Eloize Cristina Chiarreotto Ropelle

Caracterização da atividade da PTP1B em hipotálamo de roedores obesos submetidos ao exercício físico.

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Orientador: Prof. Dr. José Rodrigo Pauli

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Abstract

The food intake and energy expenditure are regulated by specific neurons in the hypothalamus. However, hypothalamic inflammation is associated with insulin and leptin resistance, hyperphagia and obesity. In this scenario, hypothalamic protein tyrosine phosphatase 1B (PTP1B) has emerged as the key phosphatase induced by inflammation responsible for the central insulin and leptin resistance. The aim of this study was evaluate the effects of exercise on PTP1B activity in the hypothalamus of obese rodents. Here, we demonstrated that acute exercise reduced inflammation and PTP1B protein level/activity in the hypothalamus of obese rodents. Exercise disrupted the interaction between PTP1B with proteins involved in the early steps of insulin (IRβ and IRS1) and leptin (Jak2) signaling, increased the anorexigenic effects of insulin and leptin in obese rats. Interestingly, the anti-inflammatory action and the reduction of PTP1B activity mediated by exercise occurred in an Interleukin-6 (IL-6)-dependent manner, because exercise failed to reduce inflammation and PTP1B protein level after the disruption of hypothalamic-specific IL-6 action in obese rats. Conversely, intracerebroventricular (ICV) administration of recombinant IL-6 reproduced the effects of exercise, improving hypothalamic insulin and leptin action by reducing the inflammatory signaling and PTP1B activity in obese rats at rest. Taken together, our study reports that physical exercise restores insulin and leptin signaling, at least in part, by reducing hypothalamic PTP1B protein level through the central anti-inflammatory response mediate by IL-6.

Resumo

A ingestão alimentar e o gasto energético são minuciosamente regulados por neurônios específicos localizados no hipotálamo. No entanto, a inflamação hipotalâmica está associada com a resistência à insulina e leptina, obesidade e hiperfagia. Neste contexto, proteína tirosina fosfatase 1B (PTP1B) hipotalâmica surgiu como a fosfatase chave responsável pela resistência à central de insulina e leptina. O objetivo do atual estudo foi avaliar o efeito do exercício físico agudo sobre a expressão da PTP1P hipotalâmica em roedores obesos. Nossos resultados demonstraram que o exercício físico reduziu a inflamação e os níveis proteicos e a atividade da PTP1B no hipotálamo de animais obesos. O exercício físico reduziu a interação entre a PTP1B com proteínas envolvidas na via de transmissão do sinal da insulina (IRβ e IRS1) e da leptina (Jak2), melhorando os sinais anorexigênicos mediados por esses hormônios. De forma interessante, o efeito anti-inflamatório e o efeito inibitório sobre a PTP1B mediados pelo exercício ocorreu de forma dependente da Interleucina-6 (IL-6), uma vez que o exercício não reduziu a inflamação e os níveis proteicos PTP1B após a inibição específica da IL-6 hipotalâmica em animais obesos. Por outro lado, a administração intracerebroventricular (ICV) do IL-6 recombinante reproduziu os efeitos do exercício, melhorando a ação da insulina e leptina hipotálamo, reduzindo a sinalização inflamatória e atividade PTP1B em ratos obesos em repouso. Coletivamente, o nossos resultados demonstraram que o exercício físico restaurou a sinalização de insulina da leptina no hipotálamo de animais obesos, pelo menos em parte, pela redução da expressão e atividade da PTP1B hipotalâmica, sendo esse efeito decorrente da resposta anti-inflamatória mediada pela IL-6.

Sumário

Páginas

Dedicatória

Ao meu esposo Eduardo,

que mesmo bem a frente, guardou meu lugar e

esperou que chegasse minha vez.

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Lista de abreviaturas:

ACTH-cortisol: Hormônio adenocorticotrófico

AgRP: Proteína relacionada ao Agouti

Akt: Proteína quinase B

CART: *cocaine-and anphetamine-regulated transcription*

CD45: Membro da família das tirosinas fosfatases

db/db: Camundongo com deficiência no gene da leptina

FoxO1: Membro da família forkhead Box O

Gp130: Glico proteína transmembrana (tradutor de sinal da IL-6)

ICV: Intracerebroventricular

IkBα: *IkappaB alpha*

IKKβ: *IkappaB kinase beta*

IL-10: Interleucina 10

IL-1ra:Interleucina 1 receptor antagonista

IL-1β: Interleucina 1 beta

IL-6 -/-: camundongo deficiente em interleucina 6

IL-6: Interleucina 6

IL-6R: Receptor de interleucina 6

IR: Receptor de insulina

IRS-1: Substrato 1 do receptor de insulina

IRS-2: Substrato 2 do receptor de insulina

IRβ: Receptor de insulina beta

Jak: Proteina Janus quinase

Jak2: Proteína Janus quinase 2

KDa: Quilodaltons

LAR: Membro da família das tirosinas fosfatase

MAPK: Proteína quinase ativadora de mitose

mRNA: Ácido ribonucleico mensageiro

NF-kB: Fator nuclear kappa B

NPY: Neuropeptídio Y

Ob/ob: Camundongo deficiente de leptina

OBR: Receptor de leptina

OBRL: Receptor de leptina de forma longa

OBRS: Receptor de leptina de forma curta

PI3K: fosfatidilinositol 3- quinase

POMC: proopiomelanocortina

PTP: Proteína tirosina fosfatase

PTP1B: Proteína tirosina fosfatase 1B

PTPs: Proteínas tirosina fosfatase

PTPα: Proteína tirosina fosfatase α

SH 2: Domínio homólogo a Src 2

SHP2: Membro da família das tirosinas fosfatase

SNC: sistema nervoso central

SOCS3: Supressor de sinalização de citocina 3

Src 2: *Steroid receptor coactivator 2*

STAT: Transdutor de sinal e ativador de transcrição

STAT3: Transdutor de sinal e ativador de transcrição 3

TNFα: Fator de necrose tumoral

α-MSH: Hormônio estimulador de melanócito alfa

1. Introdução

O hipotálamo é reconhecido como a principal estrutura anatômica do sistema nervoso central (SNC), envolvida no controle da ingestão alimentar e do gasto energético. Os núcleos hipotalâmicos arqueado e paraventricular possuem como função integrar as informações periféricas por intermédio de hormônios e nutrientes para o controle da ingestão alimentar e do gasto energético [1]. Estudos realizados na década de 40 demonstraram que lesões no núcleo ventromedial do hipotálamo de roedores induziam hiperfagia e obesidade; enquanto estímulos no núcleo hipotalâmico lateral induziam anorexia [2]. Esses achados foram determinantes na caracterização do hipotálamo como estrutura chave para o controle da homeostase energética em mamíferos.

A partir da identificação da leptina em 1994, grande avanço vem sendo obtido na caracterização dos mecanismos neurais de controle da fome e do gasto energético mediados pela ação de hormônios no hipotálamo. Durante as duas últimas décadas, a localização dos receptores da leptina em núcleos hipotalâmicos de roedores, bem como a descrição da via de transmissão intracelular disparado por este hormônio em neurônios hipotalâmicos foram determinantes para o entendimento do controle da ingestão alimentar e do gasto energético [1, 3].

Transmissão do Sinal da Leptina e da Insulina no Hipotálamo.

A leptina é produzida principalmente pelo tecido adiposo e em menores quantidades no epitélio gástrico e placenta [4-6]. A proteína do gene *ob* está presente no plasma de camundongos normais, como um monômero com peso molecular de 16 kDa, não foi detectada em plasma de camundongos *ob/ob* (camundongos com deficiência do gene da

leptina), e foi observada em concentrações elevadas em camundongos *db/db* (camundongos com deficiência do gene do receptor da leptina) [7]. A administração de leptina a camundongos *ob/ob* resulta em diminuição da ingestão alimentar, perda de peso e redução dos níveis glicêmicos [8], além de aumentar a atividade simpática em dirção ao tecido adiposo marrom, com consequente aumento do gasto energético [9]. Entretanto, o mesmo resultado não foi observado quando este hormônio foi injetado nos animais *db/db*.

Os níveis séricos de leptina correlacionam-se de forma positiva com o índice de massa corporal na grande maioria das populações estudadas [5, 10-12]. A secreção desse hormônio diminui com o jejum prolongado e estímulo β-adrenérgico [13] e aumenta em resposta à administração de insulina e glicocorticóides [14]. A leptina é secretada de forma pulsátil e inversamente relacionada à atividade do eixo ACTH-Cortisol, ou seja, ocorre diminuição da secreção de leptina ao amanhecer e aumento no final da tarde [15].

O receptor de leptina (OBR) é membro da família gp130 da classe I dos receptores de citoquinas[16] é encontrado em muitos tecidos com várias formas de *splicing*, sendo as mais encontradas a forma curta (OBRS), expressa em vários tecidos, que apresenta domínios intracelulares truncados, e a forma longa (OBRL), que apresenta domínios intracelulares longos e é expressa principalmente no hipotálamo (núcleos paraventricular, arqueado, ventromedial e dorsomedial) [17, 18]. O OBRS não tem sua função bem definida, mas parece influir no transporte da leptina através da barreira hematoencefálica e talvez contribua para a depuração da leptina atuando como uma fonte de receptor solúvel.

A homologia do receptor de leptina à classe I dos receptores de citoquinas forneceu informações importantes para a descoberta dos possíveis mediadores intracelulares da ação

da leptina. Os receptores da classe I das citoquinas agem através das famílias das proteínas Jak (*Janus Kinase*) e STAT (*Signal Transducers Activators of Transcription*) [19]. Tipicamente, as proteínas Jak estão constitutivamente associadas com sequências 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 a 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 de neurotransmissores anorexigênicos como a proopiomelanocortina (POMC) [3] (Figura 1).

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-quinase), e é possível que a capacidade do OBR controlar o peso dependa também destas vias de sinalização [3]**.**

Após a ativação dos receptores de leptina no cérebro e das proteínas envolvidas na transmissão do sinal desse hormônio, respostas neuronais integradas são necessárias para modular a ingestão alimentar e o gasto energético. Alguns neurotransmissores importantes para o funcionamento dessa rede neuronal estimulam a ingestão alimentar como o neuropeptídeo Y (NPY) [20] e o *Agouti related peptide* (AGRP) [21], enquanto outros provocam redução da ingestão alimentar como o *cocaine-and anphetamine–regulated transcription* (CART) [22], *proopiomelanocortin* (POMC) [1, 3] e o *melanocyte stimulating hormone* (α-MSH) [23]. A leptina regula o balanço energético diminuindo os níveis de neuropeptídeos anabólicos NPY e AGRP e aumentando a concentração de neuropeptídeos catabólicos CART, POMC e α-MSH.

Assim como a leptina, a insulina também é considerada um hormônio que sinaliza ao hipotálamo sobre as condições do estado energético modulando a ingestão alimentar [24, 25]. A insulina é um hormônio anabólico produzido pelo pâncreas e é secretada em resposta à elevação dos níveis glicêmicos. Uma vez na corrente sanguínea, a insulina atravessa a barreira hematoencefálica via um sistema de transporte saturável em níveis proporcionais aos plasmáticos [26]. Os receptores de insulina são expressos por neurônios envolvidos na ingestão alimentar [27-29]. A administração de insulina no sistema nervoso central reduz a ingestão alimentar e diminui o peso corporal, enquanto a deficiência desse hormônio causa hiperfagia [30].

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) (Figura 1). A fosforilação em tirosina das proteínas IRS cria sítios de reconhecimento para moléculas contendo domínios com homologia à Src 2 (SH2) [31]. Dentre estas destaca-se a fosfatidilinositol 3 quinase (PI3K). No hipotálamo, a PI-3K resulta na ativação e fosforilação da Akt. Uma vez fosforilada, a Akt fosforila e desativa o fator de transcrição FoxO1 (membro da família Forkhead Box O). A FoxO1 fosforilada é mantida no citoplasma, deixa de promover a transcrição gênica de neurotransmissores responsáveis pelo aumento da ingestão alimentar, como o NPY [32, 33], resultando em decréscimo da ingestão alimentar.

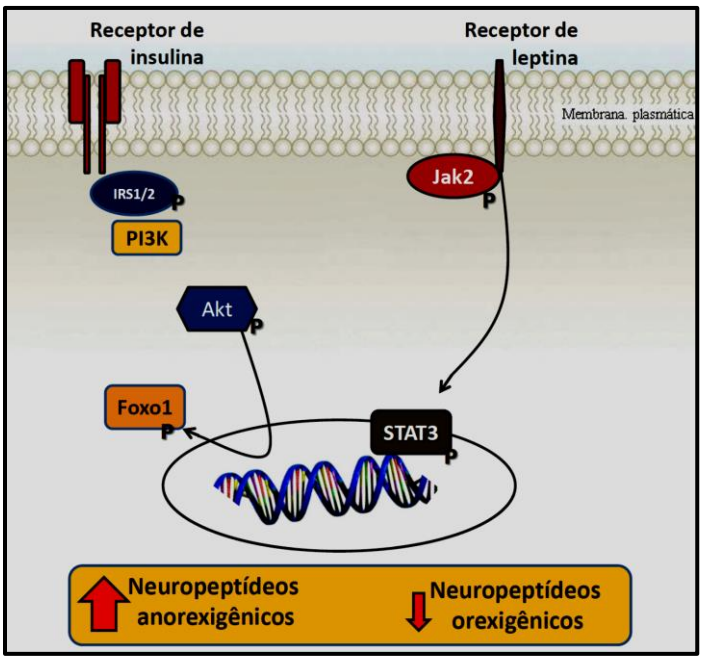

Figura 1- Desenho esquemático dos sinais anorexigênicos disparados pela insulina e pela leptina em neurônios hipotalâmicos.

Inflamação hipotalâmica e Resistência à Insulina e Leptina: O papel da PTP1B.

Inicialmente, a descoberta da leptina passou a uma nova esperança para o tratamento da obesidade, entretanto, muito do entusiasmo com a leptina se desfez com a constatação de que indivíduos obesos respondem mal ao tratamento com leptina e que a administração desse hormônio em modelos experimentais de obesidade, demonstrou a existência de resistência central a esse hormônio [34-37]. A frequente associação clínica entre diabetes mellitus tipo 2 e obesidade, aliada ao fato de que pacientes obesos são em geral hiperleptinêmicos e hiperinsulinêmicos, fomentou a hipótese de que o controle inadequado da fome e da termogênese, que predispõem ao desenvolvimento de obesidade, devessem-se a uma resistência hipotalâmica à ação da leptina. Tal suspeita foi confirmada por meio de estudos realizados em diferentes modelos animais de obesidade [34-36]. Embora a leptina não

seja a terapia anti-obesidade ideal, como esperado inicialmente, o desenvolvimento de estratégias para reduzir à resistência central a leptina pode ser um novo caminho para o tratamento da obesidade.

Na última década estudos passaram a identificar alguns dos possíveis mecanismos que induzem a resistência à insulina e leptina nos centros hipotalâmicos controladores do apetite que podem estar envolvidos com a hiperfagia e obesidade [1, 3]. Diferentes grupos evidenciaram reduzida capacidade dos sinais destes hormônios em tecido hipotalâmico em diferentes modelos experimentais de obesidade [34, 35, 38, 39]. A resistência à ação da insulina e da leptina no sistema nervoso central bloqueiam a ativação das vias anorexigênicas mediada por estes hormônios e contribuem diretamente para o desenvolvimento da obesidade [1, 3]. A hipótese de que possivelmente um processo inflamatório de baixa magnitude esteja envolvido com o descontrole dos sinais de saciedade, vem ganhando destaque. Ratos alimentados com dieta rica em gordura saturada apresentam discreto aumento da expressão de citoquinas inflamatórias como o Fator de Necrose Tumoral alfa (TNFα) e Interleucina-1 beta (IL-1β) no hipotálamo [39]. Neste cenário, algumas proteínas relacionadas à inflamação foram descritas como moduladores negativos da sinalização da insulina e leptina no hipotálamo, dentre elas destacam-se; a SOCS3 (*Supressor of Citokine Signaling 3*) [38], a proteína tirosina fosfatase 1B, PTP1B [40] [35] e o IKKβ [36]. Basicamente o mecanismo de ação destas proteínas está relacionado à diminuição da fosforilação em tirosina de proteínas como o IRβ, o IRS1, a Jak2 e a STAT-3.

O balanço entre a fosforilação e a desfosforilação de proteínas é a base para o controle de diversos eventos biológicos disparados por efetores extracelulares, como

hormônios, agentes carcingênicos, citocinas, neurotransmissores e substâncias ou metabólicos tóxicos [41]. Estima-se que 30% das proteínas intracelulares são fosfoproteínas e que cerca de 4% do genoma eucariótico codifique proteínas quinases e proteínas fosfatases [42]. Em particular, a fosforilação e desfosforilação de resíduos de treonina, serina e tirosina em proteínas são eventos chave na regulação da divisão, diferenciação e desenvolvimento celular, regulação do metabolismo e expressão gênica, contração, transporte, locomoção celular, aprendizado e memória [42]. As atividades de proteínas quinases e fosfatases são minuciosamente reguladas *in vivo* de maneira que modificações na atividade dessas enzimas podem proporcionar consequências graves, que incluem neoplasias, diabetes, inflamação e doenças resultantes de defeitos imunológicos [41].

As proteínas tirosinas fosfatases (PTPs) são importantes reguladoras de eventos de sinalização celular dependente de fosforilação em tirosina e podem representar novos alvos terapêuticos para o tratamento de várias doenças em humanos [43]. Algumas PTPs, como PTPα, PTP, CD45, SHP2, LAR, e a PTP1B, estão descritas como reguladoras negativas da sinalização da leptina e insulina [44], sendo a PTP1B, a principal proteína tirosina fosfatase implicada na regulação da ação desses hormônios [45, 46].

PTP1B é expressa em diferentes tecidos sensíveis à insulina e diferentes estudos em cultura de células e em roedores, indicam que esta enzima se associa ao receptor de insulina (IR) e aos substratos 1 e 2 do receptor de insulina (IRS-1 e IRS-2) promovendo a desfosforilação dessas proteínas atenuando o sinal da insulina [47-49]. De forma semelhante, a PTP1B também regula negativamente a transmissão do sinal da leptina através da interação direta com a proteína JAK2 [50, 51]. Esses sinais inibitórios da PTP1B em direção à sinalização da insulina e da leptina resulta em aumento do sinais orexigênicos (figura 2).

Figura 2.

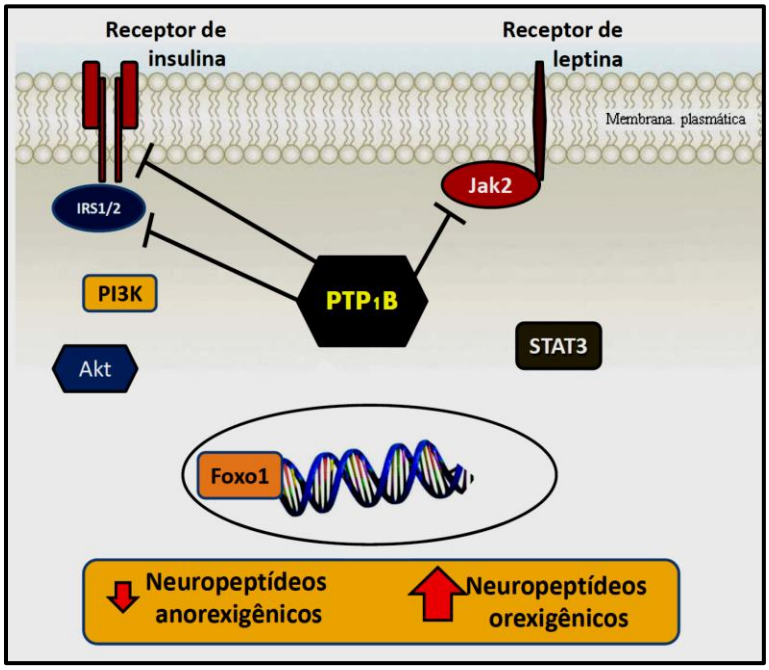

Figura 2 – Representação esquemática da ação da PTP1B sobre a desfosforilação em tirosina das proteínas IR, IRS-1 e Jak2. A ação da PTP1B no hipotálamo resulta em resistência à insulina e leptina e aumento da ingestão alimentar.

Recentemente, Bence e colaboradores descobriram que a PTP1B em neurônios hipotalâmicos regula o peso corporal, adiposidade e a ação da leptina em modelo animal [40]. Esse estudo mostrou que camundongos que não expressam a PTP1B especificamente em neurônios possuem hipersensibilidade a leptina e não desenvolvem obesidade mesmo quando alimentados com dieta rica em gordura, evidenciando a importância da participação desta proteína em células neuronais para o controle do apetite e do peso corporal em roedores. Nosso grupo demonstrou que a inibição da PTP1B hipotalâmica através do oligonucleotídeo antisense dirigido exclusivamente em regiões específicas no hipotálamo

de ratos obesos, foi capaz de restaurar a sensibilidade à insulina e leptina e reduzir significativamente a ingestão alimentar [35]. Adicionalmente, demonstramos que o processo inflamatório observado em neurônios de animais obesos é responsável pelo aumento da expressão e atividade da PTP1B no tecido hipotalâmico, demonstramos ainda que o TNF-α é a principal molécula inflamatória responsável pelo aumento da atividade da PTP1B no hipotálamo [52]. Neste sentido, estratégias com finalidade de reduzir a inflamação no sistema nervoso central são de grande interesse para diminuir a expressão da PTP1B e restabelecer a sensibilidade à ação da insulina e da leptina em neurônios do hipotálamo.

Efeitos do Exercício Físico sobre a Resistência Hipotalâmica à Insulina e Leptina.

A prática regular de exercício físico representa uma das pedras angulares para a prevenção e tratamento da obesidade e doenças associadas, melhorando a sensibilidade à insulina em tecidos periféricos [53-57]. Os efeitos decorrentes da prática de exercícios resultam em aumento do gasto energético, colaborando para a redução da adiposidade e, consequentemente, para a redução do peso corporal. Além destes efeitos, evidências acumuladas nos últimos anos apontam que o exercício físico tem participação direta na sensibilidade à ação da insulina em diferentes tecidos [58], incluindo o hipotálamo [59]. Postula-se que a prática de exercício físico seja capaz de reduzir os níveis teciduais e séricos de marcadores inflamatórios em modelos experimentais e em humanos [60, 61]. Em modelo de obesidade genética, Bi e colaboradores evidenciaram que o exercício físico aumentou a sinalização da leptina, após administração exógena do hormônio, prevenindo a hiperfagia [62].

Nos últimos anos nosso laboratório se dedicou na avaliação dos efeitos do exercício físico sobre a sensibilidade à insulina e leptina no hipotálamo [59, 63]. Recentemente demonstramos que roedores obesos submetidos à uma única sessão de exercício em esteira ou natação, apresentaram redução significativa de marcadores inflamatórios; como a ativação da via IKK/NF-kB e redução da atividade de proteínas envolvidas no estresse de retículo endoplasmático em neurônios [64]. Essa resposta anti-inflamatória, deveu-se ao aumento da Interleucina-6 e da Interleucina-10 em neurônios, principalmente no núcleo arqueado hipotalâmico. A redução da inflamação e do estresse de retículo endoplasmático proporcionou melhora da sensibilidade à insulina e à leptina em hipotálamo dos animais obesos, contribuindo para redução da ingestão alimentar e do peso corporal [64]. Esses resultados demonstram que o exercício físico pode ser uma forma eficaz para reduzir o processo inflamatório em células neuronais, e inserem uma nova óptica no entendimento do exercício como estratégia de combate à obesidade [65 66].

Coletivamente, esses estudos suportam a hipótese de que a inflamação de baixo grau em células do sistema nervoso central está associada com resistência à insulina e leptina por diferentes mecanismos, e que, estratégias antiinflamatórias, como o exercício físico, podem ser determinantes para a melhora da sensibilidade à insulina e leptina no hipotálamo. Mediante o exposto, elaboramos a hipótese de que o exercício físico, através da IL-6, poderia contribuir para a melhora da sensibilidade à ação destes hormônios através da redução da expressão e atividade da PTP1B em hipotálamo de roedores obesos.

2. Objetivos

Objetivo geral

O objetivo principal do estudo foi caracterizar os efeitos do exercício físico sobre a expressão e atividade da PTP1B hipotalâmica e relacionar esses efeitos com a sensibilidade à insulina e leptina em hipotálamo de roedores obesos.

Objetivos específicos

Etapa 1 – Efeitos do exercício sobre a PTP1B hipotalâmica

Avaliar o efeito da sessão aguda de exercício físico sobre a expressão e atividade da PTP1B no hipotálamo de ratos controle e obesos.

Analisar a associação da PTP1B com as proteínas IR, IRS-1 e Jak2 no hipotálamo de ratos exercitados.

Etapa 2 – Efeitos da IL-6 sobre a PTP1B hipotalâmica

Analisar a possível colocalização do receptor de IL-6 (IL-6R) com a PTP1B em hipotálamo de ratos.

Avaliar o efeito da infusão intracerebroventicular de Interleucina-6 sobre a expressão e atividade da PTP1B no hipotálamo de ratos obesos.

Analisar o efeito da infusão intracerebroventicular de Interleucina-6 sobre a associação da PTP1B com as proteínas IR, IRS-1 e Jak2 no hipotálamo de ratos obesos.

Determinar os efeitos da infusão intracerebroventricular do anticorpo anti-IL-6 sobre a expressão da PTP1B no hipotálamo de ratos obesos submetidos ao exercício físico agudo.

3. Capítulo – Artigo

Acute Exercise Suppresses Hypothalamic PTP1B Protein Level and Improves Insulin and Leptin Signaling in Obese Rats

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Running head: Exercise reduces PTP1B action in hypothalamus

Key words: exercise, obesity, hypothalamus, leptin, insulin

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ABSTRACT

Hypothalamic inflammation is associated with insulin and leptin resistance, hyperphagia and obesity. In this scenario, hypothalamic protein tyrosine phosphatase 1B (PTP1B) has emerged as the key phosphatase induced by inflammation responsible for the central insulin and leptin resistance. Here, we demonstrated that acute exercise reduced inflammation and PTP1B protein level/activity in the hypothalamus of obese rodents. Exercise disrupted the interaction between PTP1B with proteins involved in the early steps of insulin (IRβ and IRS1) and leptin (Jak2) signaling, increased the tyrosine phosphorylation of these molecules and restored the anorexigenic effects of insulin and leptin in obese rats. Interestingly, the anti-inflammatory action and the reduction of PTP1B activity mediated by exercise occurred in an Interleukin-6 (IL-6)-dependent manner, because exercise failed to reduce inflammation and PTP1B protein level after the disruption of hypothalamic-specific IL-6 action in obese rats. Conversely, intracerebroventricular (ICV) administration of recombinant IL-6 reproduced the effects of exercise, improving hypothalamic insulin and leptin action by reducing the inflammatory signaling and PTP1B activity in obese rats at rest. Taken together, our study reports that physical exercise restores insulin and leptin signaling, at least in part, by reducing hypothalamic PTP1B protein level through the central anti-inflammatory response.

INTRODUCTION

It has been proposed that obesity is associated with hypothalamic inflammation and dysfunction in animal models (5, 9, 46) and in humans (37, 40). An imbalance between caloric intake and energy expenditure is generated as a consequence of low-grade inflammation, leading to the hypothalamic insulin and leptin resistance through distinct intracellular mechanisms (3, 5, 6, 9, 46). During the last decade, the Protein Tyrosine Phosphatase 1 B (PTP1B) was investigated as a key phosphatase involved in insulin and leptin resistance in hypothalamic cells (18) and *in vivo* (43).

PTP1B is the prototypic member of the protein tyrosine phosphatase (PTP) family, encoded by the *Ptpn1* gene (4, 38). PTP1B is predominantly localized on the cytoplasmic face of the endoplasmic reticulum and is expressed ubiquitously in classical insulin- and leptin-targeted tissues including the hypothalamus (4). PTP1B is able to associate with and dephosphorylate the tyrosine residues from several proteins, such as, Insulin Receptor beta (IRβ), Insulin Receptor Substrate-1 (IRS-1) and Janus Kinase 2 (Jak2) (17, 20, 35, 43). High protein levels of PTP1B were found in the muscle, liver and hypothalamus of rodents in animal models of obesity (10, 27, 44, 48). Interestingly, *Ptpn1*-/- mice showed lean body weight and improved glucose homeostasis after high-fat diet treatment (11).

In the hypothalamus, a high level of PTP1B protein level is associated with insulin and leptin resistance, hyperphagia and obesity (27). Conversely, neuronal *Ptpn1*-/- mice have reduced weight and adiposity and increased activity and energy expenditure (2). In addition, PTP1B ablation in POMC neurons leads to leptin hypersensitivity and increases the energy expenditure in mice (1). Several lines of evidence suggested that TNFα/NF-κB signaling controls PTP1B protein level (28, 44), whereas, *TNF*α-/- mice displayed low PTP1B expression despite high-fat diet (HFD) treatment (44). In hypothalamic tissue, both HFD and intracerebroventricular (ICV) injection of a low dose of recombinant TNFα were sufficient to increase PTP1B activity leading to insulin and leptin resistance in rodents (28), suggesting that this phosphatase is induced during the inflammatory status.

Physical exercise is considered one of the most important non-pharmacological strategies to prevent or treat obesity. Several studies reported that exercise protects against

high-fat diet-induced hypothalamic inflammation and increases leptin signaling in the hypothalamus of rodents (19, 42, 47), however the mechanism by which exercise sensitizes the hypothalamus is not understood. It is important to note that upon contraction, skeletal muscle stimulates the production and release of cytokines, also called myokines, which can influence metabolism and modify cytokine production in tissues and organs. IL-6 is the first cytokine present in the circulation during physical exercise (26). This myokine can act as a pro-inflammatory or anti-inflammatory mediator depending on the *in vivo* environmental circumstances (26). Recently, we described that IL-6 improves insulin and leptin signaling by reducing inflammatory signaling and endoplasmic reticulum stress in the hypothalamus of obese, exercised animals (31), however, the effects of exercise on hypothalamic PTP1B activity remains unclear. Therefore, in the present study, we investigated the role of exercise and IL-6 on hypothalamic PTP1B expression and activity in obese rodents.

MATERIALS AND METHODS

Animals and diets

Male 4-wk-old Wistar rats were obtained from the University of Campinas Breeding Center. The investigation was approved by the ethics committee and followed the university guidelines for the use of animals in experimental studies and experiments conform to the Guide for the Care and Use of Laboratory Animals, published by the U.S. 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. Rats were randomly divided into two groups: control, fed on standard rodent chow (3.948 kcal.Kg^{-1}) or high-fat diet (HFD) (5.358 kcal.Kg^{-1}) *ad libitum* for 3 months. The high-fat diet composition was previously described (23).

Male (10-wk-old) *ob/ob* and *db/db* mice and their respective control C57BL/6J background were obtained from The Jackson Laboratory and provided by the University of São Paulo. The mice were bred under specific pathogen-free conditions at the Central Breeding Center of the University of Campinas and were fed on standard rodent chow (3.948 kcal.Kg^{-1}).

Antibodies and chemicals

Anti-phospho-Jak2 (rabbit polyclonal, AB3805) antibody was from Upstate Biotechnology (Charlottesville, VA, USA). Anti-JAK2 (rabbit polyclonal, SC-278), anti-phospho-IRβ (rabbit polyclonal, SC-25103), anti-IRβ (rabbit polyclonal, SC-711), anti-phospho-IRS-1 (rabbit polyclonal, SC-17199), anti-IRS-1 (rabbit polyclonal, SC-559), anti-IL-6 (rabbit polyclonal, SC-7920), anti-IL6Rα (rabbit polyclonal, sc-13947) and anti-PTP1B (goat polyclonal, SC1718) antibodies were from Santa Cruz Biotechnology, Inc. Anti-PTP1B (AB-1 mouse polyclonal) was purchased from Calbiochem (La Jolla, CA). Anti-beta tubulin (rabbit polyclonal, #2146), anti-phospho-IKKα/β (rabbit polyclonal, #2687) and anti-IκBα (rabbit polyclonal, #9242) were from Cell Signalling Technology (Beverly, MA, USA). Leptin and recombinant IL-6 were from Calbiochem (San Diego, CA, USA). Protein A-Sepharose 6 MB and Nitrocellulose paper (Hybond ECL, 0.45 mm)

were from Amersham Pharmacia Biotech United Kingdom Ltd. (Buckinghamshire, United Kingdom). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Intracerebroventricular cannulation

After intraperitoneal injection of a mix of ketamin (10 mg) and diazepam (0.07 mg) (0.2 ml/100 g body weight), the rats were stereotaxically instrumented with a chronic 26-gauge stainless steel indwelling guide cannula aseptically placed into the third ventricle at the midline coordinates of 0.5 mm posterior to the bregma and 8.5 mm below the surface of the skull of the rats using the Stoelting stereotaxic apparatus, as previously described (32). After 5 d recovery period, cannula 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 angiotensin injection were not included in the experiments.

Exercise protocols

Swimming - Animals were acclimated to swimming for 2 d (10 min per day). Water temperature was maintained at 32–34 °C. Rats performed two 3-h exercise bouts, separated by one 45-min rest period. The rats swam in groups of three in plastic barrels of 45 cm in diameter that were filled to a depth of 50 cm. This protocol was conducted between 11:00 a.m. and 6:00 p.m., as previously described (32) and mice performed four 30-min exercise bouts separated by one 5-min rest period. The mice swam in groups of four in plastic barrels of 40 cm in diameter that were filled to a depth of 20 cm. This protocol was conducted between 3:00 p.m. and 6:00 p.m. Both exercise protocols finished at 6:00 p.m. for evaluation of food intake and analysis of hypothalamic tissue as previously described (31).

Intracerebroventricular (ICV) injections

Rats were deprived of food for 2 h with free access to water and received 2 μl of bolus injection into the third ventricle, as follows:

Insulin and leptin injections. Rats received ICV infusion of vehicle (saline), insulin (200 mU), or leptin (10^{-6} M) at 6:00 p.m. to evaluate the food consumption. 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. For insulin and leptin signaling evaluation, the hypothalamic tissue was removed 15 minutes after insulin or leptin ICV injection.

Recombinant IL-6. Animals received ICV injection of vehicle (saline) or recombinant IL-6 (50, 100, or 200 ng) at 6:00 p.m. For Western blot analysis, we injected recombinant IL-6 into the third ventricle, and the hypothalamus was excised 6 h later.

IL-6 neutralizing antibody. Rats were randomly selected for saline, rabbit pre-immune serum (RPIS) or rabbit antiserum against IL-6 (50 ng) (anti-IL-6) ICV injection. Anti-IL-6 was injected into the third ventricle of the rats 30 min before the exercise protocol and after the first bout of swimming exercise.

Serum analysis

Blood was collected from the cava vein 15 min after the exercise protocols. Plasma was separated by centrifugation (1.100 x g) for 15 min at 4 °C and stored at -80 °C until the assay. RIA was employed to measure serum insulin. Leptin and IL-6 concentrations were determined using a commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit (Crystal Chem Inc., Chicago, IL). Blood lactate was measured using Accutrend Plus equipment (Roche, Egham); sample blood was obtained from the tails every 60 min during the swimming exercise.

Corticosterone analysis

Corticosterone levels were determined using urine samples obtained from rats using a specific metabolic cage for 24 h after the exercise protocols. The corticosterone level was determined using an EIA kit from Cayman Chemical Co. (Ann Arbor, MI).

Immunohistochemistry

Paraformaldehyde-fixed hypothalami were sectioned (5 mm). The sections were obtained from the hypothalami of five rats per group in the same localization (antero-posterior = 21.78 from bregma) and were subjected to regular single- or double-immunofluorescence staining using DAPI, anti-IL6 receptor alpha and anti-PTP1B antibodies, according to a previously described protocol (31). Analysis and photodocumentation of results were performed using a LSM 510 laser confocal microscope (Zeiss, Jena, Germany). The anatomical correlations were made according to the landmarks given in a stereotaxic atlas. The frequency of positive cells was determined in 100 randomly counted cells using Analysis software (Version 2.4).

Western blotting analysis and immunoprecipitation

After exercise and/or ICV treatments, the animals were anesthetized, and the hypothalamus was quickly removed, minced coarsely, and homogenized immediately in a freshly prepared ice-cold buffer (1% Triton X-100, 100 mmol/l Tris pH 7.4, 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium vanadate, 2 mmol/l phenyl methylsulphonyl fluoride, and 0.1 mg aprotinin) suitable for preserving the phosphorylation states of enzymes. Western blotting was performed as previously described (33).

The β subunits of the IR (IRβ), IRS1, Jak2 and PTP1B were immunoprecipitated from rat hypothalami. Antibodies used for immunoblotting were anti-IR, anti-IRS-1, anti-Jak2 and anti-PTP1B. Blots were exposed to preflashed Kodak XAR film. Band intensities were quantified by optical densitometry (Scion Image software, ScionCorp, Frederick, MD, USA) of the developed autoradiographs.

Protein tyrosine phosphatase activity assay

The *in vitro* PTP1B activity assay was conducted based on a protocol previously described by Taghibiglou et al. (36). After six hours of IL-6 ICV injection, hypothalami were removed and homogenized in solubilization buffer containing 1% Triton X-100, 20 mM, Tris (pH 7.6), 5 mM EDTA, 2 mM PMSF and 0.1 mg aprotinin/mL, 1 mM EGTA and 130 mM NaCl. Lysates were centrifuged (15 000 x g rpm, 40 min, 4 °C) and the

supernatants were collected for immunoprecipitation with anti-PTP1B antibody. Immunoprecipitates were washed in PTP assay buffer (100 mM HEPES (pH 7.6), 2 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl and 0.5 mg/mL bovine serum albumin). The pp60c-src C-terminal phosphoregulatory peptide (TSTEPQpYQPGENL) was added to a final concentration of 200 μM in a total reaction volume of 60 μL in a PTP assay buffer for immunoprecipitation. The activity of total extracts (125 μg) was measured in the same manner in a total reaction volume of 60 μl in a PTP assay buffer, adding the peptide to a final concentration 200 μM. 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 96-well plates, 100 μl of Biomol Green Reagent (Enzo Life Sciences, USA) was added, and the absorbance was measured at 660 nm.

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 in autoradiographs and quantified by optical densitometry (Scion Image). Statistical analysis was performed using the ANOVA test with the Bonferroni post test. Significance was established at the $p < 0.05$ level.

RESULTS

Acute exercise reduces PTP1B protein levels in the hypothalamus of obese rodents.

First, we sought to determine the effects of exercise on hypothalamic PTP1B protein levels and activity in obese animals. By using Western blotting analysis, high PTP1B protein levels were found in the hypothalamic samples of high-fat diet-fed rats, as expected (Fig. 1 A). Interestingly, four hours after the acute protocol of exercise, we observed a significant reduction in hypothalamic PTP1B levels in obese rats (about 35%), when compared to obese animals at resting condition (Fig. 1A), however, exercise did not change PTP1B protein levels in the hypothalamus of control rats (Fig. 1A). To confirm the suppressive effect of exercise on hypothalamic PTP1B in obese animals, a PTP1B activity assay was performed four hours after the acute protocol of exercise and, in fact, exercise did not change PTP1B activity in the hypothalamus of lean rats but reduced PTP1B activity in the hypothalamus of obese rats by about 30% (Fig. 1B). In addition, we employed an acute swimming exercise in *ob/ob* and *db/db* mice to evaluate the hypothalamic PTP1B protein levels four hours after the acute exercise. As observed in obese rats, swimming exercise reduced PTP1B protein levels in the hypothalamus of both, *ob/ob* and *db/db* mice (Fig. 1C).

Blood lactate analysis demonstrated that the swimming exercise protocol presented moderate intensity for both, lean and obese rats (Fig. 1D), because a maximal lactate steady state in rats was previously estimated at 5.5 mmol/L (15). In addition, acute swimming exercise did not change the total body weight in lean and obese rats when compared to the respective control groups at the rest condition (Fig. 1E). Corticosterone levels were slightly increased in obese animals when compared to lean group and the urine obtained after the exercise protocol revealed that corticosterone levels were not change after the exercise protocol when compared to obese rats at rest (Fig. 1F). These data suggest that moderate exercise was sufficient to reduce PTP1B protein levels in the hypothalamus of obese rats independent of body weight changes and stressful effects.

Exercise disrupts the association between PTP1B and IRβ, IRS1 and Jak2 in the hypothalamus of obese rats.

The effects of acute swimming exercise on PTP1B interactions with early proteins involved in insulin and leptin signaling were examined. Immunoprecipitation assays demonstrated a strong interaction between PTP1B and IRβ, IRS1 and Jak2 in the hypothalamus of obese animals, when compared to the control group (Figs. 2A–C, respectively). However, in the hypothalamic samples obtained four hours after swimming exercise, we observed that exercise disrupted the PTP1B association with IRβ, IRS1 and Jak2 in the hypothalamus (Figs. 2A–C, respectively). Exercise did not change the PTP1B interaction with IRβ, IRS1 and Jak2 in the hypothalamus of lean rats (data not chow).

Thereafter we examined the insulin and leptin sensitivity in the hypothalamus of obese rats after acute exercise. Fifteen minutes after the exercise protocol we performed an ICV injection of insulin or leptin in obese animals. We note that swimming exercise increased insulin-induced IRβ and IRS1 tyrosine phosphorylation (Figs. 2D and E) and leptin-induced Jak2 tyrosine phosphorylation in the hypothalamus of obese rats, when compared to the obese group at rest (Fig. 2F). Consistent with these results, we observed that exercise restored insulin's and leptin's anorexigenic actions by reducing food intake in obese animals (Figs. 2G and H, respectively). Exercise reduced insulin but did not change the leptin serum levels in obese rats when compared to obese animals under resting conditions (Figs. 2I and J, respectively). These data demonstrated that acute exercise reduced PTP1B association with IRβ, IRS1 and Jak2 in the hypothalamus of obese animals and improved insulin and leptin signaling in the hypothalamic tissue.

IL-6 anti-inflammatory activity reduces PTP1B protein levels in the hypothalamus.

Because potent anti-inflammatory effects of IL-6 were observed and that IL-6 was produced during exercise, we hypothesized that IL-6 could be involved in the reduction of hypothalamic PTP1B protein levels in exercised rats. Consistent with our previous data (13, 30, 31), exercise increased the hypothalamic protein levels of IL-6 in obese rats (Fig. 3A).

To determine whether IL-6 is involved in the reduction of PTP1B protein levels in the hypothalamus, we performed a time-course experiment *in vivo* by injecting recombinant IL-6 into the third hypothalamic ventricle of obese rats at rest. Six hours after the recombinant IL-6 injections, we observed that hypothalamic PTP1B protein levels were reduced in a dose-dependent manner in obese rats (Fig. 3B). Furthermore, 200 ng of recombinant IL-6 infusion reduced the PTP1B activity by 33% in the hypothalamus of obese rats, when compared to obese animals that received saline (2μL) ICV injection (Fig. 3C).

It has been proposed that NF-κB activity controls PTP1B protein levels under inflammatory conditions (28, 44). Thus, we sought to determine the role of IL-6 on upstream proteins involved in NF-κB activation. We observed that acute ICV recombinant IL-6 injection reduced IKKβ serine phosphorylation and increased IκBα protein levels in the hypothalamic tissue of obese rats (Fig. 3D and E).

Similar to the exercise protocol, acute recombinant IL-6 injection disrupted interactions between PTP1B and IRβ, IRS1 and Jak2 in the hypothalamus of obese animals, when compared to saline injection (Fig. 3F-H). IL-6 ICV injection increased insulin-induced IRβ and IRS1 tyrosine phosphorylation and leptin-induced Jak2 tyrosine phosphorylation in obese rats (Fig. 3I-K). In addition, ICV IL-6 injection increased the anorexigenic action of insulin (Fig. 3L) and leptin (Fig. 3M) by reducing the food consumption in obese rats at rest.

Next, we performed immunohistochemical analyses to evaluate the localization of IL-6R and PTP1B in obese rats. Double-staining confocal microscopy showed that IL-6Rα is expressed in a majority of cells in the arcuate nucleus. Interestingly, we observed that most cells expressing IL-6Rα in the arcuate nucleus were shown to possess PTP1B (Fig. 4A and B), suggesting a possible interaction between these molecules.

Exercise requires IL-6 hypothalamic action to reduce PTP1B protein levels.

We hypothesized that exercise requires IL-6 action to reduce PTP1B protein levels in the hypothalamus. To test this hypothesis, we developed an experimental strategy to

block the central action of IL-6 in obese animals during physical exercise. Thus, we injected an anti-IL-6 antibody or rabbit pre-immune serum (RPIS) into the third-hypothalamic ventricle in obese animals at thirty minutes before the exercise protocol and during the resting period between the two bouts of exercise (Fig. 5A). Interestingly, exercise failed to reduce PTP1B protein levels (Fig. 5B) and activity (Fig. 5C) in the hypothalamus of obese rats injected with anti-IL-6 antibody, when compared to exercised animals that received RPIS. We also observed that anti-IL-6 ICV pretreatment blunted, at least in part, the anti-inflammatory effects of exercise in the hypothalamus in obese rats (Fig. 5D and E). Moreover, high levels of PTP1B/IRβ, PTP1B/IRS1 and PTP1B/Jak2 interactions were found in the hypothalamic samples obtained from exercised obese rats that received anti-IL6 antibody, when compared to exercised animals injected only with RPSI (Fig. 5F-H). Finally, exercise failed to improve insulin and leptin signaling in the hypothalamus of obese animals that received anti-IL-6 antibody pretreatment (Fig. 5J-K). It in important to note that all these events occurred even in the presence of high levels of circulating IL-6 in exercised obese animals (Fig. 5L).

DISCUSSION

In the present study, we investigated the effects of acute exercise on PTP1B protein levels/activity in the hypothalamus of obese rodents. Our data demonstrated that swimming exercise was sufficient to reduce the protein levels of hypothalamic PTP1B in different models of obesity. In parallel, we observed that exercise reduced PTP1B interactions with proteins involved in the insulin and leptin signaling cascade, improving the anorexigenic action of these hormones. We also determined that exercise reduced hypothalamic PTP1B protein levels through the anti-inflammatory response mediated by IL-6. These data are important since inflammation inhibition in the central nervous system is a potential target therapy to combat obesity and that most anti-inflammatory therapies have limited direct effects on neuronal inflammation. Our study provides substantial evidence that long-term of moderate exercise could help to reorganize the anorexigenic signals and, therefore, aid in counteracting the energy imbalance induced by obesity reducing hypothalamic PTP1B protein levels through the central anti-inflammatory response.

PTP1B plays a critical role in the metabolic processes of mammals (4, 38). This phosphatase negatively regulates insulin signaling by directly dephosphorylating the IR and IRS1 (11, 17, 35) and leptin signal transduction by dephosphorylating Jak2 tyrosine phosphorylation *in vivo* (43). The expression of this enzyme is upregulated in different insulin- and leptin-sensitive tissues in obesity, diabetes, dyslipidemia, and metabolic syndrome (16, 38). Interestingly, PTP1B knockout mice display high insulin (11) and leptin (43) sensitivity and are resistant to developing obesity under high-fat diet treatment. Thus, PTP1B was characterized as a core inhibitor of insulin and leptin signaling in different cell types, including in hypothalamic neurons. Consistent with this notion, strategies to reduce the aberrant PTP1B activation in the hypothalamus are of great interest to improve central insulin and leptin actions and prevent or treat obesity.

Several studies were performed to elucidate the role of neuronal PTP1B in the control of food intake and energy expenditure. In an elegant study, Bence and colleagues used a genetic approach to determine that loss of PTP1B protein levels specifically in neurons caused a marked decrease in adiposity in mice (2). Rodents with POMC neuron-

specific deletion of the gene encoding PTP1B presented reduced fat mass, improved leptin sensitivity, and increased energy expenditure (1). Similar results were found in mice lacking PTP1B in leptin receptor-expressing neurons (39). Moreover, it was demonstrated that transient reduction of PTP1B in discrete hypothalamic nuclei by infusion of an antisense oligonucleotide in obese rats, increased insulin and leptin sensitivity and resulted in decreased food intake, body weight and adiposity after high-fat diet feeding (27). In addition to these genetic strategies, our data demonstrated that acute physiological stimulus, such as swimming exercise, was sufficient to reduce PTP1B protein levels in the hypothalamic tissue of obese and diabetic rodents and the PTP1B/IRβ, PTP1B/IRS1 and PTP1B/Jak2 interactions in obese rats. It is important to attempt that long-term moderate physical exercise reduced PTP1B protein levels and restored the anorexigenic effects of insulin and leptin without changing the total body weight and adiposity.

Inflammatory signaling has been proposed as the main mechanism responsible for inducing PTP1B overexpression in obese and diabetic mice. Zabolotny et al. (44) determined that TNFα administration increased PTP1B mRNA levels in adipose tissue, liver, skeletal muscle, and the hypothalamic arcuate nucleus and PTP1B protein levels in the livers of mice (44). Furthermore, it was demonstrated that TNFα induced the recruitment of NFκB subunit p65 to the PTP1B promoter *in vitro* and *in vivo* (44). In accordance with these data, we observed a consistent activation of IKKβ and IκBα degradation in the hypothalamus of rats after high-fat diet treatment with substantial elevation of PTP1B protein levels. Surprisingly, acute exercise disrupts inflammatory signaling and reduces PTP1B protein levels/activity restoring insulin and leptin anonexigenic effects in obese animals. Previously, we demonstrated that long-term acute exercise improved insulin sensitivity in the skeletal muscle of obese (8, 34) and aged animals (24) by reducing IKKβ signaling and PTP1B activity. These results suggest that physical exercise suppresses PTP1B protein levels through anti-inflammatory mechanisms in different tissues.

Physical exercise elicits the production and secretion of proteins from skeletal muscle during contraction. These molecules can induce metabolic changes in other tissues or organs, such as the liver, adipose tissue, pancreas and hypothalamus (12, 25). The anti-

inflammatory response mediated by exercise is carried out by IL-10, IL-1 receptor antagonist (IL-1ra), and soluble TNF-receptors (sTNF-R) and in particular by IL-6 (26). IL-6 is most often classified as a pro-inflammatory cytokine, although consistent data also demonstrate that IL-6 induces an anti-inflammatory response and may attenuate the inflammation of acute-phase responses (26). Recently, it has been reported that physical exercise suppressed hyperphagia in obese animals by reducing hypothalamic IKKβ/NF-κB and endoplasmic reticulum stress activation through IL-6 and IL-10 anti-inflammatory activity (31). The IL-6/IL-10 anti-inflammatory axis induced by exercise was observed in different models of rodents and humans (7, 22, 29, 45). In the present study, we observed that exercise diminished hypothalamic PTP1B protein levels, through the anti-inflammatory effects of IL-6 in obese rats. For example, exercise failed to reduce IKKβ phosphorylation and PTP1B protein levels after the disruption of hypothalamic-specific IL-6 action by anti-IL-6 antibody injection. Conversely, ICV injection of recombinant IL-6 mimicked the exercise effects in obese rats at rest, reducing the inflammatory signaling and PTP1B protein levels.

The role of hypothalamic IL-6 in the control of energy balance remains uncertain. It has been proposed that centrally acting IL-6 exerts anti-obesity effects in rodents. An interesting study investigated the impact of a loss of IL-6 on body composition in mice lacking the gene encoding IL-6 (*IL-6*$^{-/-}$ mice); the authors found that mature-onset obesity observed in *IL-6*$^{-/-}$ mice was partly reversed by IL-6 replacement, and that this effect occurred through the central action of IL-6 (41). Previously, we demonstrated that the IL-6 receptor (IL6R) is largely expressed in the arcuate nucleus of the hypothalamus of rats, in both, orexigenic (NPY and AgRP) and anorexigenic (POMC) neurons (30, 31). In the present study, immunohistochemical analyses demonstrated the colocalization of IL-6R and PTP1B in the hypothalamus of obese rats at rest, but low colocalization was found in the arcuate nucleus of obese rats after acute exercise, demonstrating that the IL-6 anti-inflammatory action occurred in the same cell types where PTP1B was expressed.

Although skeletal muscle contraction delivers IL-6 into the circulation during exercise, it is not possible to affirm the source of the protein levels of IL-6 observed in the hypothalamus of exercised animals. Alternatively, several studies have demonstrated that

exercise increases IL-6 production in the brain of rodents (14) and humans (21). In addition, we previously determined that the same exercise protocol used in the present study was sufficient to increase IL-6 mRNA and protein levels in the hypothalamus of obese rats (31). In addition, it was reported that ICV, but not intraperitoneal IL-6 treatment, increased energy expenditure in obese mice, demonstrating that centrally acting IL-6 exerts anti-obesity effects in rodents (41). Consistent with these ideas, we observed that ICV anti-IL-6 pre-treatment blocked the effects of exercise even in the presence of high levels of serum IL-6. However, the mechanism by which exercise induces IL-6 production in the brain remains unclear and requires further investigation.

Collectively, our study demonstrated that long-term moderate exercise induced an anti-inflammatory response in the hypothalamus of obese rodents, reducing PTP1B protein levels/activity, reducing PTP1B interactions with IBβ, IRS1 and Jak2, and improving insulin and leptin signaling and sensitivity in an IL-6 dependent-manner, whereas exogenous ICV infusion of IL-6 reproduced these effects in obese rats. Thus, the effects of acute exercise on PTP1B protein levels seems to depend on hypothalamic IL-6 action, and this phenomenon may help to reorganize the set point of nutritional balance during the obesity state.

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AUTHOR CONTRIBUTIONS

E.C.C.R. researched data contributed to discussion and reviewed/edited manuscript. L.S.S.P. researched data. C.K.K. researched data. G.D.P. researched data. P.K.P. researched data. V.R.R.S. researched data. D.E.C. researched data. J.B.C. contributed to discussion and reviewed/edited manuscript. E.R.R. wrote the manuscript, contributed to discussion and reviewed/edited manuscript. J.R.P wrote the manuscript, contributed to discussion and reviewed/edited manuscript.

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FIGURE LEGENDS

Figure 1. Exercise reduces hypothalamic PTP1B protein levels in different models of obesity. Representative blots show (A) Hypothalamic PTP1B protein level in control and in diet-obesity induced rats (n=6). (B) PTP1B activity assay was performed by using hypothalamic samples from Wistar rats in different experimental conditions (n=5). (C) Hypothalamic PTP1B protein level in *ob/ob* and *db/db* mice (n=6). (D) Median of blood lactate during exercise protocol. Samples were obtained each 60 minutes during swimming exercise protocol (n=6). (E) Body weight. (F) 24-h evaluation of urine corticosterone (ng/mL). Bars represent the mean ± S.E.M. of six-eight rats. * $p<0.05$, vs. the control group (chow) at rest and # $p<0.05$, vs. obese group at rest.

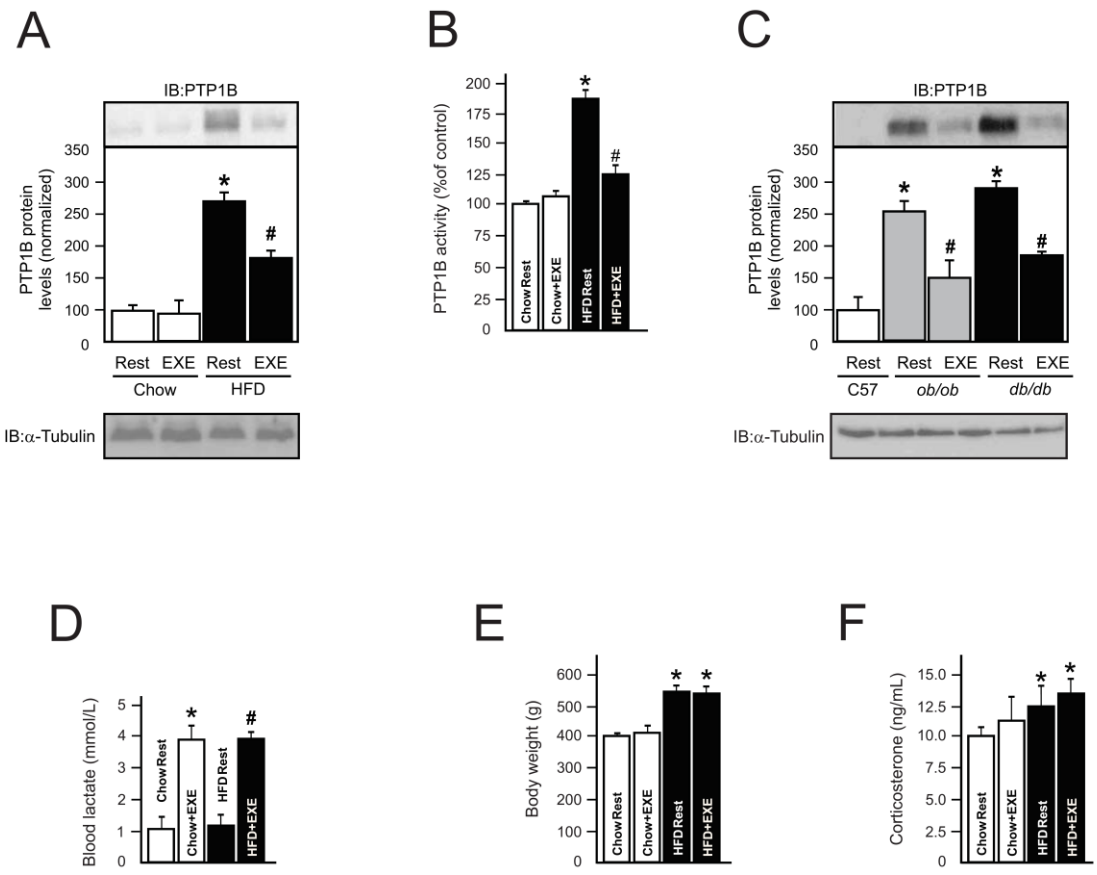
Figure 2. PTP1B association and insulin and leptin signaling in the hypothalamus of obese rats. Immunoprecipitation assay was performed to evaluate; (A) IRβ/PTP1B association, (B) IRS1/PTP1B association and (C) Jak2/PTP1B association in hypothalamic samples of Wistar rats (n=6). Representative blots show; insulin-induced (D) IRβ $^{tyr\ 1162/1163}$, (E) IRS1$^{tyr\ 971}$ and (F) leptin-induced Jak2 $^{tyr1007/1008}$ phosphorylation in hypothalamic samples of Wistar rats (n=6). 24-h of food consumption (Kcal) was monitored after intracerebroventricular injection of (G) insulin or (H) leptin (n=12). Serum levels of (I) insulin (ng/mL) and (J) leptin (ng/mL) were determined 15 minutes after exercise (n=6-8). Data were expressed by using mean ± S.E.M. * $p<0.05$, vs. the control group (chow) and # $p<0.05$, vs. HFD group.

Figure 3. IL-6 anti-inflammatory action and PTP1B expression in the hypothalamus of obese rats. Representative blots show; (A) IL-6 expression in the hypothalamus of obese rats at rest and after swimming exercise protocol (n=6). (B) PTP1B expression after ICV IL-6 injection (50-200ng) or saline in the hypothalamus of obese Wistar rats (n=6). (C) PTP1B activity assay was performed by using hypothalamic samples from Wistar rats in different experimental conditions (n=5). Representative blots show (D) Hypothalamic IKK ^{ser180} phosphorylation and (E) IκBα expression. (F-H) Immunoprecipitation assay was performed to evaluate IRβ/PTP1B, IRS1/PTP1B and Jak2/PTP1B association in hypothalamic samples of Wistar rats (n=6). Representative blots show; (I-K) insulin-induced IRβ $^{tyr\ 1162/1163}$ and IRS1$^{tyr\ 971}$ and leptin-induced Jak2 $^{tyr1007/1008}$ phosphorylation in hypothalamic samples of Wistar rats (n=6). 24-h of food consumption (Kcal) was monitored after intracerebroventricular injection of (L) insulin or (M) leptin (n=6-8). Data were expressed by using mean ± S.E.M. * p<0.05, vs. the control group (chow) and # p<0.05, vs. HFD group.

Figure 4. PTP1B and IL-6R colocalization in the hypothalamus of obese rats. (A) Confocal microscopy was performed to evaluate the colocalization of PTP1B (green) and IL-6Rα (red) in the arcuate nuclei of obese rats (upper panels) and exercised obese rats (lower panels), with 200xmagnification (scale bar, 20 μm). Blue arrows indicate PTP1B positive cells, white arrows indicate IL-6Rα positive cells and yellow arrows indicate PTP1B/IL-6Rα colocalization. (B) Positive cells were quantified in 100 randomly counted cells. Data were expressed by using mean ± S.E.M. * p<0.05, vs. PTP1B of obese group and # p<0.05, vs. merge of obese group. (n=5 animals per group).

Figure 5. Effects of ICV anti-IL-6 antibody pretreatment in obese exercised rats. (A) Schematic representation of experimental design. (B) Representative blots show PTP1B protein level in the hypothalamus of obese rats (n=6). (C) PTP1B activity assay was performed by using hypothalamic samples from Wistar rats in different experimental conditions (n=5). Representative blots show (D) Hypothalamic IKK ^{ser180} phosphorylation and (E) IκBα expression. (F-H) Immunoprecipitation assay was performed to evaluate IRβ/PTP1B, IRS1/PTP1B and Jak2/PTP1B association in hypothalamic samples of Wistar rats (n=6). Representative blots show; (I-K) insulin-induced IRβ $^{tyr\ 1162/1163}$ and IRS1$^{tyr\ 971}$ and leptin-induced Jak2 $^{tyr1007/1008}$ phosphorylation in hypothalamic samples of Wistar rats (n=6). (L) IL-6 serum levels were determined 15 minutes after the exercise protocol (n=6-8). Data were expressed by using mean ± S.E.M. * $p<0.05$, vs. HFD group and # $p<0.05$, vs. anti-IL6 exe group.

Figure 1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PTP1B protein levels (normalized)
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Chow HFD
IB:α-Tubulin
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PTP1B activity (%of control)
200
175
150
125
100
75
50
25
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Chow Rest
Chow+EXE
HFD Rest
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IB:PTP1B
PTP1B protein levels (normalized)
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Blood lactate (mmol/L)
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2
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Body weight (g)
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HFD Rest
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Corticosterone (ng/mL)
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Chow Rest
Chow+EXE
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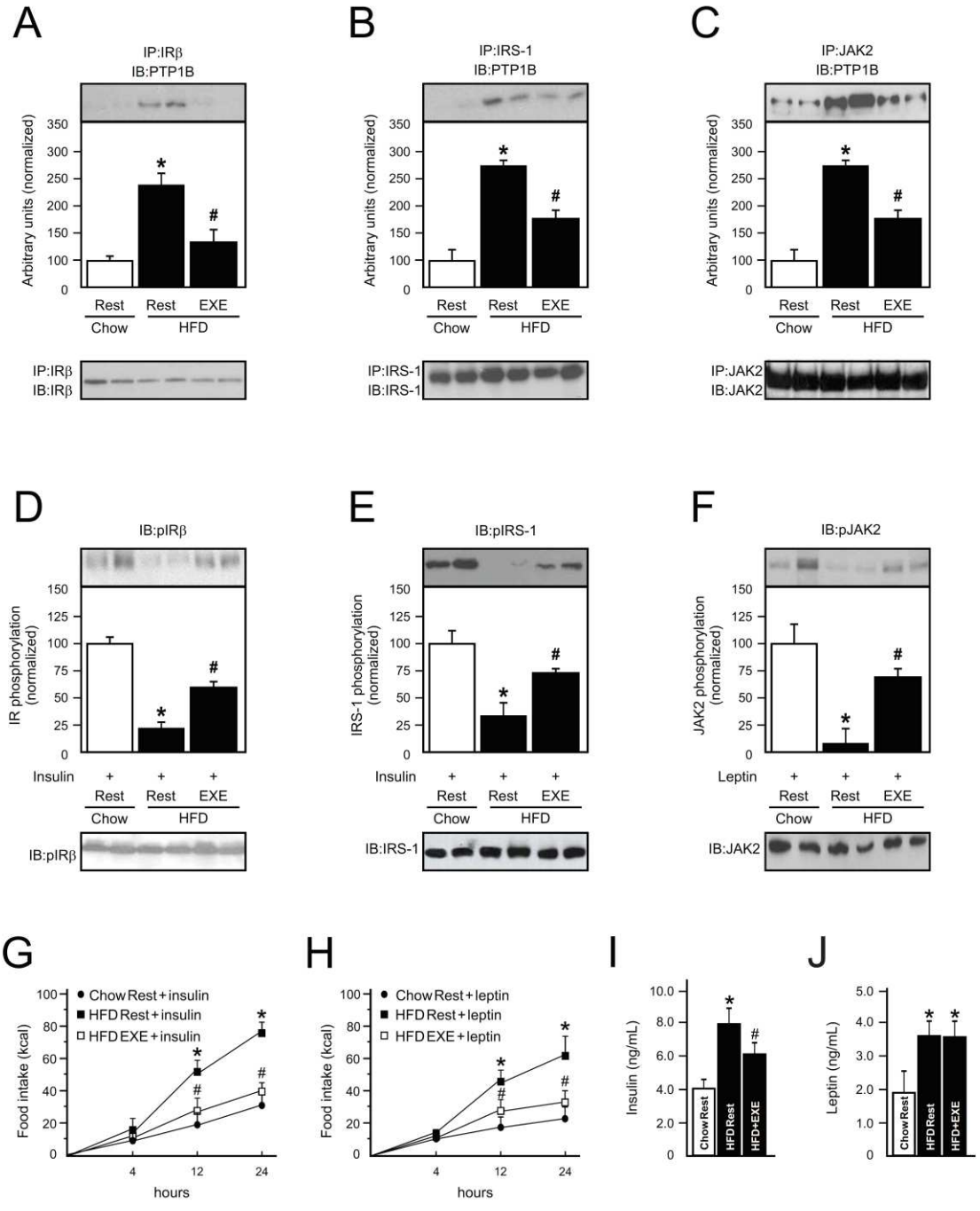
HFD+EXE

Figura 2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C
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IP:JAK2
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E
IB:pIRS-1
IRS-1 phosphorylation (normalized)
IB:IRS-1
F
IB:pJAK2
JAK2 phosphorylation (normalized)
Leptin
IB:JAK2
G
Chow Rest + insulin
HFD Rest + insulin
HFD EXE + insulin
Food intake (kcal)
hours
H
Chow Rest + leptin
HFD Rest + leptin
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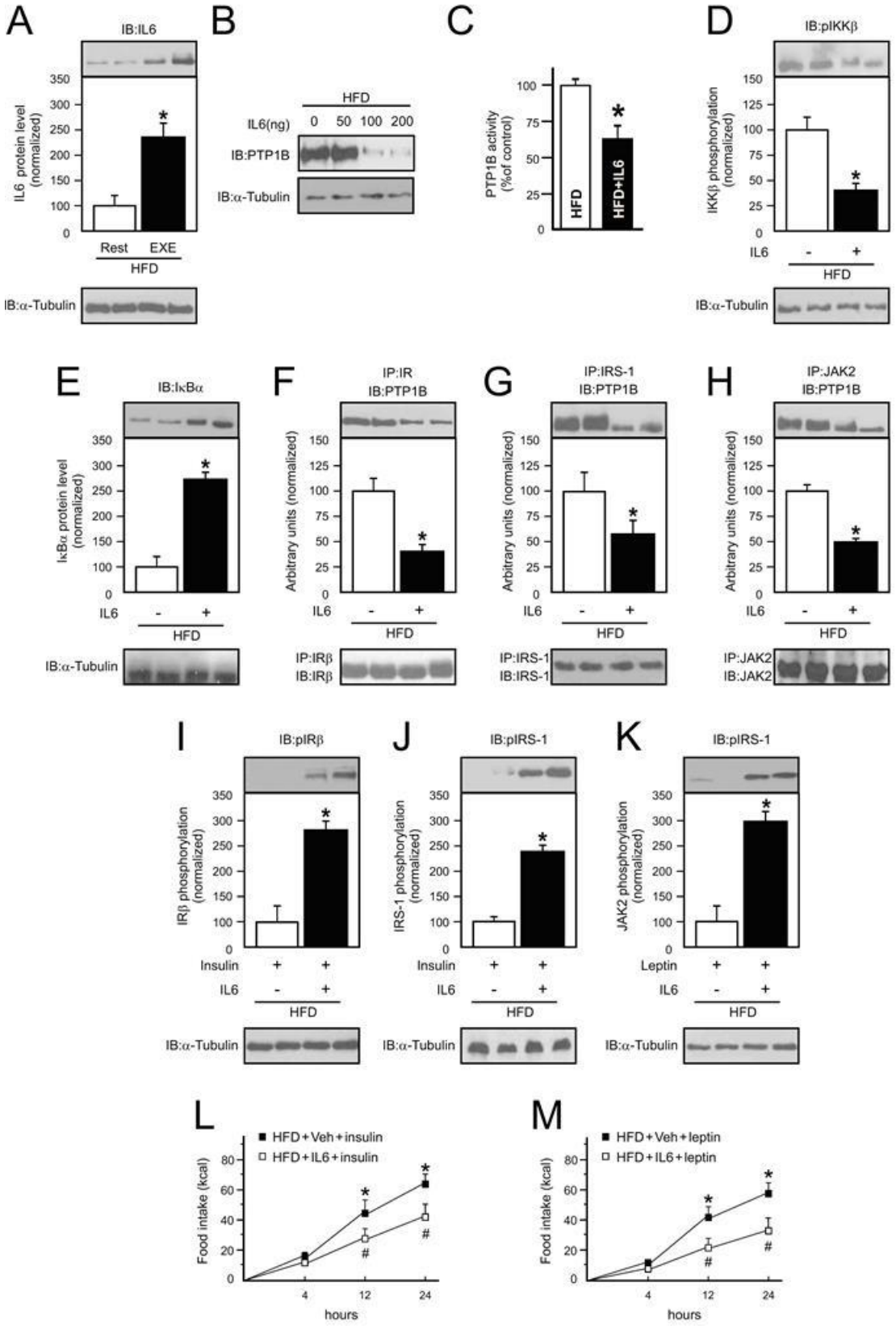
Insulin (ng/mL)
Chow Rest
HFD Rest
HFD+EXE
J
Leptin (ng/mL)

Figure 3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Rest
EXE
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HFD
IL6(ng)
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IB:PTP1B
IB:α-Tubulin
C
PTP1B activity (%of control)
HFD
HFD+IL6
D
IB:pIKKβ
IKKβ phosphorylation (normalized)
IL6
HFD
IB:α-Tubulin
E
IB:IκBα
IκBα protein level (normalized)
IL6
HFD
IB:α-Tubulin
F
IP:IR
IB:PTP1B
Arbitrary units (normalized)
IL6
HFD
IP:IRβ
IB:IRβ
G
IP:IRS-1
IB:PTP1B
Arbitrary units (normalized)
IL6
HFD
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H
IP:JAK2
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IRβ phosphorylation (normalized)
Insulin
IL6
HFD
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J
IB:pIRS-1
IRS-1 phosphorylation (normalized)
Insulin
IL6
HFD
IB:α-Tubulin
K
IB:pIRS-1
JAK2 phosphorylation (normalized)
Leptin
IL6
HFD
IB:α-Tubulin
L
HFD+Veh+insulin
HFD+IL6+insulin
Food intake (kcal)
hours
M
HFD+Veh+leptin
HFD+IL6+leptin
Food intake (kcal)
hours

Figure 4

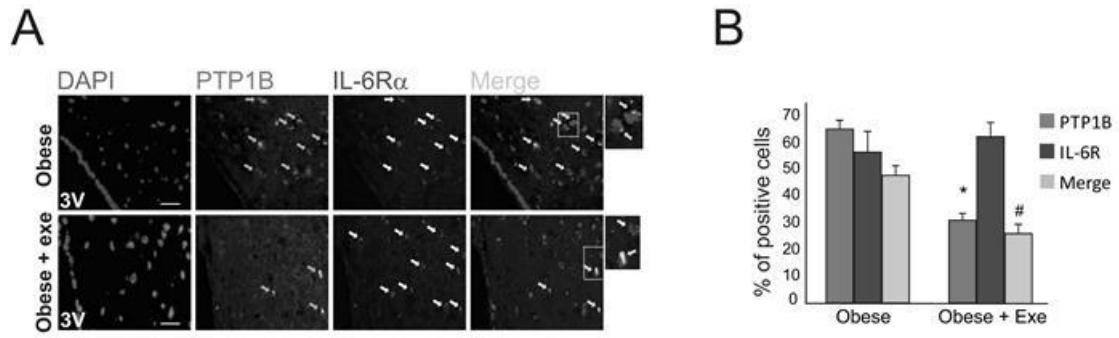
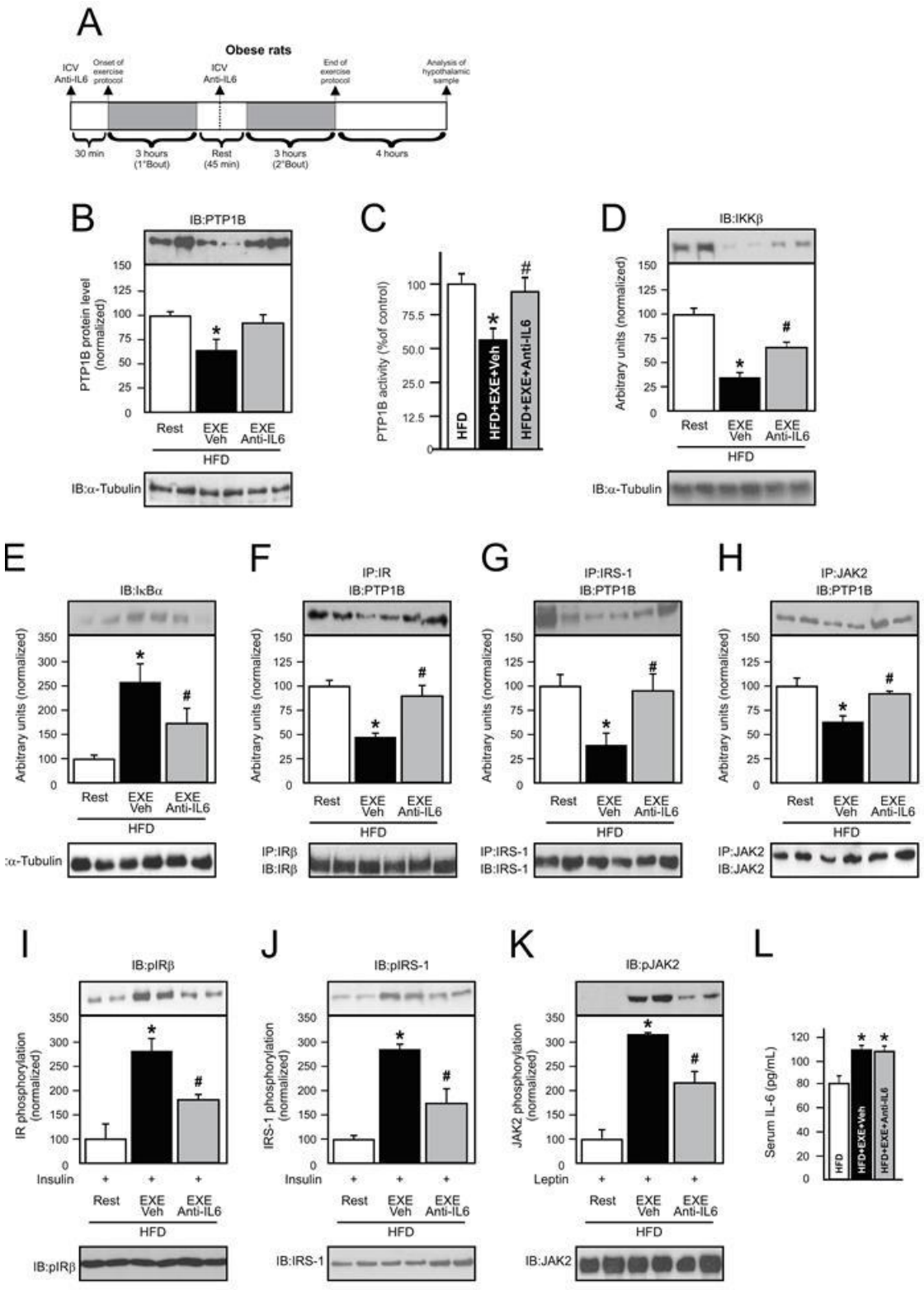


Figure 5


A
Obese rats
ICV Anti-IL6
Onset of exercise protocol
ICV Anti-IL6
End of exercise protocol
Analysis of hypothalamic sample
30 min
3 hours (1°Bout)
Rest (45 min)
3 hours (2°Bout)
4 hours
B
IB:PTP1B
PTP1B protein level (normalized)
Rest
EXE Veh
EXE Anti-IL6
HFD
IB:α-Tubulin
C
PTP1B activity (%of control)
HFD
HFD+EXE+Veh
HFD+EXE+Anti-IL6
D
IB:IKKβ
Arbitrary units (normalized)
IB:α-Tubulin
E
IB:IκBα
F
IP:IR
IB:PTP1B
IP:IRβ
IB:IRβ
G
IP:IRS-1
IB:PTP1B
IP:IRS-1
IB:IRS-1
H
IP:JAK2
IB:PTP1B
IP:JAK2
IB:JAK2
I
IB:pIRβ
IR phosphorylation (normalized)
Insulin
IB:pIRβ
J
IB:pIRS-1
IRS-1 phosphorylation (normalized)
IB:IRS-1
K
IB:pJAK2
JAK2 phosphorylation (normalized)
Leptin
IB:JAK2
L
Serum IL-6 (pg/mL)

4. Discussão

No presente estudo, investigamos os efeitos do exercício agudo sobre a expressão e a atividade da proteína PTP1B no hipotálamo de roedores obesos. Nossos dados demonstraram que uma sessão de natação ou corrida de intensidade moderada, foi suficiente para reduzir a expressão hipotalâmica desta proteína em diferentes modelos de obesidade. Em paralelo, observamos que o exercício reduziu a interação da PTP1B com proteínas envolvidas na cascata de sinalização da insulina e da leptina, promovendo aumento da fosforilação em tirosina do IR, do IRS1 e da Jak2, restaurando a sinalização anorexigênica dessas hormônios em animais obesos. Adicionalmente, demonstramos que o exercício reduziu a quantidade de PTP1B hipotalâmica através da ação central da IL-6. De forma interessante a inibição da IL-6 dirigida exclusivamente ao hipotálamo de ratos obesos, foi suficiente para bloquear os efeitos do exercício sobre a sensibilidade à insulina e leptina neste tecido. Nosso estudo demonstrou que a prática de exercícios moderados pode restaurar os sinais anorexigênicos mediados pela insulina e leptina durante a obesidade e proporcionar um reequilíbrio do balanço energético através da modulação na expressão de PTP1B no sistema nervoso central.

A PTP1B é uma fosfatase que desempenha função importante no metabolismo de mamíferos [4]. Esta fosfatase regula negativamente o sinal da insulina, ao se ligar e desfosforilar o IR e o IRS1 [45, 46, 48] e jak2 [44]. Alguns estudos reportaram aumento significativo da expressão e da atividade desta enzima em diversos tecidos sensíveis a leptina e a insulina na situação de obesidade, diabetes, dislipidemia e síndrome metabólica [43, 48]. De forma interessante, camundongos *knockout* para PTP1B, apresentam hipersensibilidade à insulina [45] e à leptina [67], apresentando elevada proteção ao

desenvolvimento da obesidade e resistência à insulina, mesmo quando expostos a dietas hipercalóricas. Desta forma, a PTP1B pode ser considerada como um potente inibidor endógeno da sinalização da insulina e leptina em diferentes células, inclusive no neurônio hipotalâmico.

Estudos anteriores elucidaram a função da PTP1B neuronal no controle da ingestão alimentar e do gasto energético. Bence e colaboradores usando uma técnica para diminuir a expressão de PTP1B especificamente em neurônios, encontraram um marcante decréscimo na quantidade do tecido adiposo em camundongos [40]. Nesse mesmo sentido, roedores com deleção neuronal específica para o gene da PTP1B em neurônios produtores de POMC, mostraram redução de massa gorda, aumento da sensibilidade a insulina e aumento de gasto energético [69]. Resultados similares foram encontrados em camundongos que apresentavam deleção da PTP1B em neurônios que expressam receptores de leptina [70]. Além disso, foi demonstrado que a redução transitória de PTP1B no núcleo hipotalâmico, através da infusão de oligonucleotídeo antisense em ratos obesos, aumentou a sensibilidade hipotalâmica à insulina e leptina, reduziu o consumo alimentar, o peso corporal e a adiposidade, mesmo quando submetidos à alimentação com alto teor de gordura [35]. Os dados do presente estudo mostraram que estímulos fisiológicos agudos, como natação e corrida, podem ser suficientes para reduzir a atividade da PTP1B neuronal e também diminuir a interação, desta tirosina fosfatase, com IRβ, IRS1 e Jak2 no tecido hipotalâmico de roedores obesos e diabéticos. Torna-se importante destacar que o exercício físico moderado de longa duração, reduziu o nível de PTP1B e restaurou os efeitos anorexigênicos da insulina e leptina, sem que houvesse alteração de peso e gordura corporal.

A sinalização inflamatória parece ser o mecanismo responsável por manter o alto nível de expressão da PTP1B em obesos e diabéticos. Zabolotny e colaboradores descreveu que a presença de TNFα, aumenta a quantidade de mRNA para PTP1B no tecido adiposo, fígado, musculo esquelético e núcleo arqueado hipotalâmico e aumenta o nível proteico da PTP1B no fígado de ratos [68]. Esses dados foram acompanhados do recrutamento da subunidade 65 e ativação do NF-κB [68]. Perante estes dados, nós observamos a ativação do IKKβ e a degradação do IkBα no hipotálamo de roedores obesos com substancial elevação do nível de PTP1B. De maneira bastante consistente, observamos que o exercício agudo interrompeu a sinalização inflamatória no hipotálamo de ratos obesos e paralelamente observamos redução tanto da atividade como da expressão de PTP1B. Esses dados estão de acordo com dados previamente publicados pelo nosso grupo, que demonstraram que o exercício agudo de longa duração, é capaz de restaurar a sensibilidade à insulina no músculo esquelético de ratos obesos [55,61] e em animais envelhecidos [53] através da redução na sinalização do IKKβ e atividade da PTP1B. Estes resultados sugerem que o exercício físico atua apenas como agente anti-inflamatório em diferentes modelos de resistência à insulina mas também suprime o nível proteico de PTP1B e em diferentes tecidos.

Sabidamente, o exercício físico produz e secreta proteínas do músculo esquelético durante a contração. Estas moléculas podem induzir alterações metabólicas em outros tecidos e órgãos, como no fígado, tecido adiposo, pâncreas e hipotálamo [71, 72]. A resposta anti-inflamatória mediada pelo exercício é produzida através da ação da IL-10, IL-1ra, receptor TNFα e em particular pela IL-6 [73]. Classicamente, a IL-6 é conhecida como uma citocina próinflamatória, no entanto, alguns achados demonstraram que em algumas situações específicas ou do microambiente em questão, a IL-6 pode promover uma resposta

anti-inflamatória significativa [73]. Recentemente, demonstramos que o exercício físico foi capaz de diminuir a hiperfagia em animais obesos, por reduzir a ativação do eixo IKKβ/NF-kB e também diminuir o estresse de retículo endoplasmático na região hipotalâmica. Esses efeitos foram decorrentes da ação anti-inflamatória promovida pela ação integrada das moléculas IL-6 e IL-10 [64]. De maneira interessante, essa resposta anti-inflamatória mediada pelo eixo IL-6/IL-10 após o exercício físico, foi observada em diferentes modelos experimentais, tanto em ratos como em humanos [74-77]. No presente estudo observamos que o exercício físico diminui a expressão proteica de PTP1B hipotalâmica, através do efeito anti-inflamatório da IL-6 em ratos obesos. No entanto, o exercício falhou em reduzir a ativação do IKKβ em animais que receberam injeção ICV do anticorpo anti-IL-6 antes da sessão de exercício. Por outro lado, a injeção do IL-6 recombinante, em hipotálamo de animais obesos, mimetizou os efeitos do exercício físico, reduzindo a sinalização inflamatória e os níveis proteicos de PTP1B em ratos obesos.

A função da IL-6 hipotalâmica no controle do balanço energético continua incerta. Postula-se que a ativação central da IL-6 em roedores, tem uma ação antiobesidade. Um elegante estudo avaliou a composição corporal em camundongos com deleção do gene codificador para IL-6 (IL-6 $^{-/-}$). Os autores observaram o desenvolvimento da obesidade de forma prematura em animais IL-6 $^{-/-}$, quando comparado aos animais controle. No entanto, a obesidade, foi parcialmente revertida com a reposição exógena de IL-6. Neste caso, os autores observaram que a reversão da obesidade através da ação da IL-6, ocorreu através da ação central da desta citocina, mas não através da sua ação sistêmica [78]. Previamente, demonstramos que o receptor de IL6 (IL6R) é altamente expresso no núcleo arqueado do hipotálamo de ratos, enquanto que o receptor de IL-6 é expresso em neurônios orexigênicos

(NPY e AgRP) e anorexigênicos (POMC) [63, 64]. No presente estudo, através de análise imunohistoquímica, pudemos observar a co-localização do receptor de IL-6 nos mesmos neurônios que expressam a PTP1B em roedores obesos em repouso, contudo, baixa co-localização dessas moléculas foi detectada no núcleo arqueado destes animais após sessão aguda de exercício, demonstrando a possível ação antiinflamatória da IL-6 em neurônios que expressam a PTP1B.

Embora a contração do musculo esquelético aumente a secreção de IL-6 na circulação durante o exercício físico, não podemos afirmar qual a exata fonte responsável pelo aumento da expressão da IL-6 no hipotálamo de animais exercitados. Porém, alguns estudos mostraram que o exercício físico é capaz de aumentar a quantidade de IL-6 no cérebro de roedores [79] e em humanos [80]. Através de trabalhos anteriores de nosso grupo de estudo, pudemos observar que o protocolo de exercício utilizado, foi suficiente para aumentar a quantidade de RNA mensageiro de IL-6 e também o nível proteico desta citocina em roedores obesos [64]. Posteriormente foi relatado que a injeção ICV, mas não o tratamento intraperitoneal de IL6, aumentou o gasto energético em roedores obesos, indicando importante ação central da IL-6 na prevenção da obesidade em roedores [76]. De acordo com esta ideia, nós observamos que o pré-tratamento ICV com o anticorpo anti-IL-6, bloqueou o efeito do exercício, mesmo na presença de elevados níveis séricos de IL-6 nos animais exercitados. Contudo, o mecanismo pelo qual o exercício induz a produção de IL-6 no cérebro permanece amplamente desconhecido e requer novos estudos.

6. Conclusão

O presente estudo demonstrou que o exercício moderado, de longa duração, induz resposta antiinflamatória no hipotálamo de roedores obesos, reduzindo a quantidade e a atividade da PTP1B em neurônios, promovendo a melhora da sinalização e sensibilidade à insulina e leptina nesses animais. Demonstramos ainda, que esses efeitos mediados pelo exercício físico aconteceram através da ação anti-inflamatória da IL-6 hipotalâmica. Coletivamente, nossos dados apontam que o exercício físico pode ser considerado um importante estímulo fisiológico capaz de modular negativamente a PTP1B no hipotálamo e recuperar a sinalização anorexigênica mediada pela insulina e leptina em animais obesos, reforçando o papel do exercício como agente preventivo e terapêutico no combate à obesidade e doenças associadas.

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C E R T I F I C A D O,

Certificamos que o projeto **"Caracterização da atividade da PTP1B em hipotálamo de roedores obesos submetidos ao exercício físico"** (protocolo nº **2598-1**), sob a responsabilidade de **Prof. Dr. José Rodrigo Pauli / Eloize Cristina Chiarreotto Ropelle**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **13 de fevereiro de 2012**.

Campinas, 17 de outubro de 2013.

2ª. VIA

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva