of IR substrates are the main mechanisms that suppress the insulin pathway (Ventre *et al.* 1997; Greene *et al.* 2003). Protein tyrosine phosphatases (PTPs) are important regulators of tyrosine phosphorylation-dependent signalling events and may represent novel targets for therapeutic intervention in a variety of human diseases (Tonks, 2003). Several PTPs, including PTPα, PTPε, CD45, SHP2, LAR and PTP1B, have been implicated as negative regulators of insulin signalling (Asante-Appiah & Kennedy, 2003). PTP1B is a major PTP implicated in the regulation of insulin action, including in the insulin-resistant state (Seely *et al.* 1996; Elchebly *et al.* 1999). c-Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein (MAP) kinase family (Weston *et al.* 2002) and can be activated by tumour necrosis factor α (TNFα) (Hirosumi *et al.* 2002) and interleukin 1β (IL 1β) (Major & Wolf, 2001). In addition, JNK might serve as a feedback inhibitor during insulin stimulation (Lee *et al.* 2003). Three JNK isoforms have been described, JNK1, 2 and 3 (Ip & Davis, 1998), of which JNK1 is most involved in the pathophysiology of obesity and insulin resistance (Hirosumi *et al.* 2002).

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Table 1. Components of rat diet and rat chow

Ingredients	Standard chow (g)	High-fat diet (g)
Casein	202	200
Sucrose	100	100
Cornstarch	397	115.5
Dextrinated starch	130.5	132
Lard	—	312
Soybean oil	70	40
Cellulose	50	50
Mineral mix American Institute of Nutrition (AIN)-93	35	35
Vitamin mix AIN-93	10	10
L-cystine	3	3
Choline	2.5	2.5

JNK activation induces inhibitory serine 307 (Ser307) phosphorylation of IRS-1, (Aguirre *et al.* 2000; Lee *et al.* 2003). Ser307 is located next to the PTB domain in IRS-1 and its phosphorylation inhibits the interaction of the PTB domain with the phosphorylated NPEY motif in the activated insulin receptor, causing insulin resistance (Aguirre *et al.* 2002). Previous studies suggest that, in addition to JNK, IκB kinase beta (IKKβ) phoshorylation also increases serine phosphorylation of IRS-1. Thus, the IKK complex appears to be another candidate that plays a key role in the phosphorylation of IRS-1 and in the regulation of insulin sensitivity.

As much of the molecular basis underlying the beneficial effects of exercise in the insulin-resistant state remains unclear, the current study was designed to investigate the effects of a single bout of exercise on PTP1B activity and IRS-1 serine phosphorylation associated with insulin resistance induced by DIO.

Methods

Experimental animals and diet

Male Wistar rats from the University of Campinas Central Animal Breeding Center were used in the experiments. All experiments were approved by the Ethics Committee of the State University of Campinas (UNICAMP).

The 4-week-old Wistar rats were divided into three groups, control rats fed standard rodent chow (Table 1), obese rats fed on an obesity-inducing diet for 3 months (DIO) (Table 1) and DIO rats submitted to a single bout of exercise (DIO + EXE).

Exercise protocol

Rats were acclimated to 10 min swimming for 2 days. The animals swam for two 3 h bouts separated by a 45 min rest period and the water temperature was maintained at ∼34°C. This exercise protocol was adaptated from a published procedure (Chibalin *et al.* 2000). After the last bout of exercise, animals were fed *ad libitum* and food was withdrawn 6 h before the tissue extraction. The rats were anaesthetized with intraperitoneal injection of sodium thiopental (40 mg (kg body weight)$^{-1}$) 8 and 16 h after the exercise protocol. Following the experimental procedures, the rats were killed under anaesthesia (200 mg kg^{-1} thiopental) following the recommendations of the NIH.

Insulin tolerance test, serum insulin quantification and glycogen formation

The rats were given an insulin tolerance test (ITT; 1.5 IU insulin (kg body weight)$^{-1}$) 16 h after the exercise protocol. Briefly, 1.5 IU kg^{-1} human recombinant insulin (Humulin R) from Eli Lilly (Indianapolis, IN, USA) was infused intraperitoneally to anaesthetized rats, the blood samples were collected at 0, 5, 10, 15, 20, 25 and 30 min from the tail for serum glucose determination. The rate constant for plasma glucose disappearance (K_{itt}) was calculated using the formula 0.693/biological half life (t_{γ_2}). The plasma glucose (t_{γ_2}) was calculated from the slope of last square analysis of the plasma glucose concentration during the linear phase of decline (Bonora *et al.* 1989). Plasma glucose level was determined using a glucose meter (Advantage, Boehringer Mannheim, USA). Plasma was separated by centrifugation (1100 *g*) for 15 min at 4°C and stored at −80°C until assayed. Radioimmunoassay was employed to measure serum insulin level, according to a previous description (Scott *et al.* 1981). Glycogen content in gastrocnemius muscle fragments was measured, according to a previously described method (Pimenta *et al.* 1989).

Hyperinsulinaemic–euglycaemic clamp procedures

HPLC-purified 2-deoxy-D-[1-^{14}C]glucose (2-[^{14}C]DG) was obtained from Amersham Biosciences Group (UK). The Harvard apparatus (model 11) and Harvard compact infusion pumps (model 975) were obtained from South Natick, MA, USA.

After 6 h of fasting, animals were anaesthetized intraperitoneally and catheters were then inserted into the left jugular vein (for tracer infusions) and carotid artery (for blood sampling), as previously described (Prada *et al.* 2000). Experiments were started when glycaemia had returned to stable levels, 30 min after the end of the surgical procedure. A 120 min hyperinsulinaemic–euglycaemic clamp procedure was conducted in anaesthetized catheterized rats, as shown previously (Prada *et al.* 2000, 2005), with continuous infusion of human insulin at a rate of 3.6 mU (kg body wt) $^{-1}$ min^{-1} to raise the plasma insulin concentration to approximately 800–900 pmol l^{-1}. Blood samples (20 μl) were collected at 5 min intervals for the immediate measurement of plasma glucose

concentrations, and 10% unlabelled glucose was infused at variable rates to maintain plasma glucose at fasting levels. To estimate insulin-stimulated glucose transport and metabolism in skeletal muscle, (2-[^{14}C]DG) was administered as a bolus (10 μCi) 45 min before the end of the clamp procedure. All infusions were performed using Harvard infusion pumps. At the end of the clamp procedure, animals were killed by an intravenous injection of ketamin and diazepam. Within 2 min, both portions of gastrocnemius from hindlimbs were removed. Each tissue, once exposed, was dissected out within 2 s, weighed, frozen with liquid N_2 and stored at -80°C for later analysis.

Analytical procedures for hyperinsulinemic-euglycemic clamping

Plasma glucose was measured using a glucometer (Advantage, Boehringer Mannheim, USA). The whole blood glucose uptake was obtained from averaged rates of the last 30 min of 10% unlabelled glucose infusion during clamp procedures. Glucose transport activity in skeletal muscle was calculated from the tissue 2-deoxy-D-glucose (2DG) profile, as described before (Ferre *et al.* 1985; McGuinness & Mari, 1997; Prada *et al.* 2005).

Protein analysis by immunoblotting

As soon as anaesthesia was assured by the loss of pedal and corneal reflexes, the abdominal cavity was opened, the cava vein exposed, and 0.2 ml normal saline or insulin (10^{-9} M) injected. At 90 s after insulin injection, both portions of gastrocnemius were ablated, pooled, minced coarsely and homogenized immediately in extraction buffer containing (mM): Tris 100 (pH 7.4), sodium pyrophosphate 100, sodium fluoride 100, EDTA 10, sodium vanadate 10 and phenylmethylsulfonyl fluoride (PMSF) 2, and 0.1 mg aprotinin ml^{-1} and 1% Triton-X 100 at 4°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 s. The extracts were centrifuged at 15 000 r.p.m. (9000 *g*) and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA, USA) for 45 min to remove insoluble material, and the supernatants of these tissues were used for protein quantification using the Bradford method (Bradford, 1976).

Proteins were denaturated by boiling in Laemmli (Laemmli, 1970) sample buffer containing 100 mM DTT, run on SDS-PAGE, transferred to nitrocellulose membranes, which were blocked, probed and developed as previously described (Saad *et al.* 1997). The β subunit of the IR (IRβ), IRS-1 and IRS-2 were immunoprecipitated from rat muscle with or without previous insulin infusion. Antibodies used for immunoblotting were anti-phosphotyrosine, anti-IR, anti-IRS-1, anti-IRS-2, anti-PTP1B, anti-PI3-K, antiphosphoserine-IRS-1307 (Upstate Biotechnology, NY, USA), antiphospho-Akt (Cell Signalling Technology, MA, USA), anti-Akt, anti-JNK, antiphospho-JNK, antiphospho-c-jun, anti-IκBα and anti-SOCS3 (Santa Cruz Biotechnology Inc., CA, USA). Blots were exposed to preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at -80°C for 12–48 h. Band intensities were quantified by optical desitometry (Scion Image software, ScionCorp, Frederick, MD, USA) of the developed autoradiographs.

Protein tyrosine phosphatase activity assay

The gastrocnemius muscles were removed and homogenized in the solubilization buffer containing (mM): Tris 20 (pH 7.6), EDTA 5, PMSF 2, EGTA 1 and NaCl 130, and 0.1 mg aprotinin ml^{-1} and 1% Triton X-100. The lysates were centrifuged (15 000 *g* for 25 min at 4°C) and the supernatants were collected for immunoprecipitation, as previously described. Immunoprecipitates were washed in PTP assay buffer containing (mM): Hepes 100 (pH 7.6), EDTA 2, DTT 1 and NaCl 150, and 0.5 mg ml^{-1} bovine serum albumin. The pp60$^{c\text{-}src}$ C-terminal phosphoregulatory peptide (TSTEPQpYQPGENL; Biomol) was added to a final concentration of 200 μM in a total reaction volume of 60 μl in a PTP assay buffer for the immunoprecipitation. The reaction was then allowed to proceed for 1 h at 30°C. At the end of the reaction, 40 μl aliquots were placed into a 96-well plate, 100 μl Biomol Green reagent (Biomol) was added, and absorbance was measured at 630 nm (Taghibiglou *et al.* 2002).

Statistical analysis

Where appropriate, the results were expressed as the means ± S.E.M. Differences between the control group and DIO and between DIO and DIO + EXE were evaluated using one-way analysis of variance (ANOVA). When the ANOVA indicated significance, a Bonferroni *post hoc* test was performed.

Results

Physiological and metabolic parameters

Table 2 shows comparative data regarding control, DIO and DIO + EXE rats. Rats fed on the high-fat diet for 12 weeks had a greater body weight, epididymal fat and fasting serum insulin than age-matched controls. No significant variations were found in body weight, epididymal fat and fasting serum insulin in DIO + EXE compared to DIO rats. The fasting glucose concentration was similar between the groups; however, the decrease in the glucose disappearance rate (K_{itt}), induced by the

Table 2. Characteristics of Wistar rats after 3 months on a high-fat diet (DIO), DIO rats submitted to acute exercise (DIO + EXE) and their age-matched controls

Groups	Number of rats (*n*)	Body weight (g)	Epididimal fat (g)	Fasting insulin (ng ml^{-1})	Plasma glucose (mg dl^{-1})	K_{itt} (% min^{-1})
Control	6	403.4 ± 21.0	5.95 ± 0.97	3.28 ± 0.15	73.7 ± 6.9	3.79 ± 0.2
DIO	8	544.7 ± 32.1*	11.85 ± 1.47**	7.97 ± 0.88*	84.4 ± 7.3	1.90 ± 0.4**
DIO + EXE	8	546.2 ± 25.2*	12.1 ± 1.34**	6.39 ± 1.9*	82.1 ± 10.0	4.69 ± 0.6#

*$P < 0.01$, **$P < 0.001$ *versus* control group and #$P < 0.001$ *versus* DIO.

high-fat diet, returned to the basal levels 16 h after acute exercise.

A hyperinsulinaemic–euglycaemic clamp procedure with tracer infusions was performed to examine the effects of acute exercise on the metabolism of glucose in skeletal muscle. The glucose infusion rate needed to clamp glycaemia at fasting levels in the presence of a constant infusion of insulin (3.6 mU (kg body weight)$^{-1}$ min^{-1}) was 4-fold lower in DIO rats than in controls and returned to control levels in DIO + EXE rats (Fig. 1*A*).

Using 2DG uptake analysis, the insulin-stimulated glucose uptake in skeletal muscle was quantified. As shown in Fig. 1*B*, DIO rats presented a significant reduction in glucose uptake in the skeletal muscle when compared to control group. In contrast, 16 h after the exercise protocol, insulin induced an increased glucose uptake of 33.8% in the muscle of DIO + EXE rats when compared to DIO rats. In addition, we evaluated the relative quantities of muscular glycogen in controls, DIO and DIO + EXE rats. The high-fat diet decreased glycogen levels in the gastrocnemius of DIO rats when compared to the control group, and returned to control levels 16 h after a single bout of exercise (Fig. 1*C*).

A single bout of exercise improves insulin signalling in the muscle of DIO rats

The effect of *in vivo* intravenous insulin infusion on IR tyrosine phosphorylation was examined in the gastrocnemius muscle of control, DIO and DIO + EXE rats. The muscles were immunoprecipitated with anti-IR antibody and then blotted with anti-phosphotyrosine antibody. Insulin induced an increase in IR tyrosine phosphorylation levels in muscle from control, DIO and DIO + EXE rats. In the control animals, insulin increased IR tyrosine phosphorylation by 9.6-fold, compared with 3.1-fold increases in the muscle of DIO rats, representing reductions in IR tyrosine phosphorylation of 4.0-fold. Insulin increased IR tyrosine phosphorylation by 6.6-fold in the muscle from DIO + EXE rats, representing an increase in IR tyrosine phosphorylation of 2.6-fold compared with DIO rats (Fig. 2*A* upper panel). There was no difference in basal levels of IR tyrosine phosphorylation between the three groups (data not shown). The protein expression of IR in the gastrocnemius muscle of control, DIO and DIO + EXE rats was quantified by immunoprecipitation and immunoblotting with anti-IR antibody.

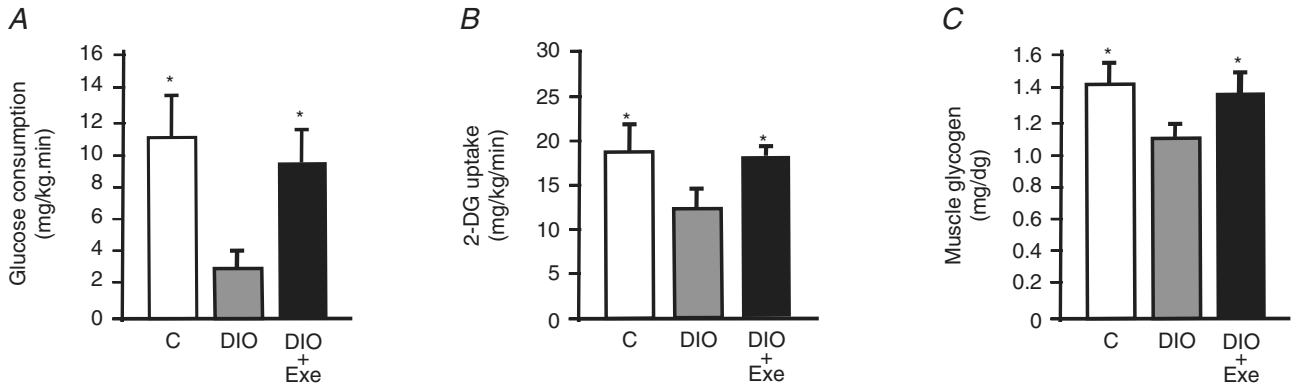

Figure 1. Effects of acute exercise on glucose uptake and glycogen content in control, DIO and DIO + EXE rats

A, steady-state glucose infusion rates obtained from averaged rates of 90–120 min of 10% unlabelled glucose infusion during hyperinsulinaemic–euglycaemic clamp procedures in the control, DIO and DIO rats submitted to acute exercise. *B*, glucose transport in gastrocnemius muscle was evaluated by 2-deoxy-D-glucose uptake during the last 45 min of the hyperinsulinaemic–euglycaemic clamp studies. *C*, muscular glycogen content is expressed as mg (100 g tissue)$^{-1}$. Bars represent means ± S.E.M. of $n = 5$ rats. *$P < 0.05$, *versus* DIO rats.

The IR protein levels were not different between the groups (Fig. 2*A* lower panel).

IRS-1 tyrosine phosphorylation and IRS-1–PI-3 kinase association increased in control animals by 10.6- and 10.1-fold following insulin administration, respectively, compared with 2.5- and 2.6-fold increases in the muscle of DIO rats (representing reductions in IRS-1 tyrosine phosphorylation and IRS-1–PI3K association of 6.4- and 5.6-fold, respectively), and increases of 4.7- and 5.0-fold in the muscle of DIO + EXE rats (representing increases in IRS-1 tyrosine phosphorylation and IRS-1–PI3K association of 2.4- and 2.5-fold, respectively, compared with DIO rats) (Fig. 2*B* upper and middle panel). IRS-2 tyrosine phosphorylation and IRS-2–PI-3 kinase association increased in control animals by 9.2- and 8.5-fold following insulin administration, respectively, compared with 2.6- and 3.0-fold increases in the muscle of DIO rats (representing reductions in IRS-2 tyrosine phosphorylation and in IRS-2–PI3K association of 5.1- and 3.7-fold, respectively), and increased 5.1- and 5.9-fold in the muscle of DIO + EXE rats (representing increases in IRS-2 tyrosine phosphorylation and in IRS-2–PI3K association of 2.5 and 2.4-fold, respectively, compared with DIO rats) (Fig. 2*C* upper and middle panel). There were no differences in basal levels of IRS-1 and IRS-2 tyrosine phosphorylation between the three groups (data not shown). The protein expression of IRS-1 and IRS-2 in the gastrocnemius muscle from control, DIO and DIO + EXE rats were quantified by immunoprecipitation and immunoblotting with anti-IRS-1 or anti-IRS-2 antibodies. The IRS-1 and IRS-2 protein levels were not different between the groups (Fig. 2*B* and *C* lower panels). Finally, in gastrocnemius muscle from control rats, insulin increased Akt serine phosphorylation by 9.3-fold, compared with 2.8-fold increase in the muscle from DIO rats (representing reductions in Akt serine phosphorylation of 4.6-fold) and increases of 5.8-fold in the muscle of DIO + EXE rats (representing an increase in Akt serine phosphorylation of 2.6-fold compared with DIO rats) (Fig. 2*D* upper panel). There were no differences between the basal levels of Akt serine phosphorylation in the three groups (data not shown). The protein expression of Akt in the gastrocnemius muscle of control, DIO and DIO + EXE rats was quantified by immunoblotting with anti-Akt antibodies. The Akt protein levels were not different between the groups (Fig. 2*D* lower panel).

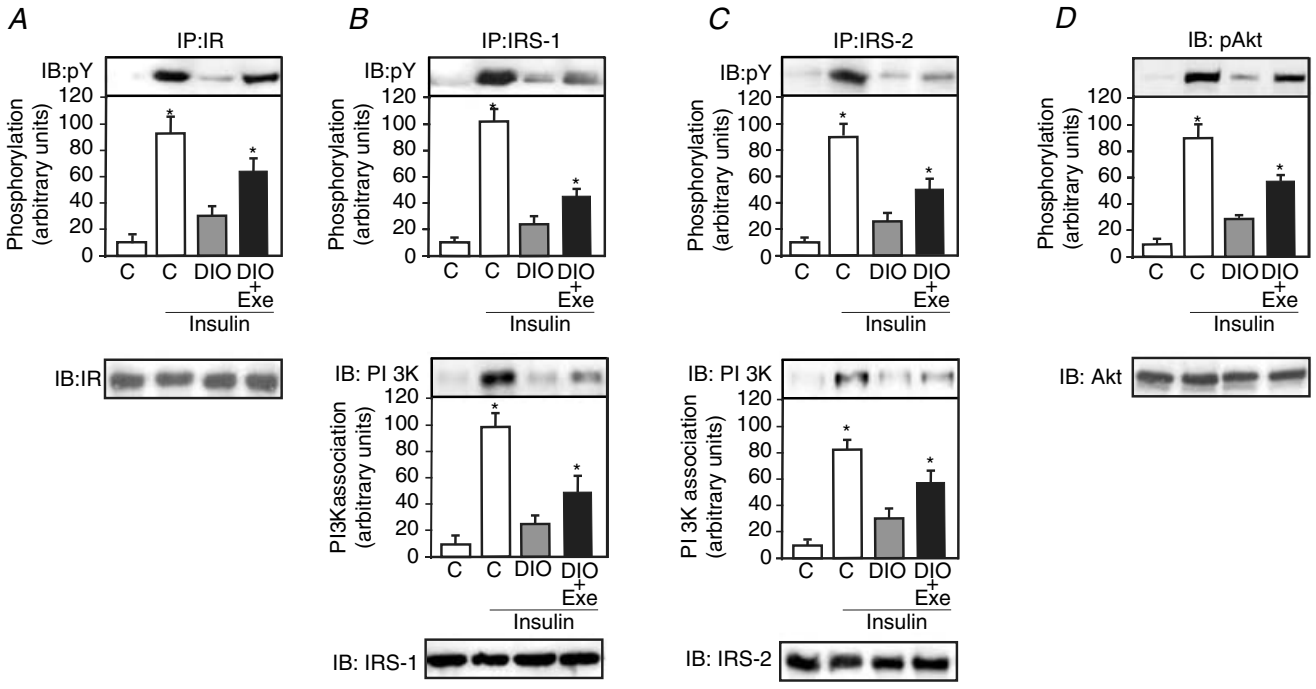

Figure 2. Insulin signalling in muscle of controls, DIO and DIO + EXE rats
Muscle extracts from rats injected with saline or insulin were prepared as described in the Methods. *A*, tissue extracts were immunoprecipitated (IP) with anti-IRβ antibody and immunoblotting (IB) with anti-PY antibody (upper panel) or anti-IRβ antibody (lower panel). *B* and *C*, tissue extracts were also IP with anti-IRS-1 and anti-IRS-2 antibodies and IB with anti-PY antibody (upper panels), anti-PI3K antibodies (middle panels) or anti-IRS-1, anti-IRS-2 antibody (lower panel). *D*, muscle extracts were IB with anti-phospho Akt and anti-Akt antibody (upper and lower panel, respectively). The results of scanning densitometry were expressed as arbitrary units. Bars represent means ± S.E.M. of $n = 6$–8 rats. *$P < 0.05$, *versus* DIO rats.

Acute exercise-mediated suppression of PTP1B activity in DIO rats

Obesity induced by diet increased the expression of PTP1B in DIO rats by 2.0-fold compared to control rats, a phenomenon that was reversed by acute exercise (Fig. 3*A*). Figure 3*B* shows that PTP1B activity increased in the muscle of DIO rats by 95% when compared to controls and acute exercise decreased PTP1B by 61% compared to DIO rats. To further explore the possibility that acute exercise mediated suppression of PTP1B activity in DIO rats, we observed that insulin induced IR tyrosine phosphorylation and IR/PTP1B interaction in muscle from DIO + EXE rats. The high-fat diet increased the IR/PTP1B association by 10.6-fold in the gastrocnemius muscle of DIO rats when compared with control rats and, in the muscle of DIO + EXE rats, IR/PTP1B association was decreased by 2.1-fold when compared with DIO rats (Fig. 3*C* upper panel). The IR protein levels were not different between the groups (Fig. 3*C* lower panel). As shown in Fig. 3*D*, insulin, in a time-dependent manner, induced increases in IR tyrosine phosphorylation in muscle from DIO rats after the exercise protocol, with a concomitant reduction of IR–PTP1B association. We also evaluated the IRS-1–PTP1B association in muscle from controls, DIO and DIO + EXE rats. The high-fat diet induced an increase in IRS-1–PTP1B association by 8.8-fold in gastrocnemius muscle of DIO rats when compared with control rats, and

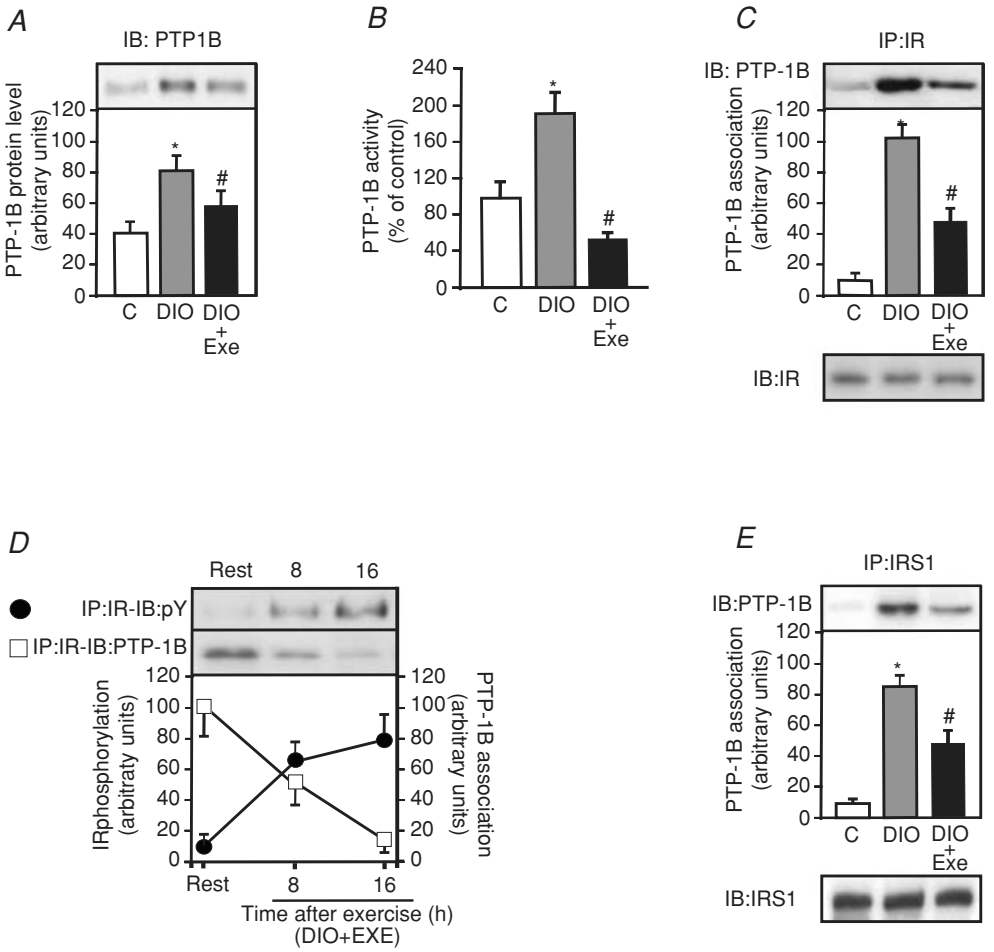

Figure 3. Effect of acute exercise on PTP1B protein levels, activity and PTP1B association with IRβ and IRS-1

A, PTP1B protein level in DIO and DIO + EXE rats were compared with control group. *B*, PTP1B assay was performed as described in the Methods. *C*, tissue extracts were immunoprecipitated (IP) with anti-IRβ followed by immunoblotting (IB) with anti-PTP1B antibody or anti-IRβ antibody (upper and lower panels). *D*, insulin-stimulated IRβ phosphorylation (●) and the IRβ–PTP1B association (□) were determined using IP with anti-IRβ and IB with anti-PY antibody and IP with anti-IRβ followed by IB with anti-PTP1B antibody. *E*, IP with anti-IRS-1 followed by IB with anti-PTP1B antibody to evaluated the IRS-1–PTP1B association (upper panel). Muscle extracts were also IP with anti-IRS-1 and IB with anti-IRS-1 antibody (lower panels). The results of scanning densitometry were expressed as arbitrary units. Bars represent means ± S.E.M. of $n = 6$–8 rats. $^*P < 0.05$, *versus* control and $\#P < 0.05$, DIO + EXE *versus* DIO.

in the muscle of DIO + EXE rats IRS-1–PTP1B association was decreased by 1.7-fold when compared with DIO rats (Fig. 3*E* upper panel). The IRS-1 protein levels were not different between the groups (Fig. 3*E* lower panel).

A single bout of exercise inhibits Ser307 phosphorylation of IRS-1, JNK activity and IκBα degradation in DIO rats

Among the serine residues that become phosphorylated in response to risk factors of insulin resistance, Ser307 has been studied extensively and Ser307 phosphorylation has become a molecular indicator of insulin resistance (Eldar-Finkelman & Krebs, 1997; Aguirre *et al.* 2002; Hirosumi *et al.* 2002; Lee *et al.* 2003); however, the effect of acute exercise on high-fat diet-induced IRS-1 serine phosphorylation has not been identified. To address this issue, we tested Ser307 phosphorylation in the gastrocnemius muscle of control, DIO and DIO + EXE rats. The muscles were blotted with anti-IRS-1 phosphoserine antibody. The high-fat diet increased IRS-1 serine phosphorylation levels in the muscle of DIO rats by 4.5-fold when compared with control rats. In the muscle of DIO + EXE rats, IRS-1 serine phosphorylation decreased by 1.7-fold when compared with DIO rats (Fig. 4*A*).

JNK activation was determined by monitoring phosphorylation of JNK (Thr183 and Tyr185) and c-Jun (Ser63), which is a substrate of JNK. The high-fat diet induced an increase in JNK phosphorylation in the muscle of DIO rats by 7.2-fold when compared with control rats. In the muscle of DIO + EXE rats, JNK serine phosphorylation decreased by 2.0-fold when compared with DIO rats (Fig. 4*B* upper panel). The JNK protein levels were not different between the groups (Fig. 4*B* lower panel). Consistent with JNK activation, c-Jun phosphorylation was 3.1-fold higher in the muscle of DIO rats when compared with control rats. In the muscle of DIO rats submitted to acute exercise, c-Jun phosphorylation decreased by 1.7-fold when compared with DIO rats

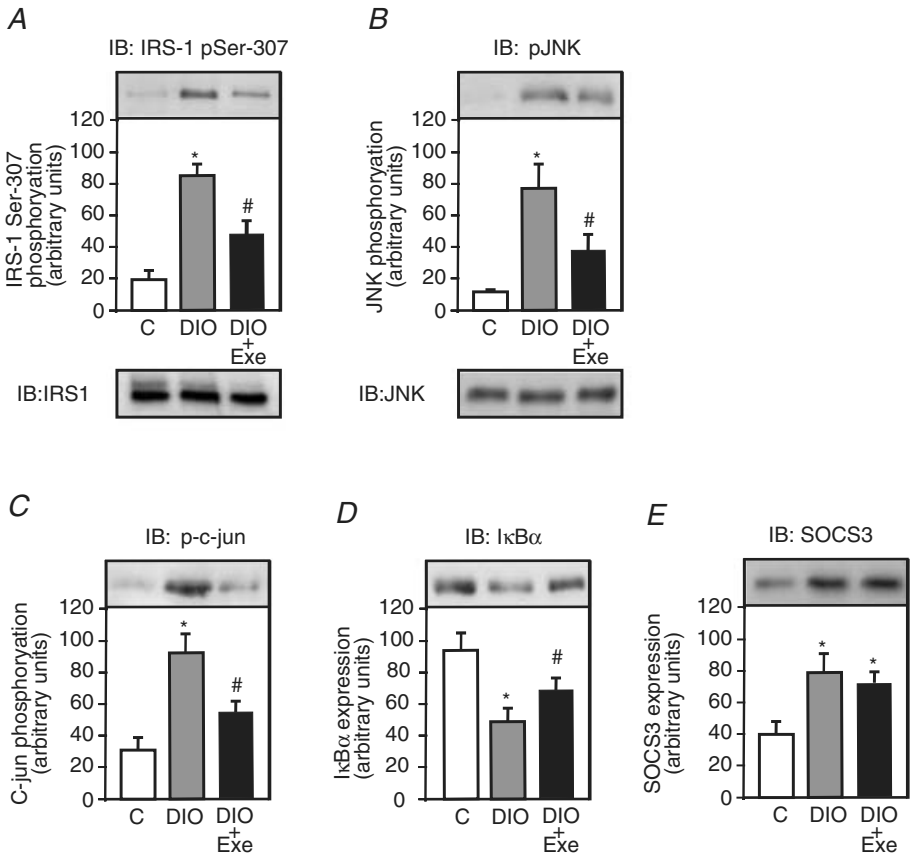

Figure 4. Effect of acute exercise on IRS-1 serine phosphorylation, JNK activity, IκBα degradation and IRS-1 and JNK protein levels in muscle of controls, DIO and DIO + EXE rats
Tissue extracts were immunoblotted (IB) with anti-IRS-1307 phosphoserine antibody (*A* upper panel), anti IRS-1 antibody (*A* lower panel), anti-phospho JNK antibody (*B* upper panel), anti-JNK antibody (*B* lower panel), antiphospho-c-Jun antibody (*C*), anti-IκBα antibody (*D*) and anti-SOCS3 (*E*) in control, DIO and DIO + EXE rats. The results of scanning densitometry were expressed as arbitrary units. Bars represent means ± S.E.M. of $n = 6$–8 rats. *$P < 0.05$, *versus* control and #$P < 0.05$, DIO + EXE *versus* DIO.

(Fig. 4*C*). Finally, we examined the IKK–NF-κB pathway, an important regulator of inflammation, in obesity- and inflammation-induced insulin resistance. The main function of the IKK complex is the activation of NF-κB through phosphorylation and degradation of IκBα (Hevener *et al.* 2003; Greten *et al.* 2004; Viatour *et al.* 2005). Thus, to assess NF-κB activation, we observed IκBα degradation in the muscle of control, DIO and DIO + EXE rats. The high-fat diet led to a decrease in IκBα expression levels in the muscle of DIO rats by 1.9-fold, compared with control rats. However, in the muscle of DIO + EXE rats, IκBα degradation was decreased by 1.4-fold when compared to DIO rats (Fig. 4*D*). The high-fat diet increased SOCS 3 expression in the muscle of DIO rats by 2.0-fold when compared to the control; however, acute exercise did not change the high-fat diet-induced modulation of SOCS 3 expression in this tissue (Fig. 4*E*).

Discussion

Impaired insulin action on whole-body glucose uptake is a hallmark feature of type 2 diabetes mellitus. Physical exercise has been linked to improved glucose homeostasis and enhanced insulin sensitivity immediately after an acute bout of exercise in humans (Devlin *et al.* 1987; Zierath, 1995) and rodents (Richter *et al.* 1982; Wallberg-Henriksson, 1987; Wallberg-Henriksson *et al.* 1988). In this study, we demonstrate that a single bout of exercise partially restored the insulin signalling in muscle of obese rats by different mechanisms. High-fat diet was observed to lead to an increase in the PTP1B protein level and in the activity and serine phosphorylation of IRS-1; it is interesting that acute exercise reversed these parameters in parallel with a reduction in JNK activity and IκBα degradation. However, the acute exercise had no effect on high-fat diet-induced SOCS3 expression.

Several mechanisms may be involved in the pathogenesis of insulin resistance in muscle. The ability of PTP1B to negatively regulate insulin receptor kinase has been established at the molecular level (Myers *et al.* 2001) and ablation of the *PTP1B* gene yields mice displaying characteristics which suggest that inhibition of PTP1B function may be an effective strategy for the treatment of diabetes and obesity (Elchebly *et al.* 1999). In accordance with this, our results show decreased activity and expression of PTP1B in DIO rats after a single bout of exercise. Furthermore, the reduction of PTP1B activity in rats submitted to acute exercise was accompanied by increased insulin sensitivity in skeletal muscle and correlates with increases in tyrosyl phosphorylation of IR, IRS-1 and IRS-2 and with reduction of IR–PTP1B and IRS-1–PTP1B association in skeletal muscle. In contrast to our results, it has been recently was reported that the amount of PTP1B associated with IR-β is not different in the muscle of normal rats at 5, 29 and 53 h after cessation of chronic voluntary exercise (Kump & Booth, 2005). These apparent contradictory results may be related to the protocol of exercise and changes in physiological and metabolic parameters in DIO rats.

Serine phosphorylation of IRS proteins is believed to be a major mechanism of suppression of IRS-1 and IRS-2 activity that contributes to insulin resistance (Saltiel & Olefsky, 1996; Saltiel & Kahn, 2001). Regulation of serine phosphorylation of IR, IRS-1 and IRS-2 proteins has been a focus of investigation in the search for the molecular mechanism of insulin resistance. Our results show a marked reduction in IRS-1 serine phosphorylation, 16 h after acute exercise in DIO rats in parallel with an increase in IR autophosphorylation. A previous study demonstrated that treatment of cultured murine adipocytes with TNF-α induces serine phosphorylation of IRS-1 and converts it into an inhibitor of the IR tyrosine kinase activity *in vitro* (Hotamisligil *et al.* 1996). The IRS-1-mediated inhibition of IR tyrosine kinase activity could occur by direct or indirect interactions between the IR and IRS-1 (Backer *et al.* 1993; O'Neill *et al.* 1994). Serine-phosphorylated IRS-1 might associate with the IR to block the autophosphorylation reaction; alternatively, serine-phosphorylated IRS-1 might act indirectly on the IR through an association with an inhibitor that acts on the IR in a stoichometric or catalytic fashion (Hotamisligil *et al.* 1996). Taken together, these data suggest that a high-fat diet mediates insulin resistance, at least in part, by inducing IRS-1 serine phosphorylation and decreasing IRS-1 and IRS-2 tyrosine phosphorylation and that this effect is inhibited by acute exercise. Studies suggest that overexpression of SOCS3 decreases insulin-induced IRS-1 and IRS-2 tyrosine phosphorylation levels, inducing insulin resistance (Ueki *et al.* 2004). However, this modulation of SOCS3 by DIO was not reversed by acute exercise. As the IR–IRS-1/2 pathway is involved in glucose uptake and glycogen synthesis in muscle, we suggest that acute exercise, by acting on this pathway, reverses insulin resistance of DIO animals.

Activation of inflammatory signalling, including of the IκB–NFκB pathway may also contribute to mediated the serine phosphorylation of IRS-1(Gao *et al.* 2002). However, few studies have examined the effect of acute exercise on the IκB–NFκB pathway. In rats, exercise activates IκB–NFκB signalling in muscle (Ji *et al.* 2004), and acute fatiguing exercise in humans reduces NFκB activity. Similar to a recent study showing that 8 weeks of aerobic exercise training reduced IκB–NFκB signalling in vastus lateralis muscle from subjects with type 2 diabetes (Sriwijitkamol *et al.* 2006), our results show that the high levels of IRS-1, phosphorylated at Ser307, in DIO rats correlated with the disappearance of IκBα. This finding is an indication of IKK activation and suggests that acute

exercise is able to reduce IKK activation and restore the IκBα expression.

Recently, JNK has been linked to the regulation of insulin signalling by several studies (Aguirre *et al.* 2000, 2002; Rui *et al.* 2001; Hirosumi *et al.* 2002; Lee *et al.* 2003). It has been suggested that JNK contributes to insulin resistance by phosphorylating IRS-1 at Ser307, and this phosphorylation leads to inhibition of the IRS-1 function (Aguirre *et al.* 2000, 2002; Rui *et al.* 2001; Lee *et al.* 2003; Prattali *et al.* 2005). However, the effect of exercise on JNK activity remains unclear. Several studies suggest that the activity of JNK intracellular signalling cascade is increased following prolonged running exercise (Boppart *et al.* 2000; Thompson *et al.* 2003). In contrast, JNK phosphorylation was reduced after resistance exercise in old men (Williamson *et al.* 2003). In this study, we observed that a single bout of exercise inhibited DIO-induced JNK activity, and that this inhibition was accompanied by a reduction in IRS-1 serine phosphorylation at Ser307.

In accordance with the results of Oakes *et al.* (1997) we observed that a single bout of exercise completely normalized the insulin action in the diet-induced obese state; however, our data show only a partial amelioration of insulin signalling. Taken together, these data suggest that the complete normalization, by acute exercise, of the insulin action in obesity induced by diet may be caused by other factors. One possibility may be associated with the increase in other insulin-independent signalling pathways. It has been postulated that AMP kinase is a important mediator of acute exercise-induced glucose uptake in muscle (Sakamoto & Goodyear, 2002; Wojtaszewski *et al.* 2002; Krook *et al.* 2004). In addition, in human subjects with type 2 diabetes, where there is impaired insulin signalling in skeletal muscle, acute exercise results in normal activation of AMP kinase (Musi *et al.* 2001; Koistinen *et al.* 2003).

In summary, a single bout of exercise improves insulin sensitivity in DIO rats by reversing high-fat diet-induced decreases in insulin-stimulated IR, IRS-1 and IRS-2 tyrosine phosphorylation. The effect of acute exercise on insulin action is further supported by our findings that DIO + EXE rats show a reduction in PTP1B activity and IRS-1 serine phosphorylation, mechanisms by which a single session of exercise may protect against high-fat diet-induced insulin resistance. Overall, these results provide new insights into the mechanism by which physical activity restores insulin sensitivity.

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