UNIVERSIDADE ESTADUAL DE CAMPINAS - UNICAMP
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CURSO DE PÓS GRADUAÇÃO EM CLÍNICA MÉDICA

TESE DE DOUTORADO

Inter-Relação entre as Vias de Transmissão do Sinal de Insulina e Leptina em Hipotálamo e Fígado de Ratos

José Barreto Campello Carvalheira

Este exemplar corresponde à versão final da Tese de Doutorado, apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutor em Clínica Médica, área de Clínica Médica, do médico JOSÉ BARRETO CAMPELLO CARVALHEIRA.

Prof.Dr. Mário José Abdalla Saad
Orientador

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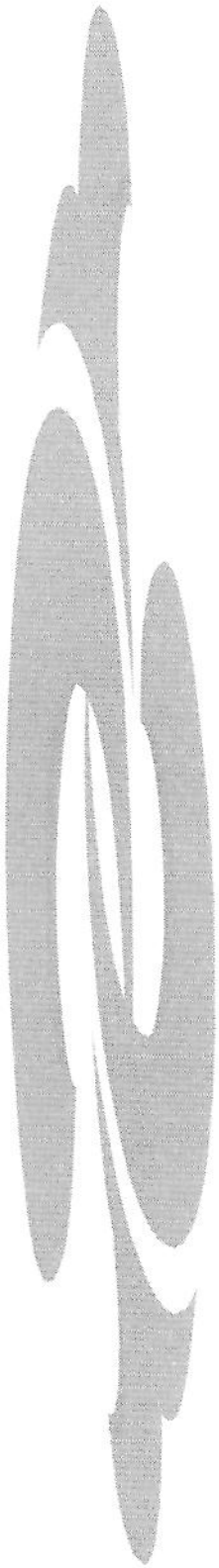
Epígrafe

"Converser pour faire se rencontrer des idées qui se mettent a flirter, à danser, à s'enlacer, à faire l'amour et puis à nous changent à jamais"

Théodore Zendin



SUMÁRIO

Lista de abreviaturas

AGL - Ácidos graxos livres.

AGRP (*Agouti Related Peptide*) – Neuropeptídeo que estimula a ingestão alimentar.

Akt (*Protein Kinase B/Akt*) - Proteína quinase B.

Camundongo *db/db* – Camundongo obeso que apresenta mutação do gene que codifica o receptor de leptina.

Camundongo *ob/ob* - Camundongo obeso que apresenta mutação do gene que codifica a leptina.

CART (*Cocaine-and Anphetamine–Regulated Transcription*) - Neuropeptídeo que inibe a ingestão alimentar.

Cbl – produto do proto oncogene Cbl.

eIF-4E/PHAS-1 (*Initiation Factor 4E for Eukaryotic Translation Inhibitor*) – Proteína que regula a translação de proteínas.

ERK (*Extracellular Signal-Regulated Kinase*) – Quinase da família das MAPK que regula a transmissão dos sinais extracelulares.

Gab-1 (*Grb2-Associated Binder-1*) – proteína que se associa ao Grb2.

gp130 (*Glycoprotein 130*) – Glicoproteína 130.

Grb2 (*Growth Factor Receptor-Bound protein 2*) – Proteína adaptadora necessária para a ativação da via MAPK por sinais extracelulares.

Icv – Intracerebroventricular.

IR (*Insulin Receptor*) - Receptor de insulina.

IRS (*Insulin Receptor Substrate*)- Substrato do receptor de insulina.

JAK2 (*Janus Kinase 2*) – Enzima quinase de tirosina associada ao receptor de leptina e outros receptores da família gp 130.

kDa – Quilodalton.

LH (*Luteinizing Hormone*) - Hormônio lutetinizante.

LIFR (*Leukemia Inhibitory Factor Receptor*) – receptor do fator inibidor de leucemia.

OBR - Receptor de leptina.

OBR_S - forma curta do receptor de leptina.

OBR_L - forma longa do receptor de leptina.

MAPK (*Mitogen-Activated Protein Kinase*) - Proteína quinase ativadora de mitose.

MODY (*Maturity Onset Diabetes of Young*) – Forma de diabetes de herança autossômica dominante.

MSH (*Melanocyte Stimulating Hormone*) – Hormônio que estimula melanócitos e também é um neuropeptídeo que inibe a ingestão alimentar.

NIRKO (*Neuron-specific IR Knockout*) - Camundongos que não têm receptor de insulina no SNC.

NPY (*Neuropeptide Y*) - Neuropeptídeo Y.

PDK (*Phosphoinositide-dependent Kinase*) – Quinase ativada por fosfoinositídeos.

PEPCK (*Phosphoenolpyruvate Carboxykinase*) - Fosfoenol piruvato carboxiquinase.

PI 3-quinase - Fosfatidilinositol 3-quinase.

PKC (*Protein Kinase C*) – Proteína quinase C.

Rato *fa/fa* – Rato Zucker obeso que apresenta mutação do receptor de leptina.

SH2 (*Src Homology 2*) - Domínios proteicos com homologia ao proto oncogene Src 2.

Shc (*Src Homology and Colagen Homology*) – Substrato do receptor de insulina

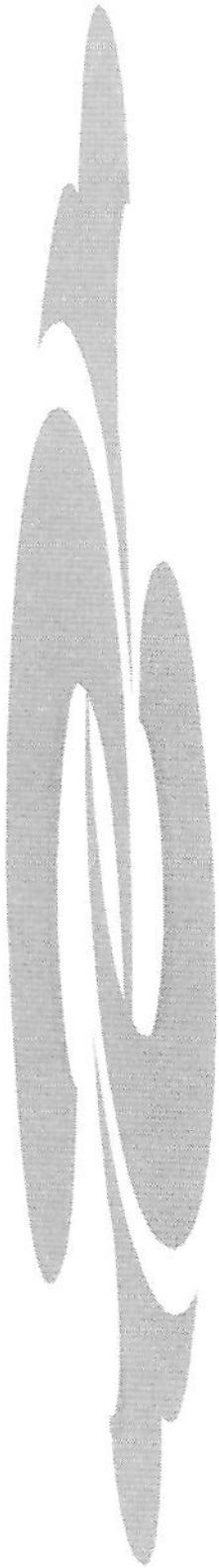
SHP2 (*Src Homology Phosphatase 2*) – Proteína tirosina fosfatase.

SNC – Sistema nervoso central.

SOCS3 (*Suppressors of Cytokine Signaling*) – Proteína que inibe a ativação da JAK2.

STAT (*Signal Transducers and Activators of Transcription*) – Proteína que transmite o sinal da superfície celular ao núcleo ativando a transcrição nuclear.

TNF –α – (*Tumor Necrosis Factor α*) - Fator de necrose tumoral α.



1. RESUMO

Insulina e leptina atuam de forma semelhante e aditiva para controlar a homeostase energética. Entretanto, a base molecular desse sinergismo permanece desconhecida. A insulina sinaliza através de um receptor tirosina quinase que fosforila e ativa seus substratos (IRSs – substratos do receptor de insulina), enquanto a leptina e sua proteína tirosina quinase associada JAK2 (*Janus Kinase 2*) medeiam a fosforilação e ativação do fator de transcrição STAT3 (*Signal Transducer and Activator of Transcription*).

Para investigar se a insulina e a leptina ativam as mesmas vias de sinalização e para determinar se esses hormônios interagem em hipotálamo, ratos Wistar machos foram estudados após implante de cânula no terceiro ventrículo através de imunoprecipitação, *immunoblotting e gel shift*.

A administração aguda intracerebroventricular (icv) de insulina resultou em aumento da fosforilação do receptor de insulina (IR), substrato 1 do receptor de insulina (IRS-1), substrato 2 do receptor de insulina (IRS-2) e MAPK (*Mitogen Activated Protein Kinase*), além da associação entre os IRSs e a PI 3-quinase e a fosforilação em serina da Akt. A administração icv de leptina resultou na fosforilação em tirosina da JAK2, OBR, STAT3, IRS-1, IRS-2 e MAPK, bem como da associação entre os IRSs e a PI 3-quinase mas não foi observada a fosforilação da Akt. O estímulo simultâneo com os dois hormônios não aumentou a fosforilação do IRS-1, IRS-2, Akt e MAPK quando comparado com a administração isolada de insulina. Em contrapartida, a insulina induziu a fosforilação da JAK2 e do receptor de leptina (OBR) que, em presença de leptina, aumentou a interação entre o STAT3 e o OBR com conseqüente aumento da ativação do STAT3.

A leptina ativou as vias de sinalização classicamente induzidas pela insulina no hipotálamo, e essas vias se intercruzaram, mas foram utilizadas de forma distinta que quando utilizadas pela insulina. Por outro lado a insulina modulou positivamente a via de sinalização da leptina. Esses resultados proporcionam uma base molecular para os efeitos coordenados da insulina e leptina no controle do apetite e peso corporal.

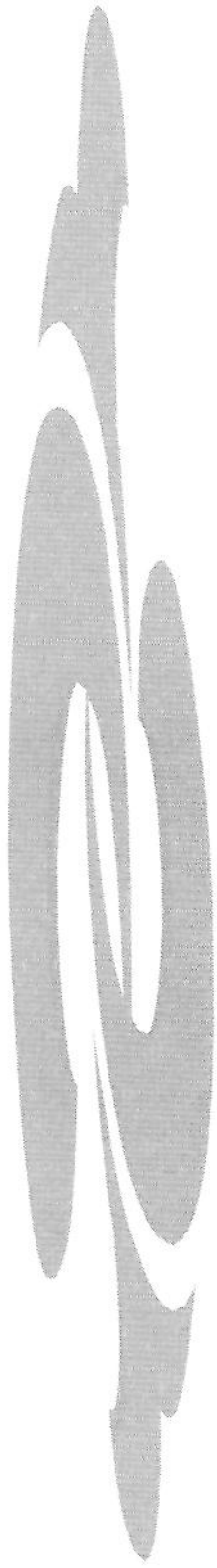
Para demonstrar interdependência entre as vias de sinalização da insulina e leptina em hipotálamo de ratos, a transmissão do sinal de insulina em direção às vias da PI 3-quinase e MAPK foi comparada em hipotálamo de ratos zucker obesos (modelo animal de resistência insulina causado pela mutação do receptor de leptina) e controle. A infusão icv de insulina reduziu a ingestão alimentar de ratos controle, o que não foi observado nos animais obesos. Constatamos redução da fosforilação do IR, IRS-1, IRS-2, da associação entre a subunidade p85 da PI 3-quinase com as proteínas IRSs, bem como da fosforilação em serina da Akt no hipotálamo de ratos obesos comparado com os ratos magros. Em contraste, a insulina estimulou a fosforilação em tirosina da MAPK de forma similar em ratos magros e obesos. Assim, documentamos resistência à sinalização da insulina em tecidos hipotalâmicos de ratos Zucker obesos. Esses resultados fortalecem a hipótese de que a insulina tem suas ações anti-obesidade através da via da PI 3-quinase e que a diminuição do sinal por esta via no hipotálamo pode contribuir para o desenvolvimento de obesidade em estados de resistência à insulina.

Originalmente, pensava-se que o hipotálamo era o único tecido a expressar a forma longa do receptor de leptina. Entretanto, evidências recentes sugerem que órgãos periféricos, entre eles o fígado, também expressam a forma longa do receptor. Assim, na

segunda parte deste estudo investigamos os efeitos rápidos e diretos da leptina em fígado e determinamos se a leptina interage com a insulina nesse órgão.

Em fígado de ratos a injeção aguda de leptina ou insulina estimulou a fosforilação da JAK2, STAT3 e STAT5b. A leptina foi menos efetiva que a insulina em estimular as proteínas IRS e a associação destas com a PI 3-quinase. O tratamento simultâneo com os dois hormônios não modificou a fosforilação máxima do STAT3, IRS-1, IRS-2 e Akt, mas causou aumento significativo da fosforilação da JAK2 e do STAT5b quando comparado com a administração isolada de insulina ou leptina. Assim, detectamos uma inter-relação positiva nas vias de sinalização desses hormônios nas proteínas JAK2 e STAT5b em fígado de ratos.

Concluindo, estes estudos caracterizaram as vias de transmissão do sinal de leptina em hipotálamo e fígado, evidenciando mecanismos para a integração da sinalização de insulina e leptina bem como a modulação destas vias em modelo animal de resistência à insulina e leptina nesses tecidos.



2. INTRODUÇÃO

A obesidade é a doença nutricional mais comum do ocidente. O aumento da massa corporal está associado ao desenvolvimento de hipertensão arterial sistêmica, diabetes mellitus, colecistopatia crônica calculosa, dislipidemia, doenças cardíacas e algumas formas de câncer (GRUNDY & BARNETT, 1990). A obesidade é uma doença atualmente de caráter epidêmico e está rapidamente se tornando um grave problema de saúde pública (KOPELMAN, 2000).

Não obstante a universalização dos estudos sobre a obesidade, seu mecanismo molecular permanece amplamente desconhecido. No início de 1953, Kennedy elaborou a seguinte teoria: quando um animal se alimenta mais que o necessário, a "gordura extra" de alguma forma sinaliza ao cérebro que o corpo está mais alimentado que o normal (KENNEDY, 1953). Com efeito, o animal passa a comer menos e gastar mais energia, o que resulta em um controle da gordura corporal preciso (variação de aproximadamente 1% ao longo de vários anos).

Dois modelos animais de síndromes de obesidade geneticamente determinada vêm sendo intensivamente investigados nos últimos 30 anos: os camundongos *ob/ob* e *db/db*. Estes camundongos têm fenótipos idênticos, pesam três vezes mais e apresentam um aumento de cinco vezes da gordura corporal comparado aos camundongos controle (mesmo quando alimentados com a mesma dieta), além de serem diabéticos.

Os primeiros estudos informativos sobre os defeitos primários nestes animais foram obtidos com experimentos em que se realizava conexão parcial dos sistemas circulatórios de camundongos através de enxertos (parabiose) (COLEMAN, 1973). A parabiose realizada entre camundongos *ob/ob* e controle magro, resulta em normalização do peso do mutante *ob/ob*. Isto sugere que o camundongo *ob/ob* é deficiente de um fator

circulante que pode ser reposto pelo sangue do animal controle. Contrariamente, a parabiose entre camundongos *db/db* e controle magro, não normaliza o peso corporal do mutante, sugerindo que o camundongo *db/db* apresenta uma alteração na capacidade de responder ao fator que induz à saciedade. Entretanto, essas conclusões não foram muito valorizadas e a sua confirmação só foi possível após a clonagem e seqüenciamento do produto do gene *ob*, a leptina, do grego *leptos*, magro (ZHANG *et al.*, 1994).

1. Transmissão do Sinal de Insulina e Leptina em Hipotálamo de Ratos: Inter-relações e Implicações Fisiopatológicas

A leptina é expressa principalmente no tecido adiposo e em menores quantidades no epitélio gástrico e placenta (BADO *et al.*, 1998; MAFFEI *et al.*, 1995; MASUZAKI *et al.*, 1997). 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*, e foi observada em concentrações elevadas em camundongos *db/db* (HALAAS *et al.*, 1995). A administração de leptina a camundongos *ob/ob* resulta em diminuição da ingestão alimentar, perda de peso e redução dos níveis glicêmicos (CAMPFIELD *et al.*, 1995), além de aumentar a atividade simpática em tecido adiposo marrom, com conseqüente aumento do gasto energético (PELLEYMOUNTER *et al.*, 1995). 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 (CONSIDINE *et al.*, 1996;

FREDERICH *et al.*, 1995; HAVEL *et al.*, 1998; MAFFEI *et al.*, 1995). A secreção desse hormônio diminui com o jejum prolongado e estímulo β-adrenérgico (AHIMA *et al.*, 1996) e aumenta em resposta à administração de insulina e glicocorticóides (DE VOS *et al.*, 1995; SALADIN *et al.*, 1995). 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 (LICINIO *et al.*, 1997).

A leptina produzida pelo tecido adiposo informa o estado nutricional do indivíduo a centros hipotalâmicos, que regulam a ingestão alimentar e o gasto energético. Assim, a redução da quantidade de tecido adiposo leva a diminuição dos níveis circulantes de leptina, estimulando a ingestão alimentar e reduzindo o gasto energético. Contrariamente, o aumento do estoque de tecido adiposo está associado a elevação dos níveis séricos de leptina, diminuindo a ingestão alimentar e aumentando o gasto energético. Através desse mecanismo, o peso do indivíduo se mantém estável durante vários anos. Por que então alguns indivíduos desenvolvem obesidade e outros não? Acredita-se que a sensibilidade à leptina seja variável e que indivíduos obesos sejam resistentes à leptina (CONSIDINE *et al.*, 1996; FRIEDMAN & HALAAS, 1998; MAFFEI *et al.*, 1995). Apenas uma ínfima parte da população obesa tem baixos níveis séricos de leptina e desenvolvem obesidade de forma semelhante ao camundongo *ob/ob* (FAROOQI *et al.*, 2001; MONTAGUE *et al.*, 1997).

Vários mecanismos podem contribuir para a resistência à leptina, como a redução do transporte de leptina através de células endoteliais e da barreira hematoencefálica impedindo a chegada da leptina no fluido intersticial cerebral. Entretanto não é claro se alterações nesse processo podem levar à obesidade, mas resultados de pesquisas indicando que humanos obesos têm menor relação entre a leptina liquórica e a plasmática comparados

com indivíduos controle são consistentes com essa possibilidade (CARO *et al.*, 1996). Outra potencial causa de resistência à leptina é a redução da sensibilidade hipotalâmica a esse hormônio. Essa alteração pode ser observada em roedores (rato Zucker (DA SILVA *et al.*, 1998) e camundongo *db/db* (LEE *et al.*, 1996)) que apresentam mutações do receptor de leptina, assim como em ratos alimentados com dieta hiperlipídica (EL-HASCHIMI *et al.*, 2000), demonstrando que fatores ambientais são capazes de modular a via de sinalização da leptina.

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 (TARTAGLIA *et al.*, 1995). 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 (MERCER *et al.*, 1996; WOODS *et al.*, 1996)).

O OBR_S 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 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*) (HELDIN, 1995). 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 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.

O OBR_L é capaz de estimular as proteínas STAT em resposta à sua ativação. Dois estudos (BAUMANN *et al.*, 1996; GHILARDI *et al.*, 1996) 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 *knockout* não resultou em obesidade (DURBIN *et al.*, 1996; MERAZ *et al.*, 1996; SHIMODA *et al.*, 1996; TAKEDA *et al.*, 1996). 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 (TARTAGLIA, 1997). Apenas a ativação do STAT3 foi detectada no hipotálamo de camundongos, após a administração exógena de leptina (VAISSE *et al.*, 1996).

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) (HELDIN, 1995), e é possível que a capacidade do OBR controlar o peso dependa também destas vias de sinalização (NISWENDER *et al.*,

2001; TARTAGLIA, 1997). Além disso, a leptina leva também à fosforilação do SHP2 (CARPENTER *et al.*, 1998), 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 (BJORBAEK *et al.*, 1998).

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) (STEPHENS *et al.*, 1995) e o *Agouti related peptide* (AGRP) (SHUTTER *et al.*, 1997), enquanto outros provocam redução da ingestão alimentar como o *cocaine-and anphetamine–regulated transcription* (CART) (KRISTENSEN *et al.*, 1998) e o *melanocyte stimulating hormone* (α-MSH) (FAN *et al.*, 1997). A leptina regula o balanço energético diminuindo os níveis de neuropeptídeos anabólicos NPY e AGRP e aumentando a concentração de neuropeptídeos catabólicos CART e α-MSH.

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 (WOODS *et al.*, 1979; WOODS *et al.*, 1985). 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 (BAURA *et al.*, 1993). Os receptores de insulina são expressos por neurônios envolvidos na ingestão alimentar (BASKIN *et al.*, 1999; BASKIN *et al.*, 1988; CHEUNG *et al.*, 1997). A administração de insulina no sistema nervoso central reduz a ingestão alimentar e diminui o peso corporal, enquanto a deficiência desse hormônio causa hiperfagia (SIPOLS *et al.*, 1995).

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 (SCHWARTZ *et al.*, 1997). Assim, à medida que o peso corporal aumenta a insulina deve aumentar para compensar a resistência à insulina e manter a homeostase de glicose (KAHN *et al.*, 1993; POLONSKY *et al.*, 1988). 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.

Várias observações indicam que a leptina desempenha uma função mais importante que a insulina no hipotálamo para controle da homeostase energética. Por exemplo, a deficiência de leptina causa obesidade grave, com hiperfagia que persiste apesar dos altos níveis circulantes de insulina. Em contraste, a deficiência de insulina não causa obesidade. Entretanto esse tipo de comparação é complicado pelo papel da insulina em promover a síntese de leptina e o armazenamento de tecido adiposo. Uma vez que o armazenamento de tecido adiposo requer insulina, não pode ocorrer ganho de peso quando a deficiência de insulina está presente, mesmo com um consumo de grande quantidade de alimento. Outro exemplo interessante é o diabetes descompensado, onde a ingestão alimentar aumenta marcadamente (LEEDOM & MEEHAN, 1989), mas a quantidade de gordura corporal está baixa, bem como os níveis plasmáticos de leptina (HATHOUT *et al.*, 1999; HAVEL *et al.*, 1998). Como tanto os níveis séricos de insulina como o de leptina estão baixos nessa situação, a "hiperfagia diabética" poderia ser resultado da diminuição da sinalização da insulina, da leptina ou de ambas. Um estudo recente esclareceu esse 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 homeostase 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 (BRUNING *et al.*, 2000).

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. 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-quinase. 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 (*knockout* 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 (ARAKI *et al.*, 1994). 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 *knockout* de IRS-1. O camundongo que não expressa o IRS-2 foi posteriormente gerado (WITHERS

et al., 1998) 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.

A fosforilação das proteínas IRSs cria sítios de ligação para a PI 3-quinase, promovendo a sua ativação. Atualmente, a PI 3-quinase é a única molécula intracelular inequivocamente considerada essencial para o transporte de glicose (CZECH & CORVERA, 1999). As proteínas alvo conhecidas dessa enzima são a Akt e as isoformas atípicas da PKC (ζ e λ), porém a função dessas proteínas no transporte de glicose ainda não está bem estabelecida (BANDYOPADHYAY *et al.*, 1997; KIM *et al.*, 1999; KITAMURA *et al.*, 1998; KOHN *et al.*, 1996; KOTANI *et al.*, 1998).

Estudos de nosso laboratório demonstraram que a insulina também fosforila e ativa a proteína JAK2 (SAAD *et al.*, 1996). Recentemente descreveu-se que a leptina é capaz de ativar as proteínas JAK2, IRS-2 e PI 3-quinase (BERTI *et al.*, 1997), com conseqüente aumento do transporte de glicose e síntese de glicogênio, sugerindo a existência de uma inter-relação entre as vias de transmissão do sinal de insulina e leptina, pelo menos nas células estudadas.

As vias de sinalização da insulina bem como o efeito molecular distal desse hormônio para regular o balanço energético ainda é desconhecido, mas envolve a redução da expressão do RNA mensageiro para o NPY (SIPOLS *et al.*, 1995). Um estudo recente (NISWENDER *et al.*, 2001) demonstrou que a ativação da PI 3-quinase é essencial para a redução da ingestão alimentar induzida por leptina. **Nesse sentido o primeiro objetivo**

deste estudo foi caracterizar a inter-relação entre as vias de sinalização da insulina e leptina em direção à via PI 3-quinase/Akt.

Apesar da significativa associação entre resistência à insulina e obesidade, a base molecular dessa associação não é conhecida. A insulina sinaliza através de um receptor do tipo tirosina quinase que fosforila e ativa as proteínas IRS, enquanto o receptor de leptina e a tirosina quinase associada, JAK2, medeiam a fosforilação e a ativação do fator de transcrição, STAT3. **Assim, o segundo objetivo deste estudo foi investigar os possíveis efeitos da insulina na via de transmissão da leptina.** Para isso, avaliamos o grau de fosforilação da JAK2, OBR, associação OBR/STAT3 e atividade do STAT3. **Ainda nessa linha de pesquisa o terceiro objetivo desse estudo foi determinar os efeitos da insulina nas suas vias de sinalização intracelular (PI 3-quinase/Akt e MAPK) em hipotálamo de um modelo animal de resistência à insulina (ratos Zucker).** Para tanto avaliamos o grau de fosforilação das proteínas IRS-1 e IRS-2 da mesma maneira que a associação das proteínas IRS com a PI 3-quinase, além da fosforilação da Akt estimulados por insulina e/ou leptina.

2. A Leptina Modula a Sensibilidade à Insulina em Tecido Hepático

A deficiência na produção de leptina, bem como mutações no seu receptor, em roedores, causam obesidade acentuada com hiperglicemia e resistência à insulina. Em alguns desses camundongos a administração de leptina exógena melhora a tolerância à glicose e a sensibilidade à insulina, independentemente de seus efeitos na ingestão alimentar, provavelmente por modular vias neuroendócrinas que controlam as ações da

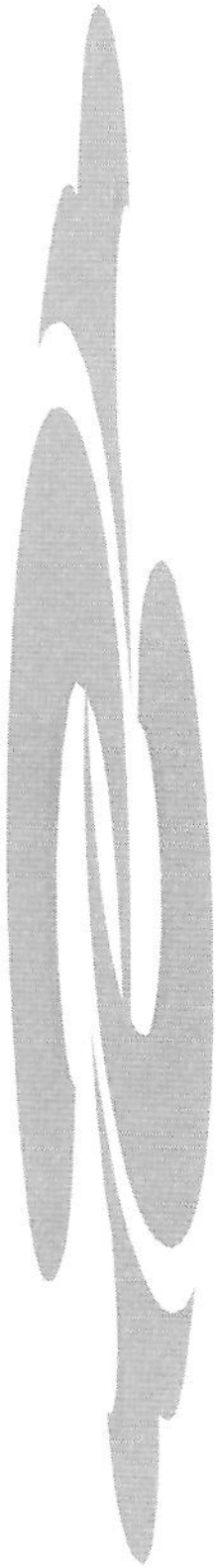
insulina no fígado (HALAAS *et al.*, 1995; SHIMOMURA *et al.*, 1999), embora a leptina possa exercer alguns de seus efeitos de forma direta nos hepatócitos (LEE *et al.*, 2001).

Evidências recentes indicam que o tratamento com leptina normaliza a glicemia em ratos diabéticos deficientes de insulina, (CHINOOKOSWONG *et al.*, 1999). Ratos transgênicos que hiperexpressam leptina paralelamente apresentam melhora da sensibilidade à isnulina (OGAWA *et al.*, 1999). Animais tratados com leptina requerem aumento da infusão de glicose durante a realização de *clamp* euglicêmico em decorrência da melhora da sensibilidade à insulina no fígado e tecidos periféricos (BARZILAI *et al.*, 1997; SHI *et al.*, 1998; SIVITZ *et al.*, 1997).

O tecido hepático tem um papel fundamental na regulação da homeostase de glicose e é um dos principais sítios da ação insulínica. No diabetes tipo 2 a resistência à insulina pode ser constatada por redução na captação de glicose induzida por insulina no músculo e tecido adiposo e principalmente pelo aumento de produção de glicose no fígado (KAHN, 1994). A leptina aumenta a capacidade da insulina em reduzir a produção de glicose, aumentando neoglicogênese e reduzindo a glicogenólise, assim como aumentando a PEPCK e diminuindo a expressão do gene da glucoquinase (LIU *et al.*, 1998; ROSSETTI *et al.*, 1997). Há controvérsia se essas ações da leptina são mediadas direta ou indiretamente. Por exemplo, a redistribuição do fluxo hepático de glicose ocorre após a injeção intracerebroventricular (icv) de leptina, sugerindo uma ação central e indireta da leptina no fígado (ROSSETTI *et al.*, 1997). Entretanto, a administração periférica da leptina (ROSSETTI *et al.*, 1997) e estudos com fígado isolado (NEMECZ *et al.*, 1999) indicam a possibilidade de uma ação direta da leptina neste tecido.

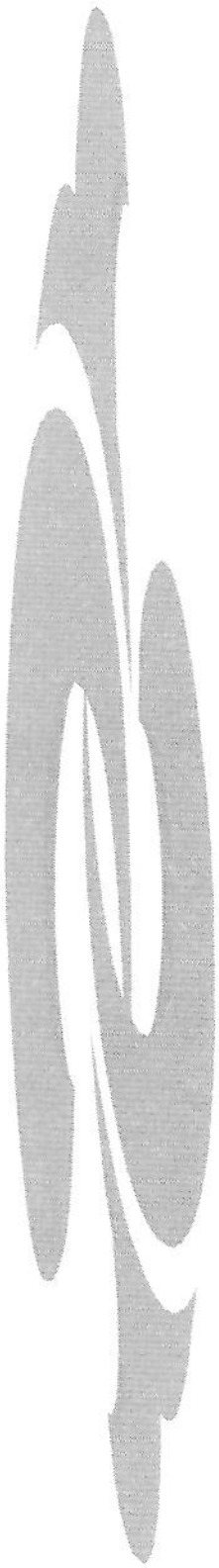
Os estudos que avaliam os efeitos da leptina nas vias de sinalização da insulina são conflitantes e parecem ser tecido específico. Alguns resultados mostram que a leptina causa resistência à sinalização da insulina, diminuindo o grau de fosforilação do IRS-1 em fibroblastos e hepatócitos (COHEN *et al.*, 1996; KRODER *et al.*, 1996). No entanto, outros estudos demonstram que a leptina mimetiza alguns efeitos da insulina no transporte de glicose e síntese de glicogênio em miotúbulos C2C12 (BERTI *et al.*, 1997) e esses efeitos seriam mediados pela PI 3-quinase (KELLERER *et al.*, 1997). Em músculo isolado ou adipócitos a incubação com leptina não estimula o transporte de glicose ou lipogênese (RANGANATHAN *et al.*, 1998; ZIERATH *et al.*, 1998). Assim, a inter-relação entre insulina e leptina no metabolismo e nas vias de sinalização da insulina ainda não está clara.

Ao que parece, o tratamento crônico com leptina em modelos animais de obesidade aumenta marcadamente a ação insulínica na captação periférica de glicose e reduz a produção hepática desse carboidrato de forma desproporcional à perda de peso corporal associada. Entretanto, o mecanismo molecular envolvido no aumento da sensibilidade à insulina nestes modelos ainda não são conhecidos. **Assim, o quarto objetivo deste estudo foi investigar o efeito direto da leptina nas proteínas envolvidas nas etapas iniciais da ação insulínica em fígado de ratos Wistar e Zucker, bem como a inter-relação entre os efeitos da insulina e leptina nestes tecidos.** Para tanto avaliamos o grau de fosforilação das proteínas IRS-1, IRS-2, OBR, JAK2, STAT3 e STAT5B, da mesma maneira que a associação das proteínas IRSs com a PI 3-quinase e a fosforilação da Akt estimulados por insulina e/ou leptina.



3. OBJETIVOS

1. Investigar o efeito da leptina e da insulina, bem como a inter-relação entre os efeitos desses hormônios, nas proteínas envolvidas na sinalização da insulina em hipotálamo de ratos Wistar. (Trabalho 1).
2. Investigar o efeito da leptina e da insulina,bem como a inter-relação entre os efeitos desses hormônios, nas proteínas envolvidas na sinalização da leptina em hipotálamo de ratos Wistar. (Trabalho 2).
3. Determinar os efeitos da insulina nas vias de sinalização intracelular PI 3-quinase/AKT e MAPK em hipotálamo de modelo animal de resistência à insulina (ratos Zucker). (Trabalho 3).
4. Investigar o efeito da leptina nas proteínas envolvidas nas etapas iniciais da ação insulínica em fígado de ratos Wistar, bem como a inter-relação entre os efeitos da insulina e leptina neste tecido, através de avaliação do grau de fosforilação das proteínas IR, IRS, AKT, OBR, JAK2, STAT1, STAT3 e STAT5b, e também da associação IRSs/PI 3-quinase, após estimulo com insulina e/ou leptina. (Trabalho 4).



4. RESULTADOS

Os resultados desta tese originaram quatro trabalhos:

1. **Overlapping of the Insulin and Leptin Signaling System in Rat Hypothalamus.** Este trabalho foi apresentado no Congresso Paulista de Diabetes e Metabolismo, realizado em maio de 1998 em Águas de Lindóia - SP; na FESBE 98, realizada em agosto de 1998 em Caxambu - MG; no 23° Congresso Brasileiro de Endocrinologia e Metabologia e III Congresso Paulista de Endocrinologia e Metabologia em outubro 1998 em São Paulo – SP; em forma de poster no Congresso Americano de Diabetes em San Diego-California, 1999 e publicado como resumo na revista *Diabetes* (48, S1:A200-A201, 1999). Encontra-se em fase final de elaboração para submissão em revista indexada.

2. **Insulin Modulates Leptin-induced STAT3 Activation in Rat Hypothalamus.** Este trabalho foi apresentado no 24° Congresso Brasileiro de Endocrinologia e Metabologia, em novembro de 2000 no Rio de Janeiro – RJ; apresentado oralmente no Congresso Americano de Diabetes em 2001 na Phyladelphia – Pennsylvania, publicado como resumo na revista *Diabetes* (50, S2:A13, 2001). Está publicado na íntegra na revista *Febs lett* (500: 1119-124, 2001.)

3. **Selective Impairment to Insulin Signaling in the Hypothalamus of Obese Zucker Rats** Este trabalho foi apresentado na FESBE 2001 (finalista do prêmio Michel Jamra), em agosto 2001 em Caxambú – MG; no XIII Congresso Brasileiro de Diabetes, realizado de 10 de a 14 de outubro de 2001 no Rio de Janeiro – RJ e apresentado

oralmente no Congresso Americano de Diabetes em 2002 em San Francisco, Califórnia, e publicado como resumo na revista *Diabetes* (51, S2:A41, 2002). Foi enviado para publicação na revista *Journal of Clinical Investigation*.

4. **Interaction Between Leptin and Insulin Signaling Pathways Differentially Affects the Jak-Stat and PI 3-Kinase-Mediated Signaling in Rat Liver.** Este trabalho foi apresentado no Congresso Paulista de Diabetes e Metabolismo realizado em abril de 2000 em Águas de São Pedro – SP; na FESBE 2000, realizada em agosto de 2000 em Caxambú – MG; no 24° Congresso Brasileiro de Endocrinologia e Metabologia, em novembro de 2000 no Rio de Janeiro – RJ e na forma de poster no Congresso Americano de Diabetes em junho de 1999, em San Diego, Califórnia e publicado como resumo na revista *Diabetes* (48, S1:A283, 1999). Foi aceito para publicação na revista *Biological Chemistry*.

4. RESULTADOS

1. Overlapping of the Insulin and Leptin Signaling System in Rat Hypothalamus

OVERLAPPING OF THE INSULIN AND LEPTIN SIGNALING SYSTEMS IN RAT HYPOTHALAMUS

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Short running title: Insulin/leptin signaling in hypothalamus

ABSTRACT

Objective: To investigate whether insulin and leptin share common intracellular signal transduction pathways and to determine whether these hormonal signaling systems modulate each other's action in rat hypothalamus.

Research Methods and Procedures: Male Wistar rats were studied after chronic implantation of an intracerebroventricular (i.c.v.) catheter into the third ventricle. Immunoprecipitation and immunoblotting were used to examine the activation of insulin and leptin signaling molecules in the rat hypothalamus.

Results: Acute i.c.v. administration of insulin resulted in a time-dependent increase in tyrosine phosphorylation of the insulin receptor (IR), insulin receptor substrate 1 (IRS-1), insulin receptor substrate 2 (IRS-2), Janus kinase-2 (JAK2) and MAPK (mitogen activated protein kinase), PI 3 kinase docking and serine phosphorylation of Akt. The i.c.v. administration of leptin resulted in tyrosine phosphorylation of JAK2, IRS-1, IRS-2 and MAPK and PI 3 kinase docking but no phosphorylation of Akt was observed. Simultaneous stimulation with both hormones did not increment tyrosine phosphorylation of IRS-1, IRS-2, Akt and MAPK when compared with isolated administration of insulin.

Discussion: Leptin rapidly activates classically insulin signaling pathways directly at the level of hypothalamus, and these pathways overlap with, but are distinct from, those engaged by insulin.

KEY WORDS

Obesity/*physiopathology

Hypothalamus/*drug effects/*metabolism

Leptin

Insulin

Protein-Tyrosine Kinase/metabolism

Receptor, Insulin/*metabolism

Phosphatidylinositol 3-Kinase/metabolism phosphatidylinositol 3-kinase

Mitogen-Activated Protein Kinase

INTRODUCTION

The discovery of leptin, a hormone produced by adipocytes that acts at the level of the brain to control appetite, has created seminal change in thinking about the nature of regulation of body mass (1-4). Human obesity is characterized by high circulating levels of leptin (5,6). Obesity is also accompanied by insulin resistance with high circulating insulin levels and a predisposition to development of type 2 diabetes (7). Thus, in obesity and type 2 diabetes, the hypothalamus is often exposed to high concentrations of both leptin and insulin. Insulin and leptin receptors are present in hypothalamus (8), raising the possibility that these two hormonal signaling systems could interact to modify each other's actions in these disease states.

The hypothalamus is thought to be the major target for leptin, which acts through receptors that bear strong sequence homology to the class I cytokine receptor family (9). The leptin receptor exists as multiple splice variants. A long form (OBRL) is expressed most abundantly in paraventricular, arcuate and ventromedial nuclei of the hypothalamus, where it mediates most of the neural signaling of leptin (10-12). Wild type OBRL transfected into different cell lines possesses a number of signaling capabilities. These include activation of the JAK-STAT (13-15) and MAPK pathways, stimulation of tyrosine phosphorylation of the insulin receptor substrates (IRS-1 and IRS-2), PI 3-kinase (PI 3-K) docking and a transient increase in c-fos and c-jun expression (16,17).

Insulin acts in the same key hypothalamic areas as leptin to suppress feeding (18). During the past decade, many of the proteins involved in insulin action have been defined at a molecular level. The insulin receptor is a protein tyrosine kinase which, when activated

by insulin binding, undergoes rapid autophosphorylation and phosphorylates intracellular protein substrates, including insulin receptor substrates (IRSs) (19-21), Shc (22) and JAK2 (23). Following tyrosine phosphorylation, the IRSs act as docking proteins for several Src homology 2 domain-containing proteins, including phosphatidylinositol 3-kinase (PI 3-kinase) and Grb2 (24,25). PI 3-kinase activates two kinases: PDK1 that phosphorylates Akt on Thr-308 and a putative PDK2 that phosphorylates Akt on Ser-473 leading to an increase in Akt kinase activity (26). Another pathway proceeds through Grb2/Sos and ras, leading to activation of the MAP kinase isoforms ERK1 and ERK2. However, a systematic evaluation of these signal transduction pathways after insulin or leptin in hypothalamus has not yet been provided.

Recently it was demonstrated that brain-specific disruption of the IR gene results in hyperphagia in female mice and causes obesity, insulin resistance, hypertriglyceridemia and hyperleptinemia in both male and female mice (27). Furthermore female mice lacking IRS-2 have increased food intake and obesity despite the high levels of leptin (28), these results suggest first a role of insulin signaling system in the brain for the control of food intake and metabolism and second that insulin resistance in the brain is also accompanied by leptin resistance reinforcing the possibility of cross-talk between the two hormones. Thus, the present study was designed to investigate whether insulin and leptin share common intracellular signal transduction pathways and to determine whether these hormonal signaling systems modulate each other's action in rat hypothalamus.

RESEARCH METHODS AND PROCEDURES

2.1. Materials

The reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad. Tris[hydroxymethyl]amino-methane (Tris), aprotinin, ATP, dithiothreitol, phenylmethylsulfonyl fluoride, Triton X-100, Tween 20, glycerol and bovine serum albumin (BSA, fraction V) were from Sigma Chemical Co. (St. Louis, MO, USA). Protein A-Sepharose 6MB and ^{125}I-protein A were from Amersham Pharmacia Biotech UK Ltd. (Buckinghamshire, UK). Nitrocellulose paper (BA85, 0.2 mm) was from Schleicher & Schuell (Keene, NH, USA). Sodium amobarbital (Amytal) and human recombinant insulin (Humulin R) were from Eli Lilly Co. (Indianapolis, IN, USA). Leptin was from Calbiochem (San Diego, CA, USA). Ketamine hydrochloride was from Fort Dodge Laboratories Inc. (Fort Dodge, IA, USA). Antibodies to IR, IRS-1, IRS-2, Erk, Erk phosphotyrosine specific, JAK2 and phosphotyrosine were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), the Akt phosphoserine 473-specific antibody was from New England Biolabs (Beverly, MA, USA) and the antibody to the p85 subunit of PI 3-Kinase was from Upstate Biotechnology (Lake Placid, NY, USA)

2.2. Animals and surgical procedures

Adult male Wistar rats (250-300 g) were used in all the experiments in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA). After an overnight fast, the rats were anesthetized with ketamine hydrochloride (100 mg/kg, ip) and positioned on a Stoelting stereotaxic apparatus using head and chin holders. An appropriate side arm holding the guide cannula was attached. The vertical coordinate of the

side arm was set at 0° in the lateral plane and at 90° in the horizontal plane. The scalp was removed in the midline to expose the saggital suture and the periosteum was then opened and reflected away from the surgical field. The lateral, anteroposterior and dorsoventral coordinates of the third ventricle were obtained from a standard atlas (29). The bregma was used as the reference point and a hole was drilled in the parietal bone at the junction of the lateral and anteroposterior coordinates. Two smaller holes were drilled around this central hole, and stainless-steel screws were inserted half-way through the holes. The cannula was positioned over the central hole and lowered carefully until the necessary dorsoventral coordinate was reached. The cannula was fixed to the skull using cranioplastic cement after ensuring hemostasis. After the cement had set, the cannula was freed from the side arm and a dummy cannula was inserted to close the outer cannula. The rats were then allowed to recover from anesthesia and the surgical procedure for 7 days.

2.3. Intracerebroventricular infusions

After a 6 h fast, the rats were anesthetized with sodium amobarbital (15 mg/kg body weight, ip), and used as soon as anesthesia was assured by the loss of pedal and corneal reflexes. Insulin (3.6 μg/μl – human insulin from E. Lilly), leptin (5 μg/μl – rat leptin from NIH), saline (2 μl in the control animals) or an equimolar mixture of insulin and leptin (3.6 μg/μl insulin, 5 μg/μl leptin) were taken up into an internal fusion cannula connected to a polyethylene supply tube. This tubing was then connected to a 2 μl syringe primed with the infusate. The dummy cannula was removed and the infusion cannula was introduced into the outer guide cannula after which the solution was injected into the third ventricle. The cranium was opened and the hypothalamus excised. In preliminary experiments we determined blood glucose and serum insulin levels in animals that received icv insulin

infusion. Insulinemia and glycemia were not altered by third ventricle insulin or saline microinjection. Under the experimental conditions, no retrograde insulin trafficking through the blood-brain barrier was observed.

2.4. Western blot analysis

The hypothalami were removed minced coarsely and homogenized immediately in the solubilization buffer containing 100 mM Tris (pH 7.6), 1% Triton X-100, 150 mM NaCl, 0.1 mg aprotinin, 35 mg PMSF/ml, 10 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, and 4 mM EDTA, using a politron PTA 20S generator 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 the antipeptide antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak Co., Rochester, NY) with Cronex Lightning Plus intensifying screens at -80°C for 12–48 h. Band intensities were quantitated by optical densitometry (Scion Image software) of the developed autoradiographs.

2.5. Statistical analysis

Where appropriate, the results were expressed as the mean ± S.E.M. accompanied by the indicated number of experiments. ANOVA factorial test was used in all statistical comparisons with $p < 0.05$ indicating significance.

RESULTS

Insulin-induced tyrosine kinase activity of the insulin receptor

To determine whether IR kinase activity was stimulated by insulin in rat hypothalamus, we measured enzyme autophosphorylation *in vitro*. A low dose of insulin was infused into the third ventricle to obtain limited tyrosine phosphorylation of IR, which was then immunoprecipitated and reacted *in vitro* with ATP to allow autophosphorylation. Subsequent Western blotting using an anti-phosphotyrosine antibody showed that insulin induced IR autophosphorylation (Fig. 1A, *lane 4*). The faint signal seen in the hypothalamic extract previously infused with a low dose of insulin but with no exogenous ATP added during the *in vitro* autophosphorylation step (Fig 1A, *lane 2*) may represent IR autophosphorylation using endogenous ATP.

Effects of insulin on IRS-1 and IRS-2 tyrosine phosphorylation and PI 3-Kinase docking

The tyrosine phosphorylation of IRS-1 and IRS-2 in rat hypothalamus was detected in immunoprecipitation experiments. Thus, 2 min after stimulation with insulin, there were significant increases in IRS-1 tyrosine phosphorylation, which suffered slight increase thereafter. To better define the levels of IRS-2 phosphorylation, we performed Western blot analysis of tyrosyl phosphorylated proteins in anti-IRS-2 immunoprecipitates before and after stimulation with insulin. Analogously, Figure 2C shows that there was a marked increase in insulin-stimulated IRS-2 phosphorylation in the rat hypothalamus, which was detectable within 2 minutes and continued to increase within the time-frame studied.

Its well established that there is a relatively stable, high affinity interaction between the IRS-1/2 and the 85 kDa subunit of PI 3-kinase, such that both proteins are

coprecipitated by antibodies to either protein (16,30). Blots that had been previously immunoprecipitated with antibodies against IRS-1 or IRS-2 were subsequently incubated with antibodies against the 85 kDa subunit of PI 3-kinase (Fig. 2 B and C). There was little PI 3-kinase immunoreactivity in the basal state of control rats. After 2 minutes of stimulation with insulin, there were significant increases in IRS-1 or IRS-2 – PI 3-kinase associations, which suffered slight increase thereafter. These observations are consistent with the formation of a stable association between IRS-1 or IRS-2 and PI 3-kinase after insulin stimulation. As predictable, there were no changes in IRS-1 and IRS-2 protein levels.

Effects of insulin on Akt serine phosphorylation in rat hypothalamus

To investigate the effects of insulin on insulin signaling downstream of PI 3-kinase, we measured the serine phosphorylation of a major metabolic intermediate target, Akt. This enzyme play multifunction roles in insulin action, and serine phosphorylation has been shown to correlate with the activity of the enzyme (31-33). As shown in Fig. 1 D, insulin induced a time-dependent increase in AKT serine phosphorylation, which paralleled the increase in PI 3-kinase binding to IRS-1 or IRS-2. In hypothalamus extracts, insulin induced increases in the serine phosphorylation of Akt after 15 min of insulin infusion into the third ventricle.

Effects of insulin on MAP kinase (ERK1-2) tyrosine phosphorylation in rat hypothalamus

ERKs tyrosine phosphorylation was assayed by immunoblot analysis with an antibody directed against the doubly phosphorylated ERKs. Representative immunoblots are shown in Figure 1E. Insulin induced a time-dependent increase in ERK-1 and ERK-2

phosphorylation, which paralleled the increase in tyrosine phosphorylation of IRS-1 or IRS-2. In hypothalamus extracts, insulin induced increases in the phosphorylation of ERKs after 15 min of insulin infusion into the third ventricle.

Effects of leptin on JAK2, IRS-1 and IRS-2 tyrosine phosphorylation and PI 3-Kinase docking.

The tyrosine phosphorylation of JAK2 in rat hypothalamus was detected in immunoprecipitation experiments. Thus, 2 min after stimulation with leptin, there was a significant increase in JAK2 tyrosine phosphorylation, which suffered slight increase within the time-frame studied. Unsurprising, there were no changes in JAK2 protein levels. Since JAK2 was activated by leptin, we evaluated the ability of leptin to stimulate tyrosine phosphorylation of IRS-1 and IRS-2. Figure 2 B shows a clear increase in leptin-stimulated IRS-1 phosphorylation, which, was maximal at 5 min followed by a strike decay. In a similar fashion to IRS-1 tyrosine phosphorylation, Comparable figure 2 C shows that there was a marked increase in leptin-stimulated IRS-2 phosphorylation in the rats' hypothalami, which was maximal at 5 min and almost vanished thereafter. We also observed a time dependent increase in the co-immunoprecipitation between IRS-1 or IRS-2 and PI 3-kinase in hypothalamus, which paralleled the increase of IRS-1 or IRS-2 phosphorylation after leptin stimulation (Fig 2 B and C).

Effects of leptin on Akt serine phosphorylation in rat hypothalamus

Using antibodies against serine-phosphorylated Akt, the levels of Akt activation were examined in hypothalamus after leptin stimulation. Akt phosphorylation in hypothalamus was unaltered by leptin injection (data not shown).

Effects of leptin on tyrosine phosphorylation of MAP kinase in rat hypothalamus

Using antibodies against tyrosine-phosphorylated ERK-1/2, the levels of ERK-1/2 activation were examined in rat hypothalamus after leptin stimulation. As shown in Figure 2 D (top), Leptin stimulated tyrosine phosphorylation of ERK-1/2 in rat hypothalamus could be detected as early as 2 minutes after leptin administration and sustained activated within the time-frame studied.

The effect simultaneous administration of insulin and leptin on IRS-1, IRS-2, Akt and MAPK tyrosine phosphorylation in rat hypothalamus.

We next examined whether simultaneous stimulation with insulin and leptin could affect the early steps of the insulin signaling pathways. At first, we chose the time-points of maximal tyrosine phosphorylation IRSs stimulated by leptin and insulin. Insulin treatment caused an increase of ~9-fold and ~7-fold in tyrosine phosphorylation of IRS-1, in hypothalamus after 2 and 15 minutes, respectively (Figure 3A), while stimulation of the hypothalamus with leptin caused an increase of ~8-fold tyrosine phosphorylation of IRS-1, in hypothalamus after 2 and no leptin induced phosphorylation was observed after 15 minutes of leptin infusion. The effect of insulin and leptin was similar to insulin alone in both time-points studied. IRS-2 tyrosine phosphorylation upon insulin and/or leptin stimulation was comparable with that obtained in IRS-1 immunoprecipitates (Fig. 3 B). Second, Akt serine phosphorylation was examined. Leptin alone had no effect on Akt tyrosine phosphorylation in hypothalamus. Insulin induced ~2- fold increase in Akt phosphorylation and no additive effect was observed with the addition of leptin (Fig 3 C). Last, we measured the tyrosine phosphorylation of ERKs. The phosphorylation of ERK in hypothalamus showed an increase ~2-fold with leptin stimulation and approximately ~2.3-

fold with leptin. The effect of insulin and leptin was similar to insulin or leptin alone (Fig. 3 D).

DISCUSSION

In the present study, we have investigated the possibility of direct interactions between insulin and leptin action in hypothalamus, focusing on some key intermediate steps on these signaling pathways. Our results show that both leptin and insulin stimulated MAPK and IRS/PI 3-kinase pathways. Akt phosphorylation were not changed by leptin but increased with insulin. Simultaneous stimulation with both hormones did not increment the maximal tyrosine phosphorylation of IRS-1, IRS-2 and MAPK and Akt serine phosphorylation.

The tyrosyl phosphorylation of the IR plays a key role in insulin signal transduction by coordinating the assembly of multicomponent signaling complexes around the activated receptor or docking proteins (34,35). IRS-1 is a major cytoplasmic substrate of the IR (36) and our results show that insulin treatment leads to rapid changes in IRS-1 tyrosine phosphorylation in hypothalamus, in agreement with studies that demonstrated that insulin stimulates tyrosine phosphorylation of a 185 kDa protein in all cells and in a variety of tissues (19,37). IRS-1 binds several SH2 proteins through its multiple tyrosine phosphorylated sites (38). The PI 3-kinase was the first SH2 protein found to be associated with IRS-1 (39). In insulin-stimulated cells, the association of PI 3-kinase with tyrosyl phosphorylated IRS-1 activates this enzyme (40). Thus, the ability of insulin to stimulate the association of IRS-1 to the 85 kDa regulatory subunit in hypothalamus suggests that insulin activates PI 3-kinase in this tissue. PI 3-kinase plays an important role in many insulin-regulated mitogenic and metabolic processes, including general and growth-specific protein synthesis (41); this can possibly be one pathway by which insulin can induce anorexia in hypothalamus. We also demonstrated that acute stimulation with insulin can

induce Akt serine phosphorylation and MAPK tyrosine phosphorylation in hypothalamus, which is in accordance with previous studies that showed that these proteins could be activated by insulin (42,43). The results of this study showing that IRS-2 is tyrosyl phosphorylated in response to insulin suggests that both IRS family members have a role in insulin signaling in hypothalamus. Clearly, there is some overlap in function between IRS-1 and IRS-2 as both bind PI 3-kinase in response to insulin.

The leptin receptor is known to act through JAK and STAT proteins (13,16,44). Inasmuch, McCowen et al (45) reported that leptin signaling differs from that of GH, and other ligands for members of this class of receptors, by the inability to stimulate JAK family proteins in the rat hypothalamus. Here we show that leptin induced JAK2 tyrosine phosphorylation in the hypothalamus and activated a pathway essential for leptin signal transduction. This discrepancy may reflect the dephosphorylation of JAK2 by one of the several phosphatases during the stocking and manipulation of the hypothalamic tissue.

Recent evidences indicate that leptin may mimic some well known effects observed after the stimulation of tyrosine kinase receptors by the activation of JAK2, including the tyrosine phosphorylation of IRSs, the activation of mitogen-activated protein kinase and PI 3-kinase (16,17). The effect of leptin stimulation on IRS-1 and IRS-2 tyrosine phosphorylation varies considerably, depending on the cell line used. Some data suggest that leptin can impair the early steps of insulin signaling including tyrosine phosphorylation of IRS-1 in hepatocytes (46). Other studies demonstrate that leptin can mimic effects of insulin such as stimulation of glucose transport and glycogen synthesis in C2C12 myotubes and that these effects may be mediated by stimulation of PI 3-kinase (16). In isolated muscle or adipocytes, short-term incubation with leptin does not stimulate glucose transport

or lipogenesis (47,48). Here we demonstrate that leptin can mediate tyrosine phosphorylation of IRS-1 and IRS-2 and their associations with PI 3-Kinase in the hypothalamus *in vivo*. At a molecular level, PI 3-kinase mediates activation of two kinases—PDK1 that phosphorylates Akt on Thr-308 and a putative PDK2 that phosphorylates Akt on Ser-473—leading to an increase in Akt kinase activity (26). Despite the associations of PI 3-kinase with IRS1 and IRS-2 we cannot detect Akt serine phosphorylation after leptin stimulation in hypothalamus. The role of leptin tyrosine kinase mediated pathway in hypothalamus in the control of energy homeostasis is not clear, but two recent studies have clarified this issue.

The present study demonstrates that leptin and insulin elicit overlapping but distinct signaling pathways. Notably, icv injection of leptin results in only a transitory phosphorylation of IRS-1 and IRS-2 and association of IRS-1 or IRS-2 with PI 3-kinase in hypothalamus and no activation of Akt compared with the sustained effects of insulin on these signaling steps. In an opposite fashion the effect of insulin on MAP kinase activation only appears after 15 min, whereas the effect of leptin is sustained. Hence, the kinetics of the effects of leptin and insulin appear to differ and the effects of leptin may be more sustained in MAPK activation and transitory in the IRSs phosphorylation. Moreover, the effects of simulataneous administration of leptin and insulin on these pathways are not additive, synergistic or inhibitory.

During the last decade, the role of leptin and insulin in regulating body weight has been emphasized. Recently, the insulin signaling pathways have also been implicated in the coordination of energy homeostasis. Thus, the brain-specific disruption of the IR gene results in hyperphagia in female mice and causes obesity, hyperleptinemia, insulin

resistance, and hypertriglyceridemia in both male and female mice (27). Furthermore, female IRS-2 knockout mice showed increased food intake and fat storage (28) and pharmacological blockade of PI 3-kinase pathway activation prevent leptin's effect on leptin-induced anorexia (49). The activation signaling pathways underlying these effects of insulin and leptin are unknown, but the present results showing a direct and rapid effect of these hormones in the hypothalamus, with the involvement of early response elements may contribute to the understanding of some of the stages of this integration.

In conclusion, we have provided evidence for rapid direct effects of insulin and leptin administration *in vivo* on intracellular signaling pathways in hypothalamus. Our data demonstrate that leptin rapidly activates classically insulin signaling pathways directly at the level of hypothalamus, and these pathways overlap with, but are distinct from, those engaged by insulin.

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FIGURE LEGENDS

Fig. 1. Insulin signaling pathway in rat hypothalamus. (A) IR tyrosine kinase activity measured by autophosphorylation. Insulin was injected into the third ventricle to stimulate partial IR autophosphorylation. The IR was then immunoprecipitated (IP) and allowed to autophosphorylate *in vitro* and in the presence of exogenous ATP (*lane 4*). Tyrosine phosphorylation was measured by immunoblotting with an antiphosphotyrosine antibody (P-Tyr). Control conditions are shown in *lanes 1-3*. *Lane 1,* the hypothalamus extract was not exposed to insulin nor was exogenous ATP added to the *in vitro* autophosphorylation reaction. *Lane 2,* insulin was infused into the third ventricle, and the hypothalamus was then extracted, but no exogenous ATP was added during the *in vitro* autophosphorylation reaction. The small signal seen in this lane probably represents IR autophosphorylation using endogenous ATP. *Lane 3*, the hypothalamus was not exposed to insulin, but ATP was added to the *in vitro* autophosphorylation reaction. The equal loading of proteins was verified by reblotting with anti-IR antibodies. Bar graphs are representative of 5 independent experiments (mean ± S.E.M.). ** $P<0.01$ compared to rats treated with saline (control) (factorial ANOVA). (B) and (C) Insulin-stimulated tyrosine phosphorylation of IRS-1, IRS-2 and their association with p85 subunit of PI 3-kinase in hypothalamus *in vivo*. Hypothalami from rats treated with insulin or vehicle for the time points indicated were lysed, and tissue extracts were immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibodies and blotted with anti-phosphotyrosine antibody. The same membranes used for IRS-1 and IRS-2 tyrosine phosphorylation were stripped and reblotted with anti-PI 3-kinase and anti IRS-1 and –2 antibodies (n = 6). (D) Hypothalamic extracts from rats that were stimulated with leptin for the time point indicated were lysed and the proteins were separated by SDS-PAGE on 12% gels and blotted with phosphoserine-specific Akt

antibodies (P-Akt). (E) Hypothalamic extracts from rats that were stimulated with leptin for the time point indicated were lysed and the proteins were separated by SDS-PAGE on 12% gels and blotted with phosphotyrosine-specific Erk antibodies.

Fig. 2. Leptin-stimulated tyrosine phosphorylation of JAK2, IRS-1 and IRS-2 and their association with p85 subunit of PI 3-kinase in hypothalamus *in vivo*. (A) Hypothalamic extracts from animals treated with leptin for the time points indicated, were prepared as described in Methods. Tissue extracts were immunoprecipitated with anti-Jak2 antibody (IP, immunoprecipitation) and immunoblotted with anti-phosphotyrosine (P-Tyr). Stripped membranes were reblotted with anti-Jak2 antibody (n = 6). (B) and (C) Leptin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2. Hypothalami from rats treated with insulin or vehicle for the time points indicated were lysed, and tissue extracts were immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibodies and blotted with anti-phosphotyrosine antibody. The same membranes used for IRS-1 and IRS-2 tyrosine phosphorylation were stripped and reblotted with anti-PI 3-kinase and anti IRS-1 and –2 antibodies (n = 6). (D) Hypothalamic extracts from rats that were stimulated with leptin for the time point indicated were lysed and the proteins were separated by SDS-PAGE on 12% gels and blotted with phosphotyrosine-specific Erk antibodies (P-Erk).

Fig. 3. Effect of insulin and leptin on IRS-1, IRS-2, Akt and Erk phosphorylation in extracts of hypothalamus from rats treated with leptin, insulin or a combination of the two (A) and (B) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated (IP, immunoprecipitation) with anti-IRS-1 or anti-IRS-2 antibodies and blotted with anti-phosphotyrosine antibody (P-Tyr). The bar graphs show the quantitative phosphorylation of IRS-1 and IRS-2 proteins (n = 6). (C) Hypothalami

from rats treated with saline, leptin, insulin or both hormones for 15 min were lysed and the proteins were separated by SDS-PAGE on 12% gels and blotted with phosphoserine-specific Akt antibodies (P-Akt). The bar graphs show the quantitative phosphorylation of Akt (n = 6). (D) Hypothalamic extracts from rats treated with saline, leptin, insulin or both hormones for 15 min were lysed and the proteins were separated by SDS-PAGE on 12% gels and blotted with phosphotirosine-specific Erk1/2 antibodies (P-Erk) (n = 6).

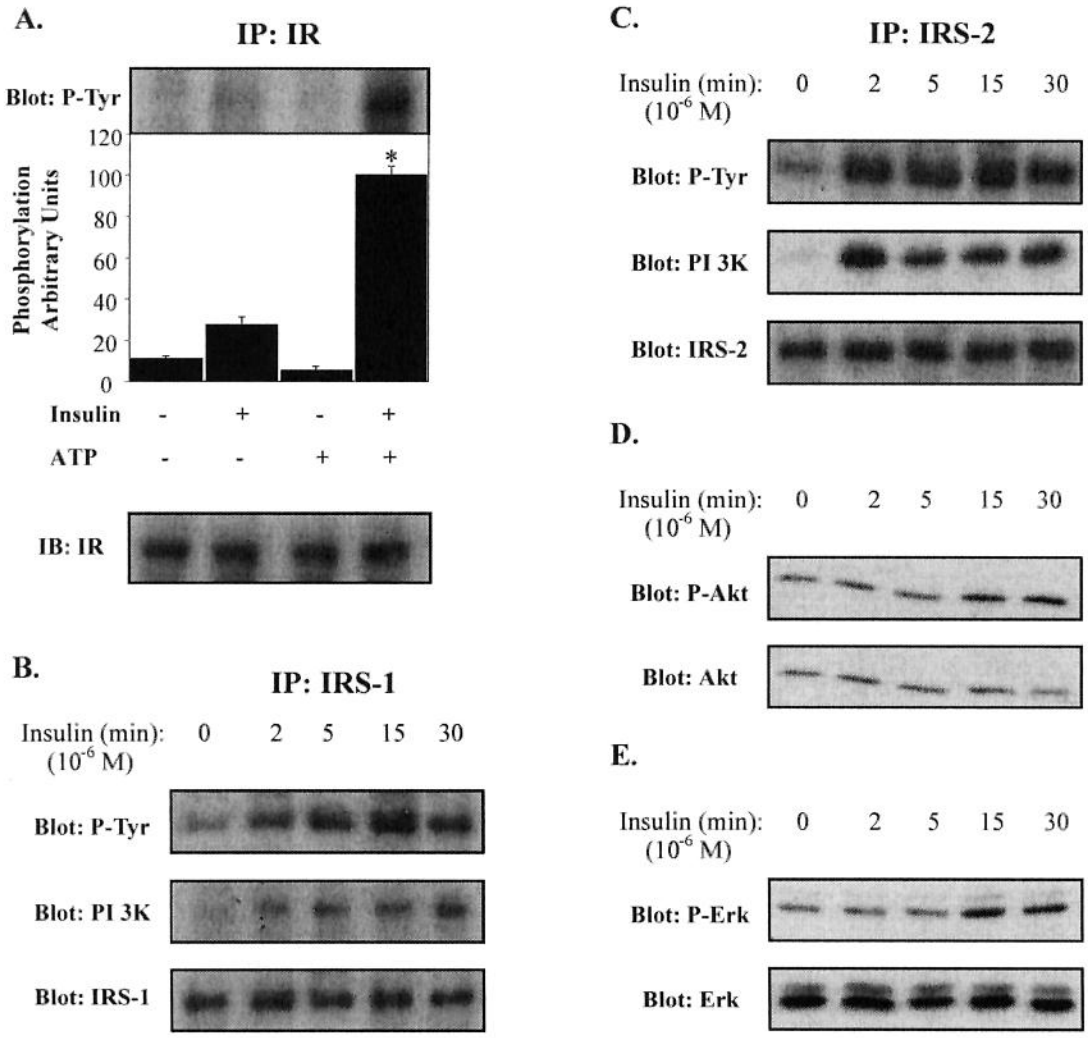

FIGURE 1 - Insulin signaling pathway in rat hypothalamus.

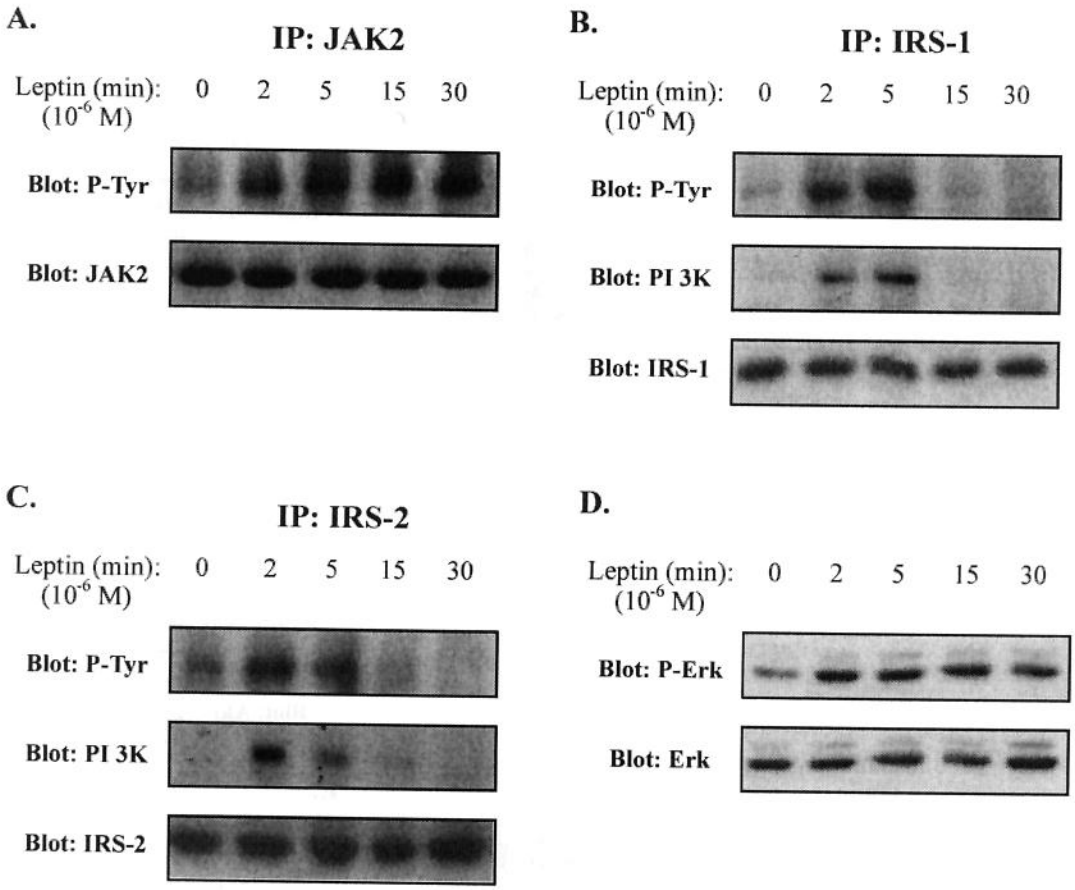

FIGURE 2 - Leptin-stimulated tyrosine phosphorylation of JAK2, IRS-1 and IRS-2 and their association with p85 subunit of PI 3-kinase in hypothalamus *in vivo*.

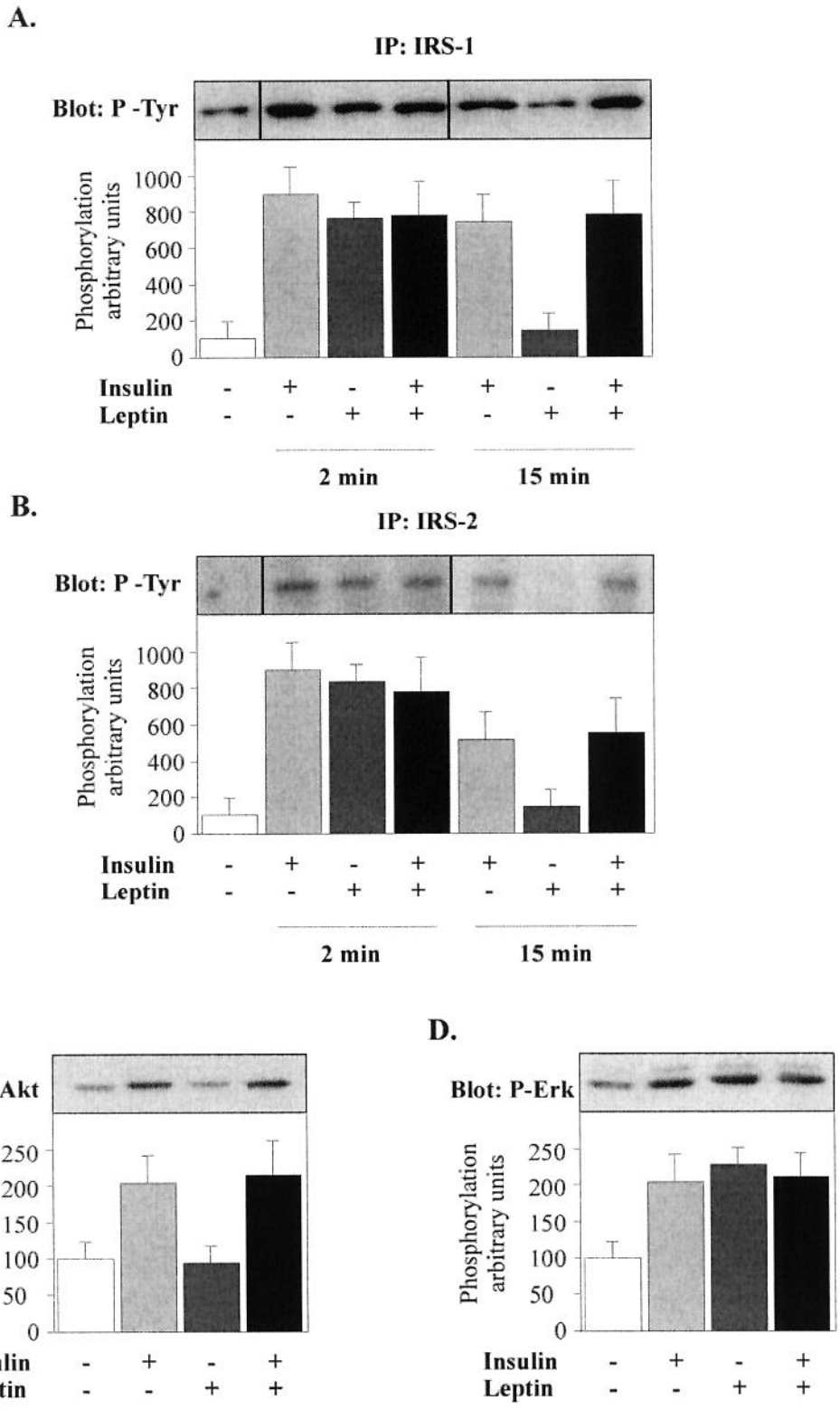

FIGURE 3 - Effect of insulin and leptin on IRS-1, IRS-2, Akt and Erk phosphorylation in extracts of hypothalamus from rats treated with leptin, insulin or a combination of the two

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Insulin modulates leptin-induced STAT3 activation in rat hypothalamus

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Abstract Insulin and leptin have overlapping effects in the control of energy homeostasis, but the molecular basis of this synergism is unknown. Insulin signals through a receptor tyrosine kinase that phosphorylates and activates the docking proteins IRSs (insulin receptor substrates), whereas the leptin receptor and its associated protein tyrosine kinase JAK2 (Janus kinase 2) mediate phosphorylation and activation of the transcription factor STAT3 (signal transducer and activator of transcription). Here, we present evidence for the integration of leptin and insulin signals in the hypothalamus. Insulin induced JAK2 tyrosine phosphorylation, leptin receptor phosphorylation which, in the presence of leptin, augmented the interaction between STAT3 and this receptor. Insulin also increased the leptin-induced phosphorylation of STAT3 and its activation. These results indicate that insulin modulates the leptin signal transduction pathway, and may provide a molecular basis for the coordinated effects of insulin and leptin in feeding behavior and weight control. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Leptin; Insulin; Tyrosine phosphorylation; Janus kinase 2; Signal transducer and activator of transcription; Long form of the leptin receptor

1. Introduction

The circulating peptide leptin, which is the product of the ob gene [1], is secreted predominantly by white adipose tissue and provides feedback information on the extent of the body's fat stores to central OB receptors (long form of the leptin receptor (OBR)) [2] that control food intake and body weight homeostasis [3,4]. The hypothalamus is thought to be the major target for leptin, which acts through receptors that bear strong sequence homology to the class I cytokine receptor family [2]. The leptin receptor exists as multiple splice variants. OBR is expressed most abundantly in paraventricular, arcuate and ventromedial nuclei of the hypothalamus, where it mediates most of the neural signaling of leptin [5–7]. Ligand binding to the OBR results in the activation of Janus kinase 2 (JAK2) by transphosphorylation and the subsequent phosphorylation of tyrosine residues on the intracellular portion of OBR [8–11]. In general, tyrosine phosphorylation of cytokine and growth factor receptors activates intracellular signals by recruiting specific signaling proteins with specialized phosphotyrosine-binding domains called src homology 2 (SH2) domains [12,13]. Tyrosine phosphorylation of OBR recruits the tyrosine phosphatase, phosphotyrosine phosphatase 2 (SHP-2) [14,15] and the signal transducer and activator of transcription (STAT3) transcription factor [14–16]. The use of these SH2 domain-containing proteins by OBR in the hypothalamus implies that they may mediate physiologically important signals [17,18]. In addition to the direct binding of SHP-2 and STAT3, OBR also mediates a number of downstream actions in a variety of cells and tissues [8].

Insulin acts in the same hypothalamic areas as leptin to suppress feeding [19]. In the past decade, many of the proteins involved in insulin action have been defined at a molecular level. The insulin receptor (IR) is a protein tyrosine kinase which, when activated by insulin binding, undergoes rapid autophosphorylation and phosphorylates intracellular protein substrates, including insulin receptor substrates (IRSs) [20–22], Shc [23] and Tub [24]. Following tyrosine phosphorylation, the IRSs act as docking proteins for several SH2 domain-containing proteins, including phosphatidylinositol 3-kinase (PI 3-kinase), Grb2, SHP2, Nck and Fyn [25–29]. A potential mitogen-activated protein kinase-independent mechanism for the regulation of gene expression is the JAK–STAT pathway. The role of insulin in the activation of the JAK/STAT pathway is controversial [30,31]. While this activation was originally thought to occur through cytokine receptors, recent work has shown that the key activating event, STAT tyrosine phosphorylation, is regulated by receptor protein tyrosine kinases [32]. Furthermore, STAT5b activation by insulin has also been reported [31]. However, there is no tyrosine phosphorylation of STAT3 after stimulation by insulin [33].

Obesity in humans and rodents is usually associated with high circulating levels of insulin and leptin as well as insulin and leptin resistance [34–38]. The interaction between insulin and leptin signaling pathways is therefore fundamental for an integrated response of the signal inputs to the hypothalamus. In this study, we examined the cross-talk between the signaling pathways of insulin and leptin leading to STAT3 activation.

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Abbreviations: IR, insulin receptor; IRS, insulin receptor substrate; OBR, long form of the leptin receptor; JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription; SH2, src homology 2; SHP-2, phosphotyrosine phosphatase 2; PI 3-kinase, phosphatidylinositol 3-kinase; EMSA, electrophoretic mobility shift assay

2. Materials and methods

2.1. Animals and surgical procedures

Adult male Wistar rats (250–300 g) were used in all the experiments in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA). After an overnight fast, the rats were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.) and positioned on a Stoelting stereotaxic apparatus using head and chin holders. An appropriate side arm holding the guide cannula was attached. The vertical coordinate of the side arm was set at 0° in the lateral plane and at 90° in the horizontal plane. The scalp was removed in the midline to expose the saggital suture and the periosteum was then opened and reflected away from the surgical field. The lateral, anteroposterior and dorsoventral coordinates of the third ventricle were obtained from a standard atlas [39]. The bregma was used as the reference point and a hole was drilled in the parietal bone at the junction of the lateral and anteroposterior coordinates. Two smaller holes were drilled around this central hole, and stainless-steel screws were inserted half-way through the holes. The cannula was positioned over the central hole and lowered carefully until the necessary dorsoventral coordinate was reached. The cannula was fixed to the skull using cranioplastic cement after ensuring hemostasis. After the cement had set, the cannula was freed from the side arm and a dummy cannula was inserted to close the outer cannula. The rats were then allowed to recover from anesthesia and the surgical procedure for 7 days.

2.2. Intracerebroventricular infusions

After a 6-h fast, the rats were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.), and used as soon as anesthesia was assured by the loss of pedal and corneal reflexes. Insulin (1.9 μg/μl – human insulin from E. Lilly), leptin (5 μg/μl – rat leptin from NIH), saline (2 μl in the control animals) or an equimolar mixture of insulin and leptin (1.9 μg/μl insulin, 5 μg/μl leptin) were taken up into an internal fusion cannula connected to a polyethylene supply tube. This tubing was then connected to a 2-μl syringe primed with the infusate. The dummy cannula was removed and the infusion cannula was introduced into the outer guide cannula after which the solution was injected into the third ventricle. The cranium was opened and the hypothalamus excised. In preliminary experiments we determined blood glucose and serum insulin levels in animals that received i.c.v. insulin infusion. Insulinemia and glycemia were not altered by third ventricle insulin or saline microinjection. Under the experimental conditions, no retrograde insulin trafficking through the blood–brain barrier was observed.

2.3. Western blot analysis

The hypothalami were removed minced coarsely and homogenized immediately in the solubilization buffer containing 100 mM Tris (pH 7.6), 1% Triton X-100, 150 mM NaCl, 0.1 mg aprotinin, 35 mg PMSF/ml, 10 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, and 4 mM EDTA, using a politron PTA 20S generator 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. Quantitative analysis of the blots was done using Scion Image software. The antibodies to: OBR (SC-1835 and SC-8325), phosphotyrosine (SC-508), STAT3 (SC-483), JAK2 (SC-294-G) were obtained from Santa Cruz Biotechnology, and the STAT3 phosphotyrosine 705-specific antibody (9131) was from New England Biolabs.

2.4. IR kinase assay

IR tyrosine kinase activity was measured by autophosphorylation. Insulin was infused into the third ventricle to stimulate limited receptor activation and partial IR autophosphorylation. IR was then immunoprecipitated and allowed to autophosphorylate in vitro in the presence of exogenous ATP. Tyrosine autophosphorylation was measured by immunoblotting with anti-phosphotyrosine antibody.

2.5. Nuclear extracts

Tissues were dissected and dounce-homogenized 10 times in 100: 1 (v:v) of buffer A (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM HEPES pH 7.9, 1 mM DTT, 1 mM $NaVO_4$, 1× complete TM protease inhibitor (Boehringer Mannheim). After centrifugation at 2000×*g* for 10 min at 4°C, the pellet was carefully resuspended in two volumes of buffer B (420 mM NaCl, 10 mM KCl, 20 mM HEPES pH 7.9, 20% glycerol, 1 mM DTT, 1 mM Na VO_4, 1× complete TM protease inhibitor) and extracted for 30 min at 4°C on a shaking rotor. After centrifugation at 16000×*g*, the supernatant was diluted 10-fold in buffer C (10 mM KCl, 20 mM HEPES pH 7.9, 20% glycerol, 1 mM DTT, 1 mM $NaVO_4$, 1× complete TM protease inhibitor) and centrifuged for 10 min at 16000×*g*. The supernatant was loaded onto a Microcon 50 ultrafiltration column (Amicon) and centrifuged for 15 min at 4000×*g* The protein concentration was determined by the Bradford assay (Bio-Rad). Samples were snap-frozen and kept at −80°C. The procedure was carried out at 4°C. DTT, $NaVO_4$ and protease inhibitors were added at the time of the experiment.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was done as described [40]. Double-stranded oligonucleotide probes were synthesized with 5′-GATC protruding ends for fill-in labeling. The M67-SIE probe sequence was 5′-CATTTCCCG-TAAATCAT-3′. 2 μg of hypothalamic nuclear extract were incubated at room temperature for 15 min in the presence of 100 pg of labeled probe (2×10^4–10^5 dpm), 2 μg de poly dI-dC (Pharmacia), 40 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES pH 7.9, 100 μM EGTA, 0.5 mM DTT and 4% Ficoll in a total volume of 12μl. Samples were run on 4% native polyacrylamide gels in 0.25×TBE at 4°C.

2.7. Statistical analysis

Where appropriate, the results were expressed as the mean ± S.E.M. accompanied by the indicated number of experiments. ANOVA factorial test was used in all statistical comparisons with $P < 0.05$ indicating significance.

3. Results and discussion

The aim of this study was to examine the cross-talk between the insulin and leptin signal transduction pathways in the hypothalamus of normal rats. The rats were studied 7 days after the implantation of a cannula in the third ventricle. Leptin, insulin or both hormones were administered i.c.v., after which the ability of leptin to activate STAT3 in the hypothalamus was assessed.

OBR has been detected in various hypothalamic regions, including the arcuate nucleus [2,41], where it completes a feedback loop that delivers information on peripheral energy stocks to the hypothalamus, thereby altering both food intake

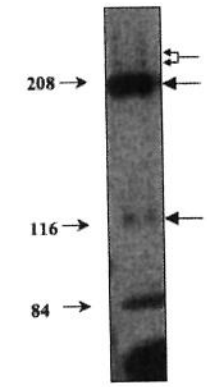

Fig. 1. Different isoforms of OBR are present in the hypothalamus. Hypothalamic lysates were run on 8% polyacrylamide gels and blotted with antibody raised against amino acids 32–51 of the extracellular domain of the OBR. Several bands representing the long and short isoforms were detected. OBR migrated as a major band just below the 208-kDa marker, whereas a band close to 116 kDa represented the short form. The bands migrating in the 60–85-kDa range may represent degradation products of the leptin receptor.

and metabolic rate. The presence of various isoforms of OBR in hypothalamic extracts was analyzed by Western blotting using a polyclonal antibody generated against amino acids 32–51 of the extracellular domain of the mouse OBR. The hypothalamus expressed the long and short isoforms of the OBR (Fig. 1). In agreement with data obtained using cells transfected with OBR-S or OBR-L [8,9], OBR migrated as a weak doublet band above the 208-kDa marker and as a major band just below this marker; the short form migrated as a minor band close to the 116-kDa marker. The bands at 60 kDa and 85 kDa were not characterized further and may represent the non-glycosylated short form of the OBR or degradation fragments as previously described in Fao cells [42].

The OBR is a member of the class I cytokine receptor family that causes a ligand-dependent increase in intracellular protein tyrosine phosphorylation which is essential for receptor function [43]. Receptors of this class lack intrinsic tyrosine kinase activity and act through receptor-associated kinases of the Janus family (JAKs). Activated JAKs can phosphorylate each other, the receptor and the recruited cellular substrates [44]. To examine leptin-induced tyrosine phosphorylation of JAK2, immunoprecipitation and Western blotting of hypothalamic extracts were done using anti-JAK2 and anti-phosphotyrosine antibodies, respectively. As shown in Fig. 2, there was an increase in the phosphorylation of JAK2 1 min after exposure to leptin. Maximal tyrosine phosphorylation of JAK2 occurred 15–30 min after leptin infusion (Fig. 2A) and then decreased dramatically (data not shown). This is the first demonstration that such signaling, previously described in cultured cells, occurs in the rat hypothalamus after the i.c.v. infusion of leptin. These results differ from those of McCowen et al. [45], who reported that leptin signaling was distinguishable from that of GH and other ligands in this class of receptors by its inability to stimulate JAK family proteins in the rat hypothalamus. This discrepancy may reflect the

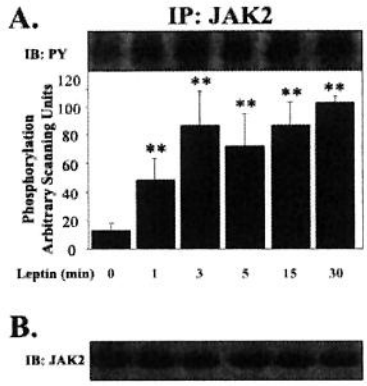

Fig. 2. Effect of leptin on JAK2 phosphorylation. Hypothalamic extracts of from rats treated with 10 μg of leptin for the indicated times, were prepared as described in Section 2. A: Tissue extracts were immunoprecipitated with anti-JAK2 (IP) and immunoblotted with anti-phosphotyrosine antibody (pY) (IB, immunoblotting). Quantitation of the phosphorylation of JAK2 protein is shown in the bar graph, and represents the mean ± S.E.M. of four independent experiments. B: The equal loading of proteins was verified by reblotting with anti-JAK2 antibodies. $^{**}P < 0.01$, compared to rats treated with saline (control) (factorial ANOVA).

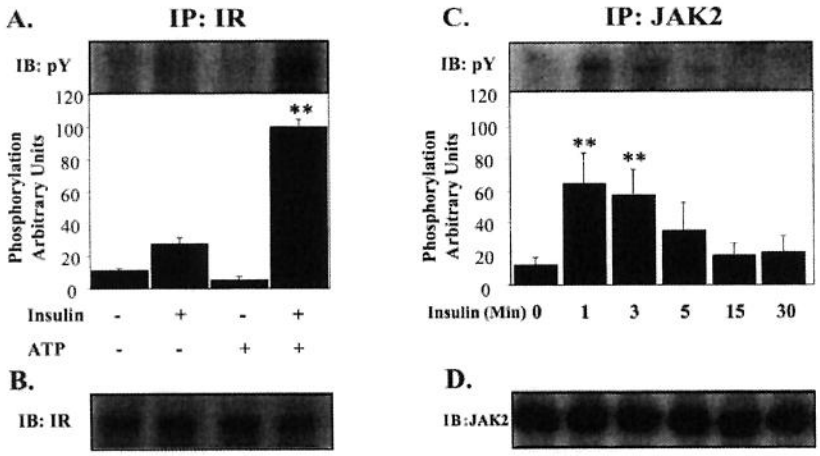

Fig. 3. Insulin induces IR tyrosine kinase activity and JAK2 phosphorylation in rat hypothalamus. A: IR tyrosine kinase activity measured by autophosphorylation. Insulin (10 ng) was injected into the third ventricle to partially stimulate IR autophosphorylation. The IR was then immunoprecipitated and allowed to autophosphorylate in vitro in the presence of exogenous ATP (lane 4). Tyrosine phosphorylation was measured by immunoblotting with an anti-phosphotyrosine antibody. Control conditions are shown in lanes 1–3. Lane 1: The hypothalamic extract was not exposed to insulin nor was exogenous ATP added to the in vitro autophosphorylation reaction. Lane 2: A low dose of insulin (10 ng) was infused into the third ventricle and the hypothalamus was then extracted, but no exogenous ATP was added during the autophosphorylation reaction in vitro. The faint signal seen in this lane probably represents IR autophosphorylation using endogenous ATP. Lane 3: The hypothalamus was not exposed to insulin, but ATP was added to the autophosphorylation reaction in vitro. B: The equal loading of proteins was verified by reblotting with anti-IR antibodies. Bar graphs are representative of five independent experiments (mean ± S.E.M.). $^{**}P < 0.01$ compared to rats treated with saline (control) (factorial ANOVA). In (C) and (D) extracts of bar graphs are representative of five independent experiments (mean ± S.E.M.). Hypothalami from rats treated with 10 μg leptin for the indicated times, were prepared as described in Section 2. C: Tissue extracts were immunoprecipitated with anti-JAK2 (IP) and immunoblotted with anti-phosphotyrosine antibody (pY) (IB, immunoblotting). Quantitation of the phosphorylation of JAK2 protein is shown in the bar graph, and represents the mean ± S.E.M. of four independent experiments. D: The equal loading of proteins was verified by reblotting with anti-JAK2 antibodies. $^{**}P < 0.01$ compared to rats treated with saline (control) (factorial ANOVA).

dephosphorylation of JAK2 by one of several phosphatases during the storage and manipulation of hypothalamic tissue.

As demonstrated in other tissues, insulin stimulated IR autophosphorylation and kinase activity in the rat hypothalamus (Fig. 3A,B). To investigate whether insulin could induce JAK2 tyrosine phosphorylation in the rat hypothalamus, as described in other tissues and cultured cells [30,46], we infused insulin i.c.v. followed by immunoprecipitation and Western blotting of hypothalamic extracts using anti-JAK2 and anti-phosphotyrosine antibodies, respectively. As shown in Fig. 3, maximal tyrosine phosphorylation of JAK2 occurred 1 min after insulin infusion and disappeared by 15 min (Fig. 3C,D).

Insulin acts in the same hypothalamic areas as leptin to suppress feeding [19]. We have performed immunohistochemical analysis of rat hypothalami using IR- and OBR-specific antibodies and the results showed high concentrations of both receptors in the arcuate nucleus and, to a lesser extent, in some periventricular neuronal bodies (data not shown). The presence of both receptors in the same hypothalamic areas may allow a cross-talk between these two hormones. To determine whether there was a direct interaction between the insulin and leptin signaling systems, insulin, leptin or insulin plus leptin were infused i.c.v. and OBR tyrosine phosphorylation was assessed. Immunoprecipitation and Western blotting of hypothalamic extracts were done using the anti-leptin receptor (SC 8325) and anti-phosphotyrosine antibodies, respectively. As shown in Fig. 4A (right side), the leptin receptor (the band just below 208 kDa) was strongly phosphorylated 15 and 30 min after an i.c.v. infusion of leptin. There was also an increase in OBR tyrosine phosphorylation as early as 5 min after exposure to insulin; maximal phosphorylation occurred at 15 min and then decreased by 30 min after insulin infusion (Fig. 4A, left side). Based on these results, we hypothesized that insulin could interfere with the leptin signaling system. When insulin was infused simultaneously with leptin, the levels of OBR phosphorylation increased by 1.7 ± 0.2-fold ($P < 0.01$) compared with leptin treatment alone. The mechanism by which insulin can induce leptin receptor phosphorylation is unknown, but may involve JAK2. In agreement with this hypothesis, the time-course of

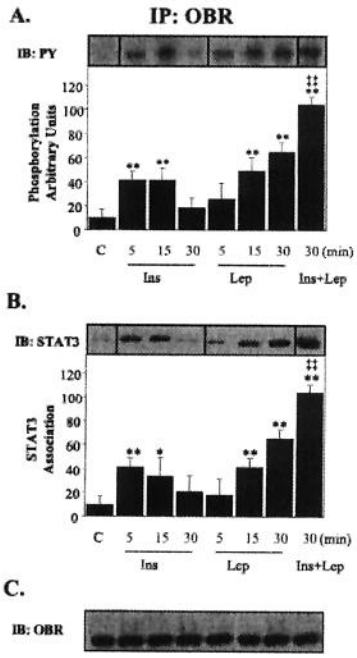

Fig. 4. Effect of insulin and leptin on OBR phosphorylation and STAT3 association in extracts of hypothalami from rats treated with leptin, insulin or a combination of the two for the indicated times. A: Western blots of anti-OBR immunoprecipitates (IP) with anti-phosphotyrosine antibody (pY) (IB, immunoblotting). The bar graph shows the quantitative phosphorylation of OBR proteins. B: The same membranes were reblotted with anti-STAT3 antibodies. The bar graph represents the quantitative association of STAT3 with OBR. The data in (A) and (B) are the means ± S.E.M. of four to six independent experiments. C: The equal loading of proteins was verified by reblotting with OBR antibodies. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; $^{\ddagger\ddagger}P < 0.01$ vs. leptin-treated rats (factorial ANOVA).

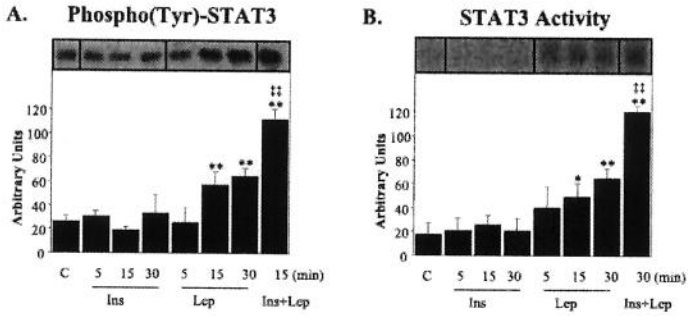

Fig. 5. Insulin (Ins) does not induce STAT3 activation but positively modulates leptin-induced activation of this protein. A: Hypothalami were stimulated with insulin, leptin (Lep), or a combination of the two for the indicated times. Hypothalami were lysed and the proteins were separated by SDS–PAGE on 8% gels and blotted with phosphotyrosine-specific STAT.3 antibodies. Leptin, but not insulin increased the phosphorylation of STAT3; combining the two hormones increased the phosphorylation compared to leptin alone. A representative Western blotting of each is shown. The bar graphs represent the means ± S.E.M. of three to four independent experiments. B: Insulin- and leptin-induced STAT3 DNA-binding activity in rat hypothalami. Rats were starved for 6 h and then received leptin, insulin or insulin plus leptin. The animals were sacrificed at the indicated times, and nuclear extractions were used for EMSA (see Section 2). Each lane represents four hypothalami. All experiments were done twice with similar results. A representative gel shift is shown for each treatment. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; $^{\ddagger\ddagger}P < 0.01$ vs. leptin-treated rats (factorial ANOVA).

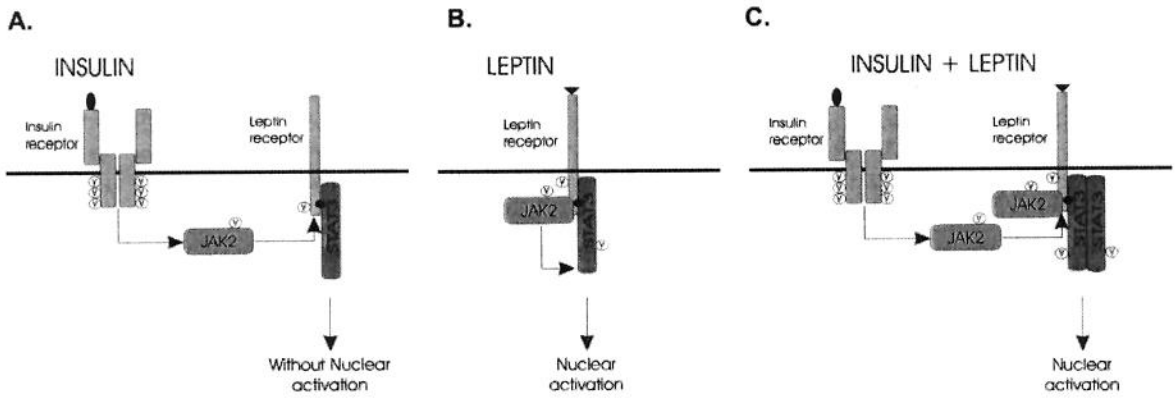

Fig. 6. Model for insulin/leptin cross-talk in rat hypothalamus. A: Insulin infusion. The phosphorylated IR binds and phosphorylates JAK2, which phosphorylates the leptin receptor. The leptin receptor binds STAT3, but there is no nuclear activation. B: Leptin infusion. The leptin receptor binds and activates JAK2, which phosphorylates the leptin receptor. The leptin receptor then binds and activates STAT3. C: Insulin+-leptin infusion. The simultaneous stimulation with insulin and leptin delivers an addictive signal, which leads to enhanced STAT3 association and nuclear activation.

insulin-induced JAK2 tyrosine phosphorylation preceded that of OBR phosphorylation.

Tyrosine phosphorylation of the OBR leads to the binding of STAT3 [47]. To detect the OBR/STAT3 association, nitrocellulose membranes containing samples previously immunoprecipitated with anti-OBR antibody were blotted with anti-STAT3 antibodies. There was an increase in OBR/STAT3 association after leptin stimulation, and this was concomitant with the increase in OBR tyrosine phosphorylation (Fig. 4B, right side). The same membrane was reprobed with anti-IR antibody, and no band was detected, demonstrating that there was not non-specific antibody interaction. Insulin was also able to recruit STAT3 to OBR (Fig. 4B, left side). This insulin-induced increase in the OBR recruitment of STAT3 indicated that insulin can modulate the OBR. The simultaneous stimulation with leptin and insulin dramatically increased this association compared to leptin treatment alone (Fig. 4B).

To determine the additive effect of insulin on the leptin signal transduction pathway, we evaluated the activity of STAT3 after the infusion of these hormones (Fig. 5A,B). Initially, we measured insulin-induced tyrosine phosphorylation of STAT3 by immunoblotting with specific phospho-STAT3 antibodies. As shown in Fig. 5A, insulin did not induce tyrosine phosphorylation of STAT3. Western blotting was also used to examine the tyrosyl phosphorylated STAT3 proteins before and after stimulation with leptin. Fig. 5A shows that there was a marked increase in leptin-stimulated STAT3 phosphorylation in rat hypothalami which was maximal at 15 min. Comparison of the bands from simultaneous stimulation revealed that the extent of STAT3 phosphorylation increased 2.1 ± 0.4-fold ($P < 0.05$) in rats receiving leptin plus insulin compared to the leptin-treated group. The results were confirmed by EMSA experiments. A time-course curve for STAT3 activation after i.c.v. leptin administration in normal rats showed an increase in STAT3 nuclear binding after stimulation with this hormone. The maximal response was obtained immediately after tyrosine phosphorylation, i.e. 15–30 min after 10 μg of leptin i.c.v. and was similar to the maximal response time observed after the iv injection of leptin [17]. In agreement with the results for STAT3 phosphorylation, there was no STAT3 activation after stimulation with insulin. However, the simultaneous administration of insulin and leptin increased the activation of STAT3 by 1.9 ± 0.5-fold compared to treatment with leptin alone. These findings indicate that insulin modulates leptin signal transduction by increasing the association of the leptin receptor with STAT3, leading to activation of the latter.

Based on these results we propose a model for the cross-talk between the insulin and leptin pathways in rat hypothalamus (Fig. 6). According to this model insulin alone induces IR tyrosine phosphorylation which is rapidly followed by JAK2 tyrosine phosphorylation. Activated JAK2 then induces the phosphorylation of OBR. This pathway leads to OBR/STAT3 association, but no activation of STAT3. Leptin on its turn, phosphorylates and activates JAK2, which leads to OBR tyrosine phosphorylation and STAT3 recruitment accompanied by its activation. Co-stimulation with insulin and leptin provides an additive signal which leads to enhanced STAT3 association and nuclear activation.

Integrated responses to insulin and leptin in the hypothalamus participate in the physiological control of food ingestion and body weight. The clinical outcomes observed in some animal models and in humans may reflect a disruption of the normal events in either of these signaling pathways. Obesity occurs in mice lacking the IR in the central nervous system (NIRKO) [19], despite elevated circulating leptin concentrations. This suggests that organ-specific insulin resistance in the central nervous system leads to central leptin resistance. On the other hand, the lack of leptin in ob/ob mice leads to obesity accompanied by hyperinsulinemia, which by itself is not sufficient to reverse the obese phenotype. Finally, in humans with type 1 diabetes, a hypoinsulinemic and a hypoleptinemic status may contribute to the ensuing hyperphagia. Thus insulin resistance, as well as a complete absence of insulin, may impair some of the leptin responses. Our results indicate a mechanism for modulation of the leptin/STAT3 signal transduction pathway by insulin. Insulin rapidly increased the phosphorylation of OBR, and improved the stimulation of leptin-mediated STAT3 phosphorylation, with a consequent gain in STAT3 signaling to the nucleus. This

mechanism may normally function to potentiate the activity of both of these pathways, and to increase stimulation in physiological processes such as the regulation of body weight and food intake, that are under the combined control of insulin and leptin. The potential for this interaction as a site for new therapeutic approaches in treating leptin resistance, and for increasing our understanding of leptin signaling and tyrosine kinase-mediated pathways, deserves further exploration.

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4. RESULTADOS

3. Selective Impairment of Insulin Signaling in the Hypothalamus of Obese Zucker Rats

SELECTIVE IMPAIRMENT OF INSULIN SIGNALING IN THE HYPOTHALAMUS OF OBESE ZUCKER RATS

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Abstract

By acting in the brain, insulin suppresses food intake. However, little is known regarding insulin signaling in the hypothalamus in insulin-resistant states. In this report, in vivo insulin signaling through the phosphatidylinositol 3-kinase (PI 3-kinase) and mitogen-activated protein (MAP) kinase pathways were compared in the hypothalamic tissues of lean (*Fa/?*) and obese Zucker *(fa/fa)* rats. Icv insulin infusion reduced food intake in lean rats but no effect was observed in obese Zucker rats. Pretreatment with PI 3-kinase inhibitors prevents insulin-induced anorexia in lean rats. Insulin-stimulated tyrosine phosphorylation of insulin receptor (IR), IR substrates (IRS-1, IRS-2), the associations of p85 subunit of PI 3-kinase to the IRS proteins and serine phosphorylation of Akt in the hypothalamus of obese rats were significantly decreased compared with the lean rats. In contrast, insulin stimulated tyrosine phosphorylation of MAP kinase was similar in lean and obese rats. The present study provides direct measurements of insulin signaling in hypothalamic tissues, and document selective resistance to insulin signaling in the hypothalamus of obese Zucker rats at the molecular level. These findings have provided support for the hypothesis that insulin may have anti-obesity actions mediated by the PI 3-kinase pathway and that the impaired PI 3-kinase signal pathway in the hypothalamus may lead to the development of obesity in insulin resistance and diabetic patients.

Mesh-Medline key words: Obesity/*physiopathology; Insulin Resistance/*physiology; Hypothalamus/*drug effects/*metabolism; Phosphatidylinositol 3-Kinase/metabolism phosphatidylinositol 3-kinase; Mitogen-Activated Protein Kinase.

1. Introduction

Insulin receptors (IR) are expressed in most tissues of the body, including the classic insulin-sensitive tissues (liver, muscle, and fat), as well as "insulin-insensitive" tissues, such as red blood cells and neurons of the CNS. In the CNS, the IR displays distinct patterns of expression in the olfactory bulb, the hypothalamus, and the pituitary (1-3), although its function in these regions remains largely unknown. Previous experiments have documented a role for insulin signaling in the regulation of food intake (4-6).

In the past decade, many of the proteins involved in insulin action have been defined at the molecular level in nonhypothalamic cells. The insulin receptor is a protein tyrosine kinase which, when activated by insulin binding, undergoes rapid autophosphorylation and phosphorylates intracellular protein substrates, including insulin receptor substrates (IRSs – IRS-1 and IRS-2 are the most important) (7-9) and Shc (10). Following tyrosine phosphorylation, the IRSs act as docking proteins for several Src homology 2 domain-containing proteins, including phosphatidylinositol 3-kinase (PI 3-kinase), Grb2, SHP2, Nck and Fyn (11-15). Downstream PI 3-kinase there is activation of a serine/threonine kinase, Akt, (16). On the other side, downstream to Grb2 activation there is activation of the mitogen-activated protein kinase, which is important in the regulation of gene-expression and cell growth (17-19).

Besides its epidemiological association with obesity, insulin resistance and hyperinsulinemia may primarily contribute to the development of obesity (20). Mechanisms to explain the weight gain caused by insulin resistance and hyperinsulinemia have been attributed to direct insulin's effects on adipocyte growth and defective insulin signaling in hypothalamus (4, 6, 20-22). To test the latter possibility, we have characterized the direct

insulin's actions on the PI 3-kinase and MAPK pathway in hypothalamic tissues from both lean (*Fa/?*) and obese Zucker *(fa/fa)* rats.

2. Materials and methods

2.1. Animals and surgical procedures

Four to five-month old male Zucker rats were used. All experiments involving animals were in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA). Room temperature was maintained between 21-23°, and a 12-hour light, 12-hour-dark cycle was used. Physiological parameters of the Zucker lean and obese rats are described in Table 1. After an overnight fast, the rats were anesthetized with ketamine hydrochloride (100 mg/kg, ip) and positioned on a Stoelting stereotaxic apparatus using head and chin holders. An appropriate side arm holding the guide cannula was attached. The vertical coordinate of the side arm was set at 0° in the lateral plane and at 90° in the horizontal plane. The scalp was removed in the midline to expose the saggital suture, and the periosteum was then opened and reflected away from the surgical field. The lateral, anteroposterior and dorsoventral coordinates of the lateral ventricle were obtained from a standard atlas (23). The bregma was used as the reference point and a hole was drilled in the parietal bone at the junction of the lateral and anteroposterior coordinates. Two smaller holes were drilled around this central hole, and stainless-steel screws were inserted halfway through the holes. The cannula was positioned over the central hole and lowered carefully until the necessary dorsoventral coordinate was reached. The cannula was fixed to the skull using cranioplastic cement after ensuring hemostasis. As soon as the cement was set, the cannula was released from the side arm and a dummy cannula was inserted to close the outer cannula. The rats were then allowed to recover from anesthesia and the surgical procedure for 7 days.

2.2. Intracerebroventricular infusions

After a 6 h fast, the rats were anesthetized with sodium amobarbital (15 mg/kg body weight, ip), and used as soon as anesthesia was assured by the loss of pedal and corneal reflexes. Insulin (2 mU – human insulin from E. Lilly), Wortmannin (100 nmol), or its saline vehicle were taken up into an internal infusion cannula connected to a polyethylene supply tube. This tubing was then connected to a 2 µl syringe primed with the infusate. The dummy cannula was removed and the infusion cannula was introduced into the outer guide cannula after which the solution was injected into the lateral ventricle. After the appropriate time interval (2 or 15 min), the cranium was opened and the hypothalamus excised. In preliminary experiments we determined blood glucose and serum insulin levels in animals that received icv insulin infusion. Insulinemia and glycemia were not altered by third ventricle insulin or saline microinjection. Under the experimental conditions, no retrograde insulin trafficking through the blood-brain barrier was observed.

2.3. Assay of satiety activity of insulin.

The satiety effect of insulin was assayed by intracerebroventricular injection into Zucker lean and obese rats. After surgery, the rats were housed in individual cages and kept on a 12-h light-dark cycle with and fed a standard rodent chow. The rats were then allowed to recover from anesthesia and the surgical procedure for 7 days. In the eighth day pretreatment with wortmannin (100 nmol icv), or vehicle was followed 30 min later, just before the onset of dark cycle, by icv administration of insulin or vehicle were injected icv (2 µl). Total, individual chow ingestion was measured in the following 12h.

2.4. Western blot analysis

The hypothalami were removed minced coarsely and homogenized immediately in the solubilization buffer containing 100 mM Tris (pH 7.6), 1% Triton X-100, 150 mM NaCl, 0.1 mg aprotinin, 35 mg PMSF/ml, 10 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, and 4 mM EDTA, using a politron PTA 20S generator 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. Quantitative analysis of the blots was done using Scion Image software. The antibodies to: IR (SC-711), IRS-1, IRS-2 (SC-8299) and anti-phosphotyrosine (SC-508) were obtained from Santa Cruz Biotechnology. Antibodies against the p85 subunit of PI 3-kinase (06-195) were from Upstate Biotechnology. Antibodies against phospho-Akt (9271L) were purchased from New England Biolabs.

2.5. Statistical Analysis

Where appropriate, the results were expressed as the mean ± S.E.M. accompanied by the indicated number of experiments. Unless indicated otherwise comparisons of data were performed using the Kruskal-Wallis 1-way ANOVA. *P* values less than 0.05 were considered to be significantly different.

3. Results

Intracerebroventricular insulin reduces food intake of lean but not obese Zucker rats. As shown in Table 1, the obese rats had higher body weight, glucose and fasting insulin levels than their age-matched lean controls. Insulin (2 mU) and its saline vehicle were infused into the lateral cerebral ventricle of male lean or obese Zucker rats. The average total food intake at 12h of the insulin-infused group was decreased by ~85% (p less than 0.01) as compared with that of the saline-infused group. On the other side, insulin infusion induced a nonsignificant reduction on food intake in the obese rats relative to their saline-infused controls (Fig. 1).

In vivo effect of insulin on tyrosine phosphorylation of IR in the hypothalamus. The effect of *in vivo* insulin on tyrosine phosphorylation of IR was examined in the hypothalamus of Zucker lean (*Fa/?*) and obese (*fa/fa*) rats. Hypothalami from insulin or vehicle –treated rats were submitted to immunoprecipitation with antibody to IR and immunoblotted with anti-phosphotyrosine antibody. As shown in Figure 2A, insulin increased IR tyrosine phosphorylation in the hypothalamus from both lean and obese rats. In lean animals, the increment was 8.4-fold above basal level, while it was only 3.2-fold in the obese rats. To determine whether this ~70% reduction in the obese rats was associated with a diminished amount of the IRβ proteins, the same membranes were reblotted with antibodies to IRβ. As shown in Figure 2B (bottom), protein levels of IR were not different between lean and obese rats.

Protein expression and tyrosine phosphorylation of IRS-1/2 and their association with the p85 subunit of PI 3-kinase in the hypothalamus. The protein expression of IRS-1 and IRS-2 in the hypothalamus from control and obese rats were quantitated by immunoprecipitation

and immunoblotting with αIRS-1 or αIRS-2 antibodies and decreases of 35% and 20%, respectively were detected, in the hypothalamus of obese rats as compared with lean controls (Figure 3A). *In vivo* experiments, insulin-stimulated IRSs phosphorylation, quantitated by immunoprecipitation with anti-IRS-1 or anti-IRS-2 antibodies and immunoblotting with αpY antibody, showed an increase of 7.8-fold and 8.1-fold in tyrosine phosphorylation of IRS-1 and IRS-2, respectively, in the hypothalamus of lean rats (Figure 3, B and C), compared with increases of 3.8- and 4-fold, respectively, in the hypothalamus of obese rats ($P < 0.05$), representing decreases of ~50% in the obese rats. The same membranes used to detect tyrosine phosphorylation of IRS-1 and IRS-2 were reblotted with antibodies against the p85 subunit of PI 3-kinase. In the lean rats insulin led to increases of 10- and 9-fold in p85 association to IRS-1 and IRS-2, respectively, (Figure 3, D and E), whereas only 4- and 4.5-fold increases were observed in the hypothalamus of obese rats, representing reductions of ~60% in these animals ($P < 0.05$).

Role of PI 3-kinase in anorectic response to insulin. Pharmacological inhibitors of PI 3-kinase activity, such as wortmannin, can block insulin activity *in vitro* (24), suggesting that PI 3-kinase-dependent signaling is needed for certain intracellular responses to insulin. We tested whether the inhibitory effects of insulin on food intake depend on PI 3-kinase, by icv infusion of wortmannin into lean rats. Treatment with insulin markedly reduced food intake at 12 h in rats pre-treated with vehicle, but we did not detect insulin-induced anorexia after pretreatment with wortmannin (Fig.4A).

Akt is a serine/threonine kinase that is serine phosphorylated and can be activated by insulin through the PI 3-kinase pathway (16). In the hypothalamus from lean rats, insulin increased serine phosphorylation of Akt by 8.4-fold over the basal levels (Figure 4B). The

basal levels of serine phosphorylation of Akt were significantly higher in hypothalamus of obese rats than lean animals ($P < 0.05$; $n = 6$), and insulin increased Akt serine phosphorylation by only 1.2-fold in these animals. By comparison to lean rats there was a reduction of ~45% in insulin-induced Akt phosphorylation in hypothalamus of obese rats. To confirm the biological efficacy of PI 3-kinase blockage with wortmannin we determined the effect of insulin in the activation of AKT in rats pretreated with wortmannin. As shown in figure 4B wortmannin impedes insulin-induced serine phosphorylation of Akt in the hypothalamus of lean rats.

Tyrosine phosphorylation of MAP kinase (ERK-1/2) in hypothalamic tissues. Using antibodies against tyrosine-phosphorylated ERK-1/2, the levels of ERK-1/2 activation were examined in hypothalamus after insulin stimulation. As shown in Figure 5, insulin stimulated tyrosine phosphorylation of ERK-1/2 equally in the hypothalamus of lean and obese rats. Insulin increased tyrosine phosphorylation of ERK-1/2 by 4.5-fold in the hypothalamus of lean rats, and 4.3-fold increases in the obese rats. The protein levels of ERK-1/2 were not significantly different in the hypothalamus between lean and obese animals.

4. Discussion

In the present study we have characterized insulin signaling in the hypothalamus of lean and obese Zucker rats. Obese Zucker rats have insulin resistance, hyperinsulinemia, hyperlipidemia and glucose intolerance, features similar to those present in obesity-related insulin resistance and type 2 diabetes mellitus (25). Here we show that treatment with insulin markedly reduced food intake at 12 h in lean rats pre-treated with vehicle, but we did not detect insulin-induced anorexia after pretreatment with a PI 3-kinase inhibitor. On the other side, in accordance with a previous report (22), our results show that central insulin administration fails to suppress food intake and body weight in the obese rats. Defective insulin action in the brain may thus contribute to the pathogenesis of obesity in the *fa/fa* rats. In addition, the results of the present study demonstrate an impairment of insulin activation of the PI 3-kinase-Akt pathway in the obese and insulin-resistant animals. However, insulin stimulated tyrosine phosphorylation of ERK-1/2 MAP kinase similarly in both phenotypes.

The sites of insulin-signaling impairment appear to be at the IR and postreceptor levels in the hypothalamus of obese Zucker rats. Although the total amount of receptor protein is not changed, IR tyrosine phosphorylation induced by insulin is significantly lower in the hypothalamus of obese than in the lean rats. The reduction in IR tyrosine phosphorylation in the hypothalamus is similar to that observed in liver and vascular cells (26).

As observed in liver (27), a moderate decrease in IRS-1 and IRS-2 protein expression occurs in the hypothalamus of the obese rats. In parallel, there is also a decrease in IRS-1 and IRS-2 tyrosine phosphorylation, reflecting the decrease in protein levels as

well as a decrease in the stoichiometry of phosphorylation. These reductions are accompanied by a decrease in insulin-induced IRS-1 and IRS-2 association with PI 3-kinase and Akt phosphorylation. However, the basal level of Akt serine phosphorylation is significantly higher in the hypothalamus of obese animals, probably as a consequence of the hyperinsulinemia, but only a mild increase occurs after acute insulin infusion.

There are probably several mechanisms that can cause decrease in insulin signaling pathway in the hypothalamus of obese insulin-resistant rats. First, increase in serine and threonine phosphorylation of IR may reduce receptor kinase activity and autophosphorylation. It has been reported that activation of PKC induces serine phosphorylation of IR, which can inhibit its tyrosine kinase activity, leading to a decrease of insulin-induced PI 3-kinase activity (28, 29). Activation of protein kinase C-α, -β, -ε, and -δ have been reported in skeletal muscle of obese Zucker and diabetic Goto-Kakizaki rats (30). Second, tumor–necrosis factor-α (TNF –α) has been suggested as important mediator of insulin resistance in obese animals through its overexpression from fat tissue (31). TNF-α has been shown to produce serine phosphorylation of IRS-1, resulting in reduced insulin receptor kinase activity and insulin resistance (32). In rodents, anti-TNF-α reagents significantly improved insulin resistance (33). Last, altered phosphotyrosine phosphatase activity might lead to reduction in tyrosine phosphorylation of IR and IRS protein (34-36).

Selective impairment of insulin signaling on PI 3-kinase pathway in the hypothalamus could be pathophysiologically important in the development of obesity. A recent study by Niswender KD *et al* (37) showed that activation of the PI 3-kinase pathway could be involved in leptin's anorexigenic effect. Taken together with the present data in an

insulin-resistant state, this finding may indicate that the anti-obesity actions induced by insulin could be blunted due to the partial inhibition of the PI 3-kinase pathway and that neuronal PI 3K is important for the effects of insulin on food intake. If the mechanism used by insulin to reduce food intake is PI 3-kinase-dependent, as our results suggest, the defective activation of PI 3-kinase in hypothalamic neurons may reduce the ability of insulin to promote weight loss in the obese rodents.

The profound insulin resistance on the PI 3-kinase pathway contrasts markedly with the ability of insulin to stimulate the MAP kinase pathway in the hypothalamus of the obese rats. Insulin resistance does not affect the MAP kinase pathway, since insulin administration increased ERK1/2 phosphorylation to the same extent in lean and obese rats. This finding of selective insulin resistance has been recently observed in the vasculature of Zucker rats (26). Two possible reasons for this difference may be proposed: alternate insulin signaling pathways and differential signal amplification. With regard to the former, the MAP kinase pathway can be activated through Grb2/Sos interaction with IRS-1/2 or Shc. Because IRS-1 tyrosine phosphorylation is dramatically reduced in the obese animals, it is possible that insulin activation of the MAP kinase pathway *in vivo* primarily occurs through Shc activation. Evidence from *in vitro* and *in vivo* studies supports this concept (38, 39). Maintenance of insulin stimulation of the MAP kinase pathway in the presence of insulin resistance on the PI 3-kinase pathway may be important in the development of insulin resistance. ERKs can phosphorylate IRS-1 on serine residues (40), and serine phosphorylation of IRS-1 and of the insulin receptor itself as described above, can be implicated in desensitizing insulin receptor signaling (41).

In summary our data have provided direct evidence that insulin can activate both the PI 3-kinase-Akt and MAP kinase pathways in the hypothalamus and that the ability of

insulin to reduce food intake is PI 3-kinase-dependent. In an insulin-resistant state, PI 3-kinase pathway appears to be selectively blunted in hypothalamic tissues, compared with MAP kinase. These findings support the hypothesis that insulin may have anti-obesity actions mediated by the PI 3-kinase pathway and that the impaired PI 3-kinase signaling pathway in the hypothalamus may lead to the development of obesity in insulin resistance and diabetic patients.

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Figure Legends

Figure 1. Effect of insulin on food ingestion in lean (L) and obese (F) Zucker rats *in vivo*. Insulin was injected icv at the onset of dark cycle and total, individual chow ingestion was measured in the following 12h. ** $P < 0.001$, insulin lean vs. vehicle lean. * $P < 0.05$, insulin lean vs. insulin obese.

Figure 2. IR protein expression and tyrosine phosphorylation in the hypothalamus of lean (L) and obese (F) Zucker rats *in vivo*. (A) Hypothalamic extracts from lean and obese rats treated with insulin or vehicle for 15 min were prepared as described in Methods. Tissue extracts were immunoprecipitated with anti-IR antibody (IP, immunoprecipitation) and immunoblotted with anti-phosphotyrosine antibody (pY) (IB, immunoblotting). (B) Stripped membranes were reblotted with anti-IR antibody. Data (mean ± SEM; $n = 4$) are expressed as relative to control, assigning a value of 100% to the lean control mean. *$P <$ 0.05, insulin lean vs. insulin obese.

Figure 3. IRS-1 and IRS-2 protein expression and tyrosine phosphorylation, and their association with the P85 subunit of PI 3-kinase in the hypothalamus of lean (L) and obese (F) Zucker rats *in vivo*. (A) Protein levels of IRS-1 and IRS-2. Isolated hypothalami were homogenized, and equal amounts of protein were subjected to immunoprecipitation (IP) with αIRS-1 or αIRS-2, separated by SDS-PAGE, and immunoblotted (IB, immunoblotting) with the same antibody. The bar graph shows the protein levels of IRS proteins. Data (mean ± SEM; $n = 4$) are expressed as relative to control, assigning a value of 100% to the lean insulin mean. *$P < 0.05$ lean vs. obese. (B and C) Insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2. Hypothalami from rats treated with insulin or

vehicle for 15 min were lysed, and tissue extracts were immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibodies and blotted with anti-phosphotyrosine antibody (pY). The bar graph shows the quantitative phosphorylation of IRS proteins. (D and E) The same membranes used for IRS-1 and IRS-2 tyrosine phosphorylation were stripped and reblotted with anti-PI 3 kinase antibodies. The bar graph represents the quantitative association of PI 3 kinase with IRS-1 or IRS-2. Data (mean ± SEM; $n = 6$) are expressed as relative to control, assigning a value of 100% to the lean insulin mean. * $P < 0.05$ insulin lean vs. insulin obese.

Figure 4. Role of PI 3-kinase signaling in the anorectic response to insulin in lean Zucker rats. (A) Pretreatment with wortmannin (100 nmol) or vehicle was followed 30 min later, just before the onset of the dark cycle, by i.c.v. administration of insulin, individual chow ingestion was measured in the following 12h. *$P < 0.05$, relative to controls. (B) Insulin-stimulated serine phosphorylation of Akt in hypothalamus of lean (L) and obese (F) Zucker rats *in vivo*. Pretreatment with wortmannin (100 nmol) or vehicle was followed 30 min later by i.c.v. administration of insulin, or vehicle for 15 minutes, the hypothalamus was lysed and the proteins were separated by SDS-PAGE on 12% gels and blotted with phosphoserine-specific AKT antibodies (IB, immunoblotting). A representative western blotting of each group is shown. The bar graph represents the quantitative serine phosphorylation of AKT. Data (mean ± SEM; $n = 4$) are expressed as relative to control, assigning a value of 100% to the lean insulin mean. # $P < 0.05$, vehicle lean vs. vehicle obese, * $P < 0.05$, insulin lean vs. insulin obese

Figure 5. Effects of insulin on tyrosine phosphorylation of MAP kinase (ERK-1/2) in hypothalamus of lean (L) and obese (F) Zucker rats *in vivo*. (A) Hypothalami from rats treated with insulin or vehicle for 15 minutes were lysed and the proteins were separated by

SDS-PAGE on 12% gels and blotted with phosphotyrosine-specific ERK antibodies (IB, immunoblotting). (B) Stripped membranes were reblotted with anti-ERK antibody. A representative western blotting of each is shown. The bar graph represents the quantitative tyrosine phosphorylation of MAP-kinase. Data (mean ± SEM; $n = 4$) are expressed as relative to control, assigning a value of 100% to the lean insulin mean. * $P < 0.05$, insulin lean vs. insulin obese.

Table 1. Characteristics of obese Zucker (*fa/fa*) rats and their age-matched lean Zucker (*Fa/?*) controls.

Groups	Number of rats (n)	Body weight (g)	Blood glucose (mg/dl)	Serum insulin (ng/ml)
Lean (*Fa/?*)	**10**	**262 ± 8**	**80 ± 5**	**11 ± 2**
Obese (*fa/fa*)	**10**	**394± 20***	**111 ± 4***	**253 ± 16***

*** $P < 0.001$ vs. lean group; unpaired Student's *t* test.**

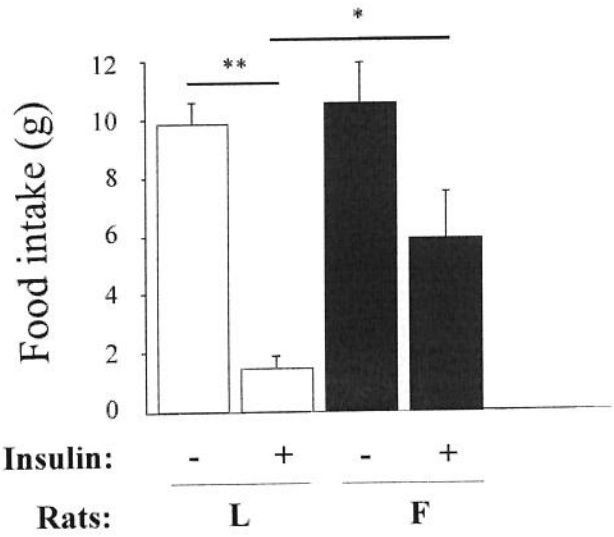

FIGURE 1 - Effect of insulin on food ingestion in lean (L) and obese (F) Zucker rats *in vivo*.

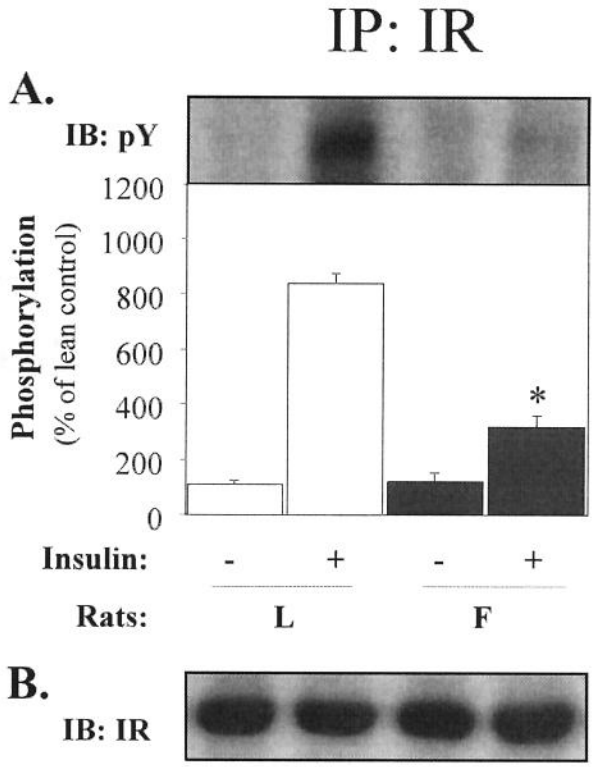

FIGURE 2 - IR protein expression and tyrosine phosphorylation in the hypothalamus of lean (L) and obese (F) Zucker rats *in vivo*.

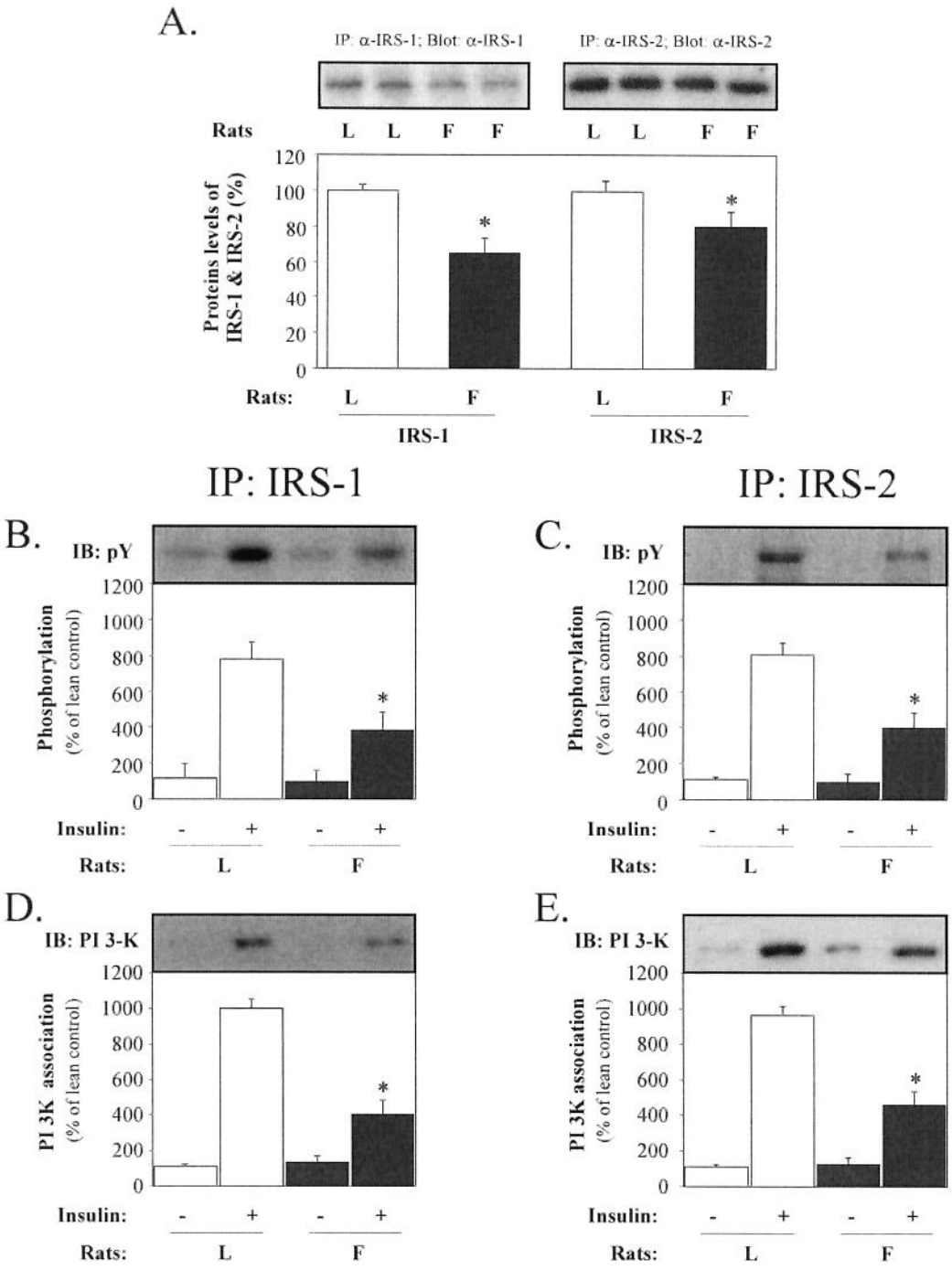

FIGURE 3 - IRS-1 and IRS-2 protein expression and tyrosine phosphorylation, and their association with the P85 subunit of PI 3-kinase in the hypothalamus of lean (L) and obese (F) Zucker rats *in vivo*.

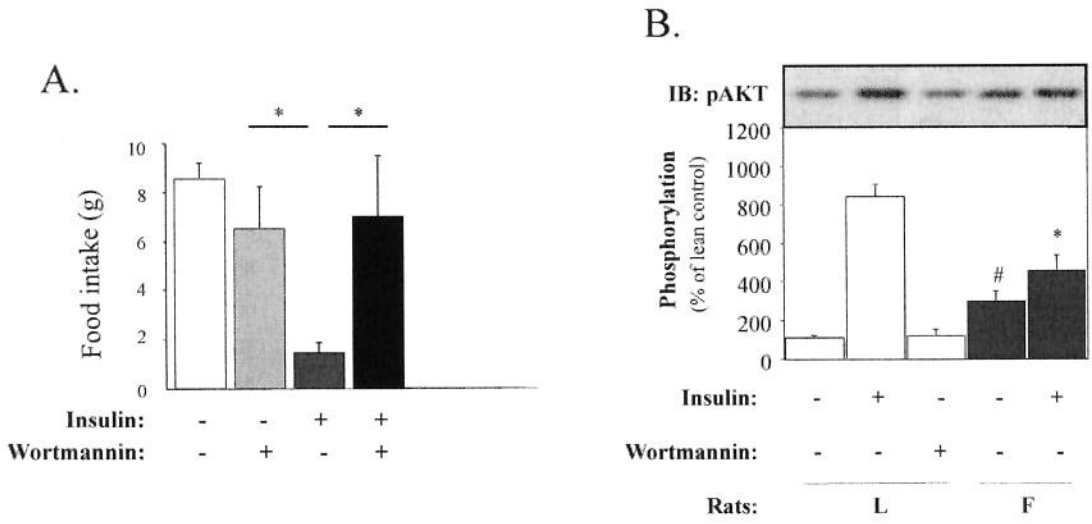

FIGURE 4 - Role of PI 3-kinase signaling in the anorectic response to insulin in lean Zucker rats.

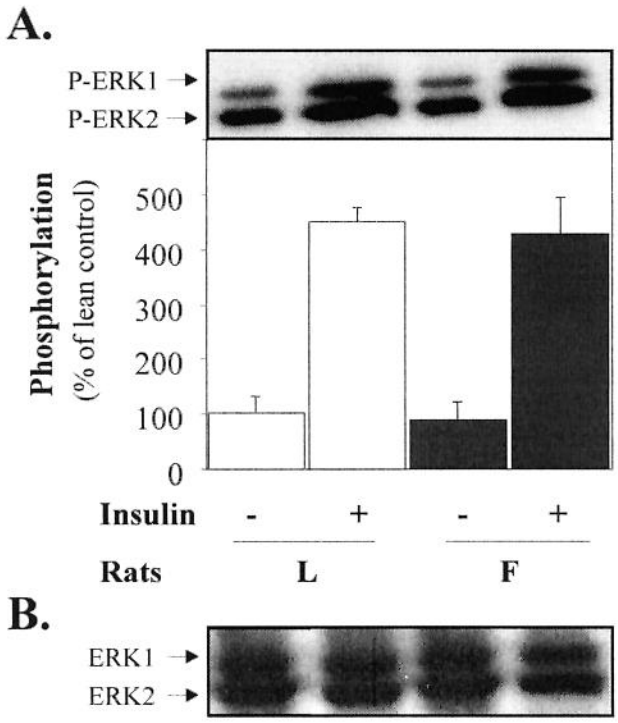

FIGURE 5 - Effects of insulin on tyrosine phosphorylation of MAP kinase (ERK-1/2) in hypothalamus of lean (L) and obese (F) Zucker r*ats in vivo*.

4. RESULTADOS

4. Interaction Between Leptin and Insulin Signaling Pathways Differentially Affects the JAK-STAT and PI 3-Kinase in Rat Liver

Interaction Between Leptin and Insulin Signaling Pathways Differentially Affects the JAK-STAT and PI 3-Kinase–Mediated Signaling in Rat Liver

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Running title: Leptin/Insulin signaling in Rat Liver

SUMMARY

Chronic leptin treatment markedly enhances the action of insulin on hepatic glucose production out of proportion to the body weight loss and increased insulin sensitivity. In the present experiments the cross-talk between insulin and leptin was evaluated in rat liver. Leptin, upon stimulation of JAK2 tyrosine phosphorylation, induced JAK2 co-immunoprecipitation with STAT3, STAT5b, IRS-1 and IRS-2. This phenomenon parallels the leptin-induced tyrosine phosphorylation of STAT3, STAT5b, IRS-1 and IRS-2. Acutely injected insulin stimulated a mild increase in tyrosine phosphorylation of JAK2, STAT3 and STAT5b. Leptin was less effective than insulin at stimulating IRSs phosphorylation and their associations with PI 3-kinase. Simultaneous treatment with both hormones promoted no change in maximal phosphorylation of STAT3, IRS-1, IRS-2 and Akt, but led to marked increase in tyrosine phosphorylation of JAK2 and STAT5b when compared with isolated administration of insulin or leptin. Thus, there is a positive cross-talk between insulin and leptin signaling pathways at the level of JAK2 and STAT5b in rat liver.

KEY WORDS

Insulin/*physiology / Leptin/*pharmacology / Liver/metabolism /Protein-Tyrosine Kinase/metabolism / DNA-Binding Proteins/metabolism / Signal Transduction/drug effects.

1. Introduction

Leptin, the protein encoded by the *ob* gene, is a hormone that is produced by adipocytes and acts through distinct receptors in target organs to control food intake and energy metabolism (Tartaglia *et al.*, 1995; Zhang *et al.*, 1994). Insulin resistance characterizes states of severe leptin deficiency or resistance, such as *ob/ob* or *db/db* mice, or genetic models of lipoatrophic diabetes. In some of these, administration of exogenous leptin improves glucose tolerance and insulin sensitivity independently of effects on food intake, probably by affecting neuroendocrine pathways that modulate insulin action in the liver and muscle (Halaas *et al.*, 1995; Shimomura *et al.*, 1999), inasmuch this cytokine might also have direct effects on hepatic cells (Lee *et al.*, 2001). Furthermore, recent evidences indicate that leptin treatment in insulin deficient diabetic rats restores normoglycemia (Chinookoswong *et al.*, 1999), and the transgenic mice overexpressing leptin shows increased insulin sensitivity (Ogawa *et al.*, 1999). These observations, taken together, suggest a potent antidiabetogenic effect of leptin *in vivo*.

Direct cross-talk between the leptin and insulin signaling systems remain unclear. Some data suggest that leptin can impair the early steps of insulin signaling including tyrosine phosphorylation of IRS-1 in hepatocytes (Cohen *et al.*, 1996). Other studies demonstrate that leptin can mimic effects of insulin such as stimulation of glucose transport and glycogen synthesis in C2C12 myotubes and that these effects may be mediated by stimulation of PI 3-kinase (Berti *et al.*, 1997; Kellerer *et al.*, 1997). In isolated muscle or adipocytes, short-term incubation with leptin does not stimulate glucose transport or lipogenesis (Ranganathan *et al.*, 1998; Zierath *et al.*, 1998).

Leptin exerts its effects through specific receptors of which five isoforms have been described, arising through alternative splicing of the primary transcript (Lee *et al.*, 1996). The leptin receptor is a member of the cytokine I receptor family of which gp 130 is a prototype. This class of receptors stimulates gene transcription via activation of cytosolic STAT proteins (Darnell 1996; Darnell 1997). The long form of the leptin receptor (OBRb) has the capacity to activate the JAK/STAT (Baumann *et al.*, 1996; Ghilardi and Skoda 1997; Ghilardi *et al.*, 1996; Tartaglia 1997; Vaisse *et al.*, 1996; White *et al.*, 1997) and MAPK (Bjorbaek *et al.*, 1997) pathways, stimulate tyrosine phosphorylation of IRS-1 (Bjorbaek *et al.*, 1997), and increase transcription of *fos*, *jun* (Bjorbaek *et al.*, 1997; Murakami *et al.*, 1997). OBRb is highly expressed in the hypothalamus, the primary site where leptin is thought to act (Tartaglia *et al.*, 1995). Leptin has been shown to activate STAT3 in the hypothalamus via JAK2 (Carvalheira *et al.*, 2001). Tyrosine-phosphorylated STAT is translocated to the nucleus where it is thought to bind to specific DNA sequences and activate genes important for energy homeostasis. Leptin may also exert direct effects at the level of gene expression or cellular function on nonhypothalamic target tissues including hematopoietic cells, T cells, the endocrine pancreas, the pituitary, the ovary, adipocytes, skeletal muscle, and hepatocytes either through the short or long forms of the receptor (Bennett *et al.*, 1996; Cusin *et al.*, 1998; Emilsson *et al.*, 1997; Liu *et al.*, 1998; Lord *et al.*, 1998; Shimabukuro *et al.*, 1997; Siegrist-Kaiser *et al.*, 1997; Yu *et al.*, 1997).

Therefore, it is not completely resolved whether, and by what mechanisms, a component of the important metabolic effects of leptin could be exerted directly at the level of peripheral tissues, as opposed to indirectly though the central nervous system. In the present study we have characterized leptin signal transduction in liver and determined whether insulin and leptin share common intracellular signal transduction pathways.

3. Results

3.1. In vivo effect of leptin on tyrosine phosphorylation of JAK/STAT pathway in liver

To determine leptin-induced tyrosine phosphorylation of JAK2 we performed immunoprecipitation and Western blotting of liver extracts with anti JAK2 and anti-phosphotyrosine antibodies, respectively. The time course experiments were performed injecting 10^{-6}M of leptin through the portal vein of rats and collecting liver specimens in different time points. As shown in Fig. 1 JAK2 is strongly phosphorylated after 30 seconds of leptin treatment, with maximal phosphorylation occurring at 90 seconds and decaying after 3 minutes (Fig. 1A).

To assess the ability of leptin to activate tyrosine phosphorylation of STAT3 and –5b we determined the time course for this activation. Initially, we measured leptin-induced tyrosine phosphorylation of STAT3 by immunoblotting with specific phospho-STAT3 antibodies. Fig. 1B shows that there was a moderate increase in leptin-stimulated STAT3 phosphorylation in rat liver which was maximal at 3 min. Co-immunoprecipitation between JAK2 and STAT3 in liver was observed in a similar fashion of JAK2 tyrosine phosphorylation. Subsequently we measured leptin-induced tyrosine phosphorylation of STAT5b, by immunoprecipitation experiments using specific STAT5b antisera and then the immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. There was a time dependent increase in STAT5b phosphorylation after leptin injection, which paralleled the increase of JAK2 phosphorylation after leptin stimulation (Fig. 1C). Similar to STAT3, there was also Co-immunoprecipitation between STAT5b and JAK2 after leptin stimulation. As expected, there was no alteration in STAT5b protein expression after leptin infusion.

Our results show a very rapid, and presumably direct effect of leptin to phosphorylate STAT5b in liver. To confirm these data we measured leptin induced STAT5b tyrosine phosphorylation in the liver of fa/fa rat, which has a mutation that decreases the cell-surface expression and leptin binding affinity to leptin receptor as a negative control (da Silva et al., 1998; White et al., 1997). In in vivo experiments, there was an increase of 6.9-fold in leptin induced tyrosine phosphorylation of STAT5b in the liver of lean rats (Figure 1, D), compared with increases of 2.4-fold in the liver of obese rats ($P < 0.05$), representing decreases of ~70% in STAT5b phosphorylation in obese rats.

3.2. Effects of Leptin on IRS-1 and IRS-2 Tyrosine Phosphorylation, PI 3 kinase docking and Akt serine phosphorylation in rat liver.

IRS proteins represent an important multifunctional interface between many receptors and intracellular pathways. A possible pathway towards the IRS proteins phosphorylation is via JAK kinases (Berti et al., 1997; Bjorbaek et al., 1997; Carvalheira et al., 2001; Kellerer et al., 1997). Since JAK2 was activated by leptin, we evaluated the ability of leptin to stimulate JAK2/IRS-1 and JAK2/IRS-2 associations. Co-immunoprecipitation between JAK2 and IRS-1 or IRS-2 in liver were detected. In immunoprecipitates of JAK2 that were blotted with anti-IRS-1 or anti IRS-2 antibodies, there was an evident association between these proteins after leptin stimulation. These results demonstrate that JAK2 interacts with IRS-1 and IRS-2 developing stable complexes in rat liver (Fig 2A).

Figure 2B shows a clear increase in leptin-stimulated IRS-1 phosphorylation, which was maximal at 90 sec and almost vanished after 3 minutes. To better define the levels of IRS-2 phosphorylation, we performed Western blot analysis of tyrosyl-phosphorylated

proteins in anti-IRS-2 immunoprecipitates before and after stimulation with leptin. Analogously, Figure 2C shows that there was a marked increase in leptin-stimulated IRS-2 phosphorylation in the rat liver, which was maximal at 90 sec followed by a striking decay.

Previous studies (Backer et al., 1992; Folli et al., 1992) have suggested that there is relatively stable, high affinity interaction between IRS-1 or IRS-2 and the 85 kDa subunit of the PI 3-kinase such that both proteins can be co-precipitated by antibodies to either protein. Blots that had been previously immunoprecipitated with antibodies against IRS-1 or IRS-2 were subsequently incubated with antibodies against the 85 kDa subunit of PI 3-kinase (Fig. 2 B and C). There was little PI 3-kinase immunoreactivity in the basal state of control rats. After 30 seconds of stimulation with leptin, there were a significant increases in IRS-1 or IRS-2 – PI 3-kinase associations, which suffered slight increase thereafter and reduced to basal levels after 3 minutes. These observations are consistent with the formation of a stable association between IRS-1 or IRS-2 and PI 3-kinase after leptin stimulation. As predictable, there were no changes in IRS-1 and IRS-2 protein levels.

Using antibodies against serine-phosphorylated Akt, the levels of Akt activation were examined in liver after leptin stimulation. As shown in Figure 2D, Akt phosphorylation in liver was unaltered by leptin injection.

3.3. The effect of simultaneous administration of leptin and insulin on JAK/STAT pathway

At first, we measured the tyrosine phosphorylation of JAK2 in the liver from rats that were stimulated with insulin, leptin or both hormones for 90 seconds. The phosphorylation of JAK2 in liver showed an increase of approximately 3.8-fold with insulin stimulation and of approximately 3.6-fold with leptin. Insulin and leptin together

showed ~8.4-fold increase in JAK2 tyrosine phosphorylation, representing increases of ~230% above the effects of leptin alone (Fig. 3 A). Second, we evaluated insulin and/or leptin stimulated JAK2/STAT3 association and STAT3 tyrosine phosphorylation in the liver from rats that were stimulated with insulin, leptin or both hormones for 3 minutes. Insulin-stimulated STAT3 association with JAK2 showed an increase of ~3.5-fold and STAT3 phosphorylation 2.0-fold over the basal levels, leptin led to ~6.2 and 3.3-fold increase in JAK2/STAT3 association and STAT3 tyrosine phosphorylation, respectively. However, simultaneous stimulation did not display additive effect on JAK2/STAT3 association and STAT3 tyrosine phosphorylation (Fig. 3 B and 3 C). Last, we assessed the association between JAK2 and STAT5b and STAT5b tyrosine phosphorylation in the liver from rats that were stimulated with insulin, leptin or both hormones for 3 minutes. Insulin led to increases of ~2.5 fold in JAK2/STAT5b association and 4.6-fold in tyrosine phosphorylation of STAT5b over the basal levels, whereas leptin induced ~4.6-fold increases in JAK2/STAT5b association and 6.1-fold increases in STAT5b tyrosine phosphorylation. Insulin and leptin together showed ~9.3 increase in JAK2/STAT5b association and ~10-fold increase in STAT5b tyrosine phosphorylation, representing increases of ~200 % and ~170%, respectively, above the effects of leptin alone (Fig. 3 D and 3 E).

3.4. The effect of simultaneous administration of leptin and insulin on PI 3-kinase/Akt pathway

We next examined whether simultaneous stimulation with leptin and insulin could affect the early steps of the insulin signal transduction pathways. We measured the tyrosine phosphorylation of IRS proteins in the liver from rats that were stimulated with insulin,

leptin or both hormones for 90 seconds. The phosphorylation of IRS-1 in liver showed an increase approximately 9.0-fold with insulin stimulation and approximately 3.3-fold with leptin. The effect of insulin and leptin was similar to insulin alone (Fig. 4 A). IRS-2 tyrosine phosphorylation upon insulin and/or leptin stimulation was comparable with that obtained in IRS-1 immunoprecipitates (Fig. 4 B). Akt serine phosphorylation was examined in the liver from rats that were stimulated with insulin, leptin or both hormones for 5 minutes. Leptin alone had no effect on Akt tyrosine phosphorylation in liver. Insulin induced ~4- fold increase in Akt phosphorylation and no additive effect was observed with the addition of leptin (Fig 4 C).

3. Discussion

In the present study, we have investigated the possibility of direct interactions between insulin and leptin action in liver, focusing on some key intermediate steps on these signaling pathways. Our results show that leptin, upon stimulation of JAK2 tyrosine phosphorylation, induced JAK2 co-immunoprecipitation with STAT3, STAT5b, IRS-1 and IRS-2. These phenomena parallel the leptin-induced tyrosine phosphorylation of STAT3, STAT5b, IRS-1 and IRS-2. Insulin stimulated a mild increase in tyrosine phosphorylation of JAK2, STAT3 and STAT5b in liver. Leptin was much less effective than insulin at stimulating IRS/PI 3-kinase pathways. Akt phosphorylation were not changed by leptin but increased with insulin. Whereas there was no change in maximal phosphorylation of IRS-1, IRS-2, Akt and STAT3, simultaneous stimulation with both hormones resulted in a marked increase in tyrosine phosphorylation of JAK2 and STAT5b when compared with isolated administration of insulin or leptin. Thus, there is direct cross-talk between the insulin and leptin signaling pathways at the level of JAK2 and STAT5b, such that the simultaneous administration of both hormones modulates the signal that is transduced through the common elements of these pathways.

The molecular mechanisms by which OBR couples to tyrosine phosphorylation events in liver are not known. In the present study the rapid tyrosine phosphorylation and association of JAK2 with IRS-1, IRS-2, STAT-3 and –5b proteins, suggests that a large signaling complex is formed with the OBR upon leptin treatment. Co-immunoprecipitation of JAK2 and IRSs, STAT-3 and –5b could be due the direct association of JAK2, IRSs, STAT3 and STAT5b with the OBR, or indirect association of IRSs, STAT3 and –5b with the JAK2 kinase. There are several mechanisms by which JAK2, IRSs and STATs may

associate to OBR. One possibility is that JAK2 initially associates with the OBR and leads to recruitment of IRSs, STAT3 and –5b proteins. A second is that OBR recruit IRSs and/or STAT proteins, which serve as adapter molecules for binding JAK2. A third possibility is that both JAK2, IRSs and STATs associate with the receptor, and upon ligand binding to the receptor, JAK2 phosphorylates the associated IRSs and STAT proteins. Further studies (mutational analysis of the OBR and or experiments in JAK2 deficient cell lines) will be required to assess this issue.

A previous *in vivo* study implicated only STAT3 in hypothalamic leptin signaling, whereas experiments involving transfected cells provide evidence for the activation of other STAT isoforms, depending on the cellular model (Baumann *et al.*, 1996; Ghilardi *et al.*, 1996; Vaisse *et al.*, 1996; White *et al.*, 1997). The nature of the STAT isoforms required for transducing the leptin signal is still controversial and may depend on the cellular context and the concomitant presence of other stimuli *in vivo*. Here we show that not only tyrosine phosphorylation of STAT3, but also STAT5b, is robustly activated in hepatic tissue by leptin administration. These results are in accordance with a recent study showing that leptin administration iv induces STAT5b gel shift in nuclear extracts of hepatocytes (Briscoe *et al.*, 2001). The current study is the first demonstration of leptin-induced STAT5b activation in liver *in vivo*.

Originally, it was thought that the hypothalamus was the only tissue expressing OBRb. However, recent evidence at the level of messenger RNA expression and cellular function suggest that peripheral organs including adipose tissue also express OBRb (Cohen *et al.*, 1996; Emilsson *et al.*, 1997; Siegrist-Kaiser *et al.*, 1997; Yu *et al.*, 1997). The short isoform of the leptin receptor (OBRa), unlike OBRb, appears incapable of activating

STATs, although it can mediate tyrosine phosphorylation of JAK2 (Bjorbaek *et al.*, 1997). Several studies have analyzed alteration in leptin signaling induced by the *fa* mutation in OBR, demonstrating a defective activation of STAT5b by the mutant receptor (White *et al.*, 1997). Thus, our data showing leptin-induced STAT5b phosphorylation in liver of lean rats, but a mild STAT5b phosphorylation in liver of Zucker *fa/fa* rats support the notion that hepatocytes express OBRb as previous suggested (Kim *et al.*, 2000).

Intracellular interactions between different signaling systems may function as mechanisms for enhancing or counter-regulating hormone action. In the case of insulin, the cross-talk with leptin-mediated pathways resulted in direct interactions between insulin and leptin signaling systems at the level of JAK2 and STAT5b. Simultaneous stimulation with both hormones led to increased tyrosine phosphorylation of JAK2 and STAT5b and the association of JAK2 with STAT5b. In contrast, no effect on STAT3 phosphorylation or JAK2/STAT3 association was observed when compared with acute insulin or leptin administration. These results suggest that the positive cross-talk between insulin and leptin signaling was due to additive effects on JAK2 activation and divergence of association between JAK2 and STAT3 and -5b. Another possible reason for this difference is differential insulin/leptin signal amplification. STAT5b can be activated either by insulin through the insulin receptor in a JAK-independent fashion (Sawka-Verhelle *et al.*, 1997; Sawka-Verhelle *et al.*, 2000) or with OBRb by leptin (Briscoe *et al.*, 2001). In contrast the role of insulin in the activation of the STAT3 is controversial (Carvalheira *et al.*, 2001; Ceresa and Pessin 1996; Kim *et al.*, 2000) and may be tissue specific. Here we show a mild activation of STAT3, but this effect was not additive with leptin.

Although leptin has an insulin-sensitizing effect, which is evident from the rapid reduction of glucose and insulin levels in leptin-deficient, insulin-resistant ob/ob mice after leptin administration (Pelleymounter *et al.,* 1995) and the enhanced insulin-stimulated glucose disposal in normal rats infused with leptin (Sivitz *et al.,* 1997), in the present study we find that leptin and insulin elicit overlapping but distinct signaling pathways towards PI 3-kinase/AKT pathway. Notably, iv injection of leptin results in phosphorylation of JAK2 and a mild phosphorylation of IRS-1 and IRS-2 with a parallel increase in IRSs/PI 3-kinase association compared with the large effects of insulin on these signaling steps. In liver, Akt have enhanced serine phosphorylation after insulin, but not after leptin stimulation.

The effect of leptin stimulation on IRS-1 and IRS-2 tyrosine phosphorylation and its effects on insulin-induced signaling have a broad range of variation that is dependent of the cell line utilized. Cohen *et al.* (1996) reported that leptin caused attenuation of several insulin-induced activities, including tyrosine phosphorylation of the IRS-1 on human hepatic cells. On the other hand Wang *et al* (1997) showed that leptin treatment increase IRS-1 and IRS-2 tyrosine phosphorylation on hepatoma cells. Furthermore Kellerer et al (1997) demonstrated that leptin have insulin like effects on glucose transport and glycogen synthesis in C_2C_{12} myotubes and these effects were dependent on IRS-2 tyrosine phosphorylation. In accordance with Kim et al (2000) here we show that in the liver the treatment with the combination of the two hormones is not additive, synergistic or inhibitory in the PI 3-Kinase-Akt pathway.

In conclusion, we have provided evidence for rapid direct effects of leptin administration *in vivo* on intracellular signaling pathways in the liver and we observe a convergence of leptin and insulin signaling at the level of PI 3-Kinase-Akt pathway without

synergism. Moreover, our results indicate a direct and positive cross-talk between insulin and leptin at the level of JAK2 and STAT5b tyrosine phosphorylation and association. This mechanism may serve to potentiate the activity of both insulin and leptin pathways and to increase stimulation in physiological processes such as the regulation of carbohydrate and lipid metabolism, that are under the combined control of insulin and leptin.

2. Materials and methods

2.1. Materials

The reagents for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonylfluoride, aprotinin, dithiothreitol, Triton X-100, Tween-20, glycerol, affinity-purified rabbit anti-mouse IgG and bovine serum albumin (BSA, fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO). Leptin was acquired from Calbiochem (La Jolla, CA). Sodium amobarbital and human recombinant insulin (Humulin R) were purchased from Eli Lilly & Co. (Indianapolis, IN). Protein A-Sepharose 6Mb, [^{125}I]Protein A and Nitrocellulose paper (Hybond ECL, 0.45 μm) were obtained from Amersham (Piscataway, N). Antibodies to IRS-1 (SC-559), IRS-2 (SC-8299), JAK2 (SC-294G), STAT3 (SC-483), STAT5b (SC-835) and phosphotyrosine (SC-508) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against the p85 subunit of PI 3-kinase (06-195) were from Upstate Biotechnology (Lake Placid, NY). Anti-Akt-Ser-473 (9271L) and anti-STAT3 phosphotyrosine 705-specific antibodies (9131) were from New England Biolabs (Beverly, MA).

2.2. Animals

Eight-week-old male Wistar and Zucker rats were allowed access standard rodent chow and water ad libitum. All experiments involving animals were in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA). Room temperature was maintained between 21-23°, and a 12-hour light, 12-hour-dark cycle was used.

2.3. Surgical procedures and tissue preparation

After 7 hours fast, rats were anesthetized with sodium amobarbital (15 mg/kg body weight, intraperitoneally), and submitted to the surgical procedure as soon as the anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein was exposed, and in vivo stimulation of the liver was obtained by injection of 500 µl of normal saline (0,9% NaCl), insulin (10^{-6}M), leptin (10^{-6} M) or an equimolar mixture of insulin (10^{-6} M) and leptin (10^{-6} M) into the portal vein. Fragments of the livers were excised in a time dependent manner. The tissue was minced coarsely, and homogenized immediately in extraction buffer [1% Triton-X 100, and 100 mM Tris (pH 7.4) containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonylfluoride, and 0.1 mg aprotinin/ml] at 4° C with a Polytron PTA 20S generator (model PT 10/35, Brinkmann Instruments, Inc., Westbury, NY) operated at maximum speed for 30 sec The extracts were centrifuged at 30,000 X g and 4 C in a Beckman Coulter, Inc. 70.1 Ti rotor (Palo Alto, CA) for 20 min to remove insoluble material, and the supernatant of these tissues was used for immunoprecipitation with the indicated antibodies.

2.4. Protein analysis by immunoblotting

The precipitated proteins were treated with Laemmli sample buffer (Laemmli 1970) containing 100 mM dithiothreitol and heated in a boiling water bath for 4 min, after which they were subjected to SDS-PAGE in a Bio-Rad miniature slab gel apparatus (Mini-Protean, Bio-Rad Laboratories, Inc., Richmond, CA). Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described (Towbin et al., 1979) except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high molecular mass proteins.

Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter for 2 h at room temperature at 4° C in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween-20). The nitrocellulose blot was incubated with the indicated antibodies; diluted in blocking buffer (0.3% BSA instead of nonfat dry milk) overnight at 4° C; and then washed for 20 min with blocking buffer without milk. An amplification step with rabbit anti-mouse IgG (1:1000 final dilution) was added to the standard protocol when monoclonal anti-phosphotyrosine antibodies were employed in Western blotting experiments. The blots were subsequently incubated with 2 mCi [^{125}I]Protein A (30 mCi/mg) in 10 ml blocking buffer for 2 h at room temperature and then washed again for 20 min as described above. [^{125}I]Protein A bound to the antipeptide antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak Co., Rochester, NY) with Cronex Lightning Plus intensifying screens at -80°C for 12–48 h. Band intensities were quantitated by optical densitometry (Scion Image software) of the developed autoradiographs. To exclude unspecific interactions between the investigated proteins and protein-A sepharose control-precipitations without the specific antibodies were performed, followed by immunoblotting with anti-JAK2, anti IRS-1, anti-IRS-2, antiSTAT3 and anti-STAT5b and no band was detected. The results indicated that the immunoprecipitation performed in the experiments described herein do not allow the detection of unspecific interactions.

2.5. Statistical analysis

Where appropriate, the results were expressed as the mean ± S.E.M. accompanied by the indicated number of experiments. Kruskal-Wallis 1-way ANOVA test was used in the statistical comparisons with $p < 0.05$ indicating significance.

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Figure Legends

Fig. 1. Effect of Leptin on JAK2, STAT3 and -5b tyrosine phosphorylation and STAT3 and –5b association with JAK2 in the liver *in vivo*. (A) Liver extracts from animals treated with leptin for the time points indicated, were prepared as described in Methods. Tissue extracts were immunoprecipitated with anti-JAK2 antibody (IP, immunoprecipitation) and immunoblotted with anti-phosphotyrosine antibody (P-Tyr). Stripped membranes were reblotted with anti-JAK2 an0tibody (n = 6). (B) Leptin-stimulated tyrosine phosphorylation of STAT3. Tissue extracts from rats treated with leptin for the time points indicated were immunoprecipitated with anti-STAT3 and blotted with anti-phospho-specific STAT3 (P-Tyr-STAT3). The same membranes used for STAT3 tyrosine phosphorylation were stripped and reblotted with anti-JAK2 and anti STAT3 antibodies. (n = 6). (C) Leptin-stimulated tyrosine phosphorylation of STAT5b. Tissue extracts from rats treated with leptin for the time points indicated were immunoprecipitated with anti-STAT5b and blotted with anti-phosphotyrosine antibody. The same membranes used for STAT5 b tyrosine phosphorylation were stripped and reblotted with anti-JAK2 and anti STAT5b antibodies (n = 6). (D) Liver extracts from lean (L) and fat (F) rats treated with leptin for 90 seconds, were prepared as described in Methods. Tissue extracts were immunoprecipitated with anti-STAT5b and immunoblotted with anti-phosphotyrosine antibody. The bar graphs represent the quantitative tyrosine phosphorylation of STAT5b. Data (mean ± S.E.M. n = 4) are expressed as relative to control, assigning a value of 100% to the lean control before leptin stimulation. $^*P < 0.01$, leptin lean vs. leptin obese.

Fig. 2. Leptin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 and their association with p85 subunit of PI 3-kinase in liver *in vivo*. (A) Liver extracts from animals treated

with leptin for the time points indicated, were prepared as described in Methods. Tissue extracts were immunoprecipitated with anti-JAK2 antibody (IP, immunoprecipitation) and immunoblotted with anti-IRS-1 and anti IRS-2. Stripped membranes were reblotted with anti-JAK2 antibody (n = 4). (B) Leptin-stimulated tyrosine phosphorylation of IRS-1. Tissue extracts from rats treated with leptin for the time points indicated were immunoprecipitated with anti-IRS-1 and blotted with anti-phosphotyrosine antibody (P-Tyr). The same membranes used for IRS-1tyrosine phosphorylation were stripped and reblotted with anti-PI 3-kinase and anti IRS-1 antibodies (n = 6). (C) Leptin-stimulated tyrosine phosphorylation of IRS-2. Tissue extracts from rats treated with leptin for the time points indicated were immunoprecipitated with anti-IRS-2 and blotted with anti-phosphotyrosine antibody. The same membranes used for IRS-2 tyrosine phosphorylation were stripped and reblotted with anti-PI 3-kinase and anti IRS-2 antibodies (n = 6). (D) Leptin-stimulated serine phosphorylation of Akt. Liver extracts from rats that were stimulated with leptin for the time point indicated were lysed and the proteins were separated by SDS-PAGE on 12% gels and blotted with phosphoserine-specific Akt antibodies.

Fig. 3. Effect of insulin and leptin on JAK/STAT pathway in extracts of livers from rats treated with leptin, insulin or a combination of the two. (A) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-JAK2 and blotted with anti-phosphotyrosine antibody. The bar graph shows the quantitative phosphorylation of JAK2. Data (mean ± S.E.M. n = 6) are expressed as relative to control, assigning a value of 100% to the control mean. (B) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-STAT3 and blotted with anti-JAK2 antibody. The bar graph shows the quantitative association of

STAT3 with JAK2. Data (mean ± S.E.M. n = 6) are expressed as relative to control, assigning a value of 100% to the control mean. (C) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated (IP, immunoprecipitation) with anti-STAT3 and blotted with anti-phospho-specific STAT3 (P-Tyr-STAT3). The bar graph shows the quantitative phosphorylation of STAT3. Data (mean ± S.E.M. n = 6) are expressed as relative to control, assigning a value of 100% to the control mean. (D) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-STAT5b and blotted with anti-JAK2 antibody. The bar graph shows the quantitative association of STAT5b with JAK2. Data (mean ± S.E.M. n = 6) are expressed as relative to control, assigning a value of 100% to the control mean. (E) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-STAT5b and blotted with anti-phosphotyrosine antibody (P-Tyr). The bar graph shows the quantitative phosphorylation of STAT5b. Data (mean ± S.E.M. n = 6) are expressed as relative to control, assigning a value of 100% to the control mean. * P<0.05 vs. leptin-treated rats

Fig. 4. Effect of insulin and leptin on IRS-1, IRS-2 and Akt phosphorylation in extracts of livers from rats treated with leptin, insulin or a combination of the two (A) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-IRS-1 and blotted with anti-phosphotyrosine antibody. The bar graph shows the quantitative phosphorylation of IRS-1. Data (mean ± S.E.M. n = 6) are expressed as relative to control, assigning a value of 100% to the control mean. (B) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-IRS-2 and blotted with anti-phosphotyrosine antibody. The bar graph shows the quantitative phosphorylation of IRS-2. Data (mean ± S.E.M. n = 6) are expressed as relative

to control, assigning a value of 100% to the control mean. (C) Western blots of livers' extracts from rats treated with leptin for 5 minutes with phosphoserine-specific Akt antibodies. The bar graph shows the quantitative phosphorylation of Akt. Data (mean ± S.E.M. $n = 6$) are expressed as relative to control, assigning a value of 100% to the control mean.

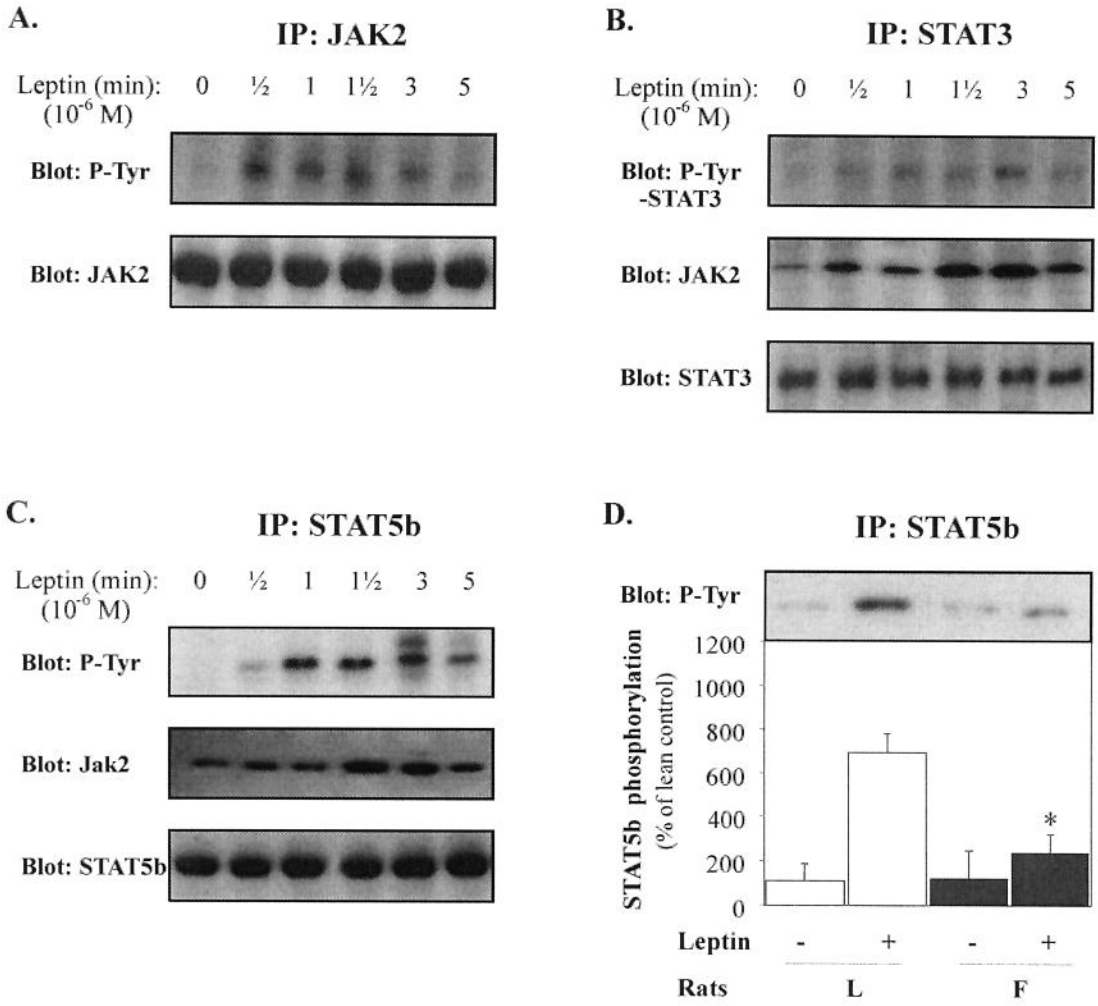


FIGURE 1 - Effect of Leptin on JAK2, STAT3 and -5b tyrosine phosphorylation and STAT3 and 5b association with JAK2 in the liver *in vivo*.

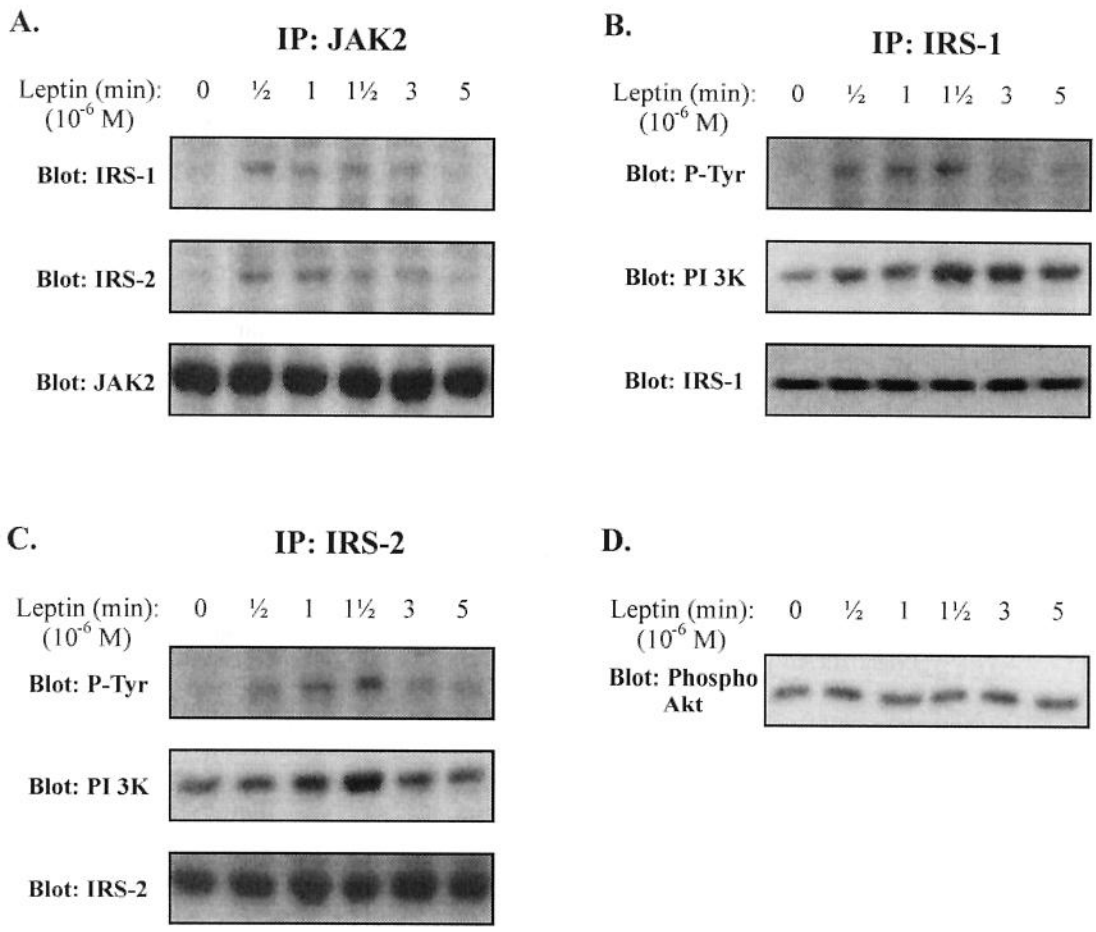


FIGURE 2 - Leptin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 and their association with p85 subunit of PI 3-kinase in liver *in vivo*.

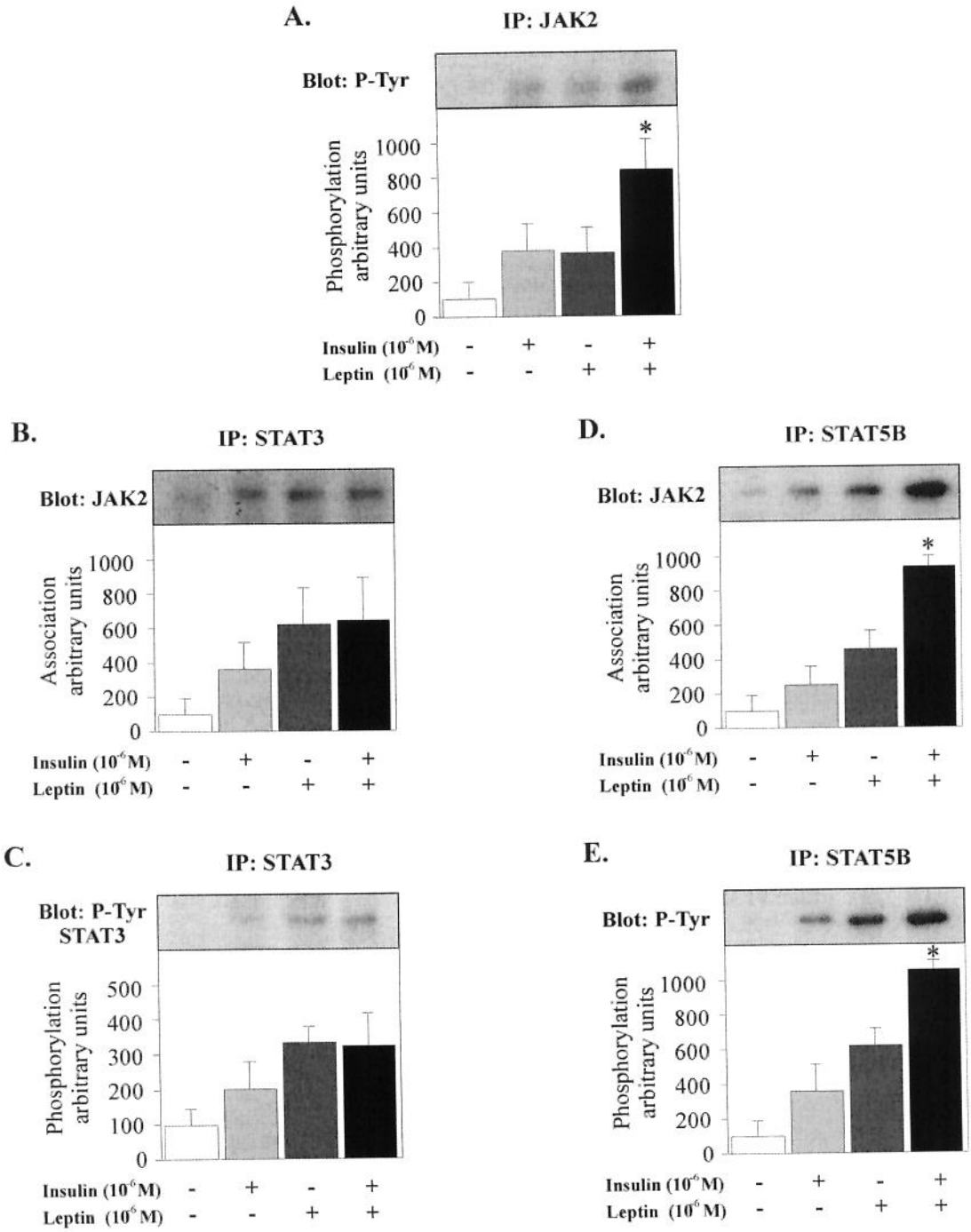


FIGURE 3 - Effect of insulin and leptin on JAK/STAT pathway in extracts of livers from rats treated with leptin, insulin or a combination of the two.

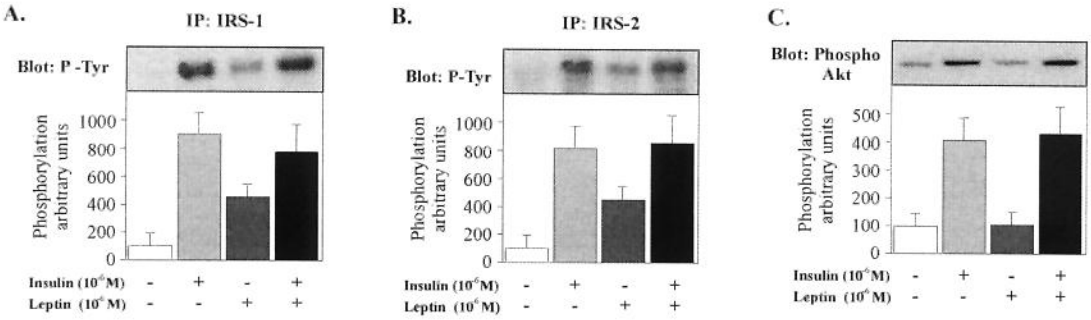
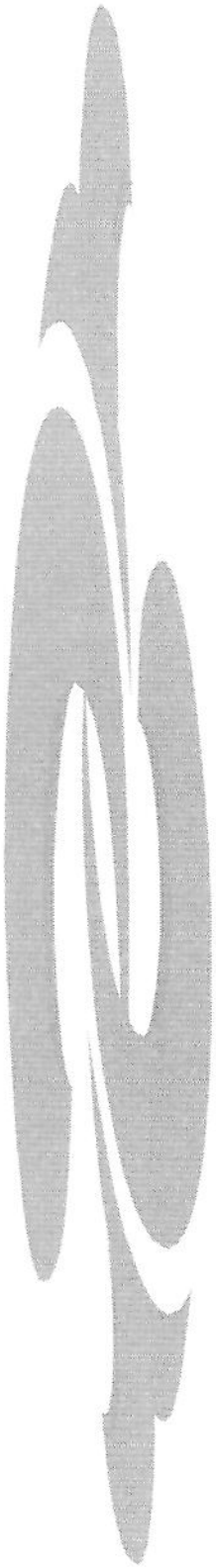


FIGURE 4 - Effect of insulin and leptin on IRS-1, IRS-2 and Akt phosphorylation in extracts of livers from rats treated with leptin, insulin or a combination of the



5. DISCUSSÃO

1. Transmissão do Sinal de Insulina e Leptina em Hipotálamo de Ratos: Inter-relação e Implicações Fisiopatológicas (Trabalhos 1 e 2)

Na obesidade e no diabetes tipo 2, os tecidos estão freqüentemente expostos a altas concentrações de insulina e leptina (CONSIDINE *et al.*, 1996; REAVEN *et al.*, 1993). O receptor de insulina (IR) e algumas formas do receptor de leptina (OBR) estão presentes em áreas similares do sistema nervoso central (SNC) e na maioria dos tecidos corporais, levantando a possibilidade de que esses dois sistemas de sinalização possam interagir e modificar a ação isolada de cada um desses hormônios nessas morbidades.

A inter-relação entre as diversas vias de sinalização é um elemento chave na determinação da resposta celular. Como a insulina e a leptina desempenham funções ora similares (redução da ingestão alimentar, aumento do gasto energético, diminuição da produção hepática de glicose) ora antagônicas (secreção de insulina) e atuam em várias proteínas intracelulares de sinalização semelhantes, neste estudo nós caracterizamos a transmissão do sinal de leptina e interação com insulina nos principais órgãos alvos desses hormônios.

Inicialmente, caracterizamos sistematicamente as vias de sinalização da insulina e leptina em hipotálamo de ratos através da avaliação da capacidade da insulina e leptina de ativarem suas proteínas de sinalização no hipotálamo; em seguida descrevemos a inter-relação entre os dois hormônios nesse tecido e finalmente examinamos o comportamento da transmissão do sinal de insulina no hipotálamo de ratos Zucker (modelo animal de resistência à insulina).

No hipotálamo, rapidamente após estímulo intracerebroventricular (icv) com insulina, a subunidade β do IR sofre autofosforilação. Como conseqüência, ocorre um aumento na fosforilação do IRS-1 e IRS-2 e na associação desses substratos com a PI 3-quinase. Além disso, observamos aumento na fosforilação da JAK2 e ativação da Akt e ERK1/2 após a administração de insulina. Estes dados demonstram um efeito direto da insulina no hipotálamo, com o envolvimento das proteínas classicamente associadas à transmissão do sinal da insulina em tecidos como hepático, muscular e adiposo (SAAD *et al.*, 1994; SAAD *et al.*, 1993; WHITE & YENUSH, 1998).

Durante os últimos dez anos o papel da insulina na regulação do peso corporal vem sendo enfatizado. As evidências disponíveis indicam que a insulina não é sintetizada em cérebro de ratos adultos em quantidades significativas (DEVASKAR *et al.*, 1994), mas é derivada da insulina circulante, a qual entra no SNC através de processos especializados de transporte (BAURA *et al.*, 1993). Acredita-se que no SNC a insulina afete a ingestão de alimentos alterando a expressão de NPY no hipotálamo (SCHWARTZ *et al.*, 1992). A síntese e liberação de NPY apresentam-se aumentadas em condições metabólicas associadas com baixos níveis de insulina, incluindo o jejum e diabetes tipo 1. O NPY é sintetizado no núcleo arqueado do hipotálamo e transportado para o núcleo paraventricular, onde sua liberação é um potente estímulo para a alimentação (SCHWARTZ *et al.*, 1992).

Recentemente, as vias de sinalização da insulina em hipotálamo de ratos foram relacionadas à coordenação da regulação da reprodução e homeostase energética. As fêmeas dos camundongos sem IRS-2 têm várias alterações no eixo hipotálamo-hipófise-ovário, além de aumento tanto da ingestão alimentar como da gordura corporal (BURKS *et al.*, 2000). Os camundongos sem IR apenas no SNC (BRUNING *et al.*, 2000) apresentam

obesidade, hiperleptinemia, resistência à insulina e hipertrigliceridemia. As vias de sinalização usadas pela insulina para reduzir a ingestão alimentar não são conhecidas, mas os resultados obtidos nesta pesquisa podem contribuir para a formulação da caracterização inicial destes efeitos.

A homologia do OBR à classe I dos receptores de citoquinas revelou imediatamente importantes pistas para os possíveis mediadores da ativação dos receptores de leptina. A classe I dos receptores de citoquina é conhecida por agir por meio da ativação das proteínas JAK e STAT (KELLERER *et al.*, 1997; VAISSE *et al.*, 1996). Tipicamente as proteínas JAK estão constitutivamente associadas ao receptor, se autofosforilam e fosforilam o receptor após a ligação hormônio-receptor. A fosforilação do receptor leva à formação de um sítio de ligação para as proteínas STAT as quais são ativadas após se ligarem ao receptor. As proteínas STAT ativadas são translocadas para o núcleo e estimulam a transcrição. Nossos resultados mostram que a leptina é capaz de induzir a fosforilação em tirosina da JAK2 no hipotálamo, uma proteína essencial para a transmissão do sinal de leptina, diferentemente dos resultados encontrados por McCowen *et al.* (1998). Entretanto, essa discrepância de resultados provavelmente deveu-se à falha em estimular respostas mensuráveis da fosforilação da proteína JAK2 ou à desfosforilação da JAK2 por uma das várias fosfatases durante o estoque e manipulação do tecido hipotalâmico no estudo de McCowen *et al* (1998).

Os resultados deste trabalho demonstram que a leptina é capaz de fosforilar o OBR, a ERK 1/2 e o STAT3 com conseqüente ativação do último, no entanto não se conseguiu detectar a ativação do STAT1 e STAT5b. Em cultura de células, muitas cascatas de sinalização mostraram-se ativadas após tratamento com leptina, incluindo STAT1

(linhagem de células derivadas de adenocarcinoma renal humano, hepatoma, e células COS expressando intensamente a forma longa do OBR), STAT5 e STAT6 (linhagem de células COS), MAPK (linhagem de células embrionárias de camundongos e células COS expressando HA-ERK1) (BJORBAEK *et al.*, 1997; GHILARDI & SKODA, 1997; ROSENBLUM *et al.*, 1996; TAKAHASHI *et al.*, 1996; WANG *et al.*, 1997), mas a relevância destes resultados no animal intacto *in vivo* é incerta. Tem sido mostrado repetidamente que resultados de sinalização obtidos em culturas de células que freqüentemente têm expressão aumentada do receptor ou de componentes da cascata de sinalização não são reproduzidos *in vivo*. Nossos resultados confirmam as observações de Vaisse *et al.* (1996) e McCowen *et al.* (1998) de que a leptina ativa apenas o STAT3 e a MAPK.

Este estudo demonstra pela primeira vez que a leptina, além de induzir a fosforilação em tirosina da via JAK2/STAT3, pode também mediar a fosforilação em tirosina do IRS-1 e IRS-2 e associação desses substratos com a PI 3-quinase em hipotálamo de ratos *in vivo*. Outros membros da família dos receptores de citoquinas, ao qual OBR pertence, possuem esta função (HELDIN, 1995).

O efeito do estímulo da leptina sobre o IRS-1 e o IRS-2 tem um grande espectro de variação que é dependente da linhagem celular utilizada. Embora, Cohen et al. (1996) tenham reportado que a leptina causasse atenuação de várias atividades induzidas pela insulina, incluindo a fosforilação em tirosina do IRS-1, dois relatos mais recentes (BJORBAEK *et al.*, 1997; WANG *et al.*, 1997) não foram capazes de reproduzir os resultados de Cohen *et al.* (1996) mostrando inclusive a fosforilação do IRS-1 em resposta à leptina. Kellerer *et al.* (1997) descreveram efeitos semelhantes a insulina no transporte de

glicose e síntese de glicogênio em células de miotúbulos de ratos. Esses efeitos foram dependentes da fosforilação do IRS-2. Nossos resultados demonstram que a leptina é capaz de fosforilar as proteínas IRS-1 e IRS-2 no hipotálamo provavelmente via JAK2, uma vez que estudos com receptores da mesma família do OBR, incluindo LIFR/OSMR, são capazes de fosforilar as proteínas IRSs (ARGETSINGER *et al.*, 1996; WHITE & YENUSH, 1998) via JAKs (ARGETSINGER *et al.*, 1996; JOHNSTON *et al.*, 1995).

A PI 3-quinase medeia a ativação de duas quinases (PDK1 que fosforila a treonina 308 da Akt e a PDK2 que fosforila a serina 473) levando ao aumento da atividade da Akt (KOBAYASHI & COHEN, 1999). Apesar de termos detectado a fosforilação do IRS-1 e IRS-2 e a associação destes com a PI 3-quinase, não detectamos fosforilação da Akt após a administração de leptina. Um estudo recente (NISWENDER *et al.*, 2001) mostrou que a ativação da PI 3-quinase está envolvida na redução de apetite induzida pela leptina. Associando este resultado com os nossos podemos sugerir que o efeito da leptina na redução de apetite seja independente da ativação da Akt.

A integração das respostas da insulina e leptina no hipotálamo tem participação fundamental no controle da ingestão alimentar e do peso corporal. Embora a insulina seja secretada pelas células β do pâncreas, suas concentrações circulantes são proporcionais à quantidade de tecido adiposo (POLONSKY *et al.*, 1988). Insulina e leptina entram no SNC via um sistema mediado por receptor, em que o transporte dos hormônios ocorre de forma saturável através das células endoteliais do cérebro (BAURA *et al.*, 1993). Os receptores de insulina e leptina estão localizados nos mesmos núcleos hipotalâmicos (BASKIN *et al.*, 1988). Finalmente, os dois hormônios reduzem o peso corporal de forma dose dependente

quando administrados no SNC, e não provocam sintomas de fraqueza (CHAVEZ *et al.*, 1995; THIELE *et al.*, 1997; WOODS *et al.*, 1996; WOODS *et al.*, 1979).

Os nossos resultados demonstram que a insulina e a leptina utilizam vias distintas, mas que se conectam durante a ativação das vias da MAPK e IRS-PI 3-quinase, e apresentam inter-relação direta e positiva na via JAK/STAT. Notavelmente, a injeção de leptina resultou em fosforilação transitória do IRS-1 e IRS-2 e associação do IRS-1 ou IRS-2 com a PI 3-quinase e não ativou a Akt comparado com os efeitos duradouros da insulina nessa via de sinalização. Em contrapartida, o efeito da insulina na ativação da MAPK só foi observado quinze minutos após o estímulo, enquanto o efeito da leptina foi duradouro e já estava presente dois minutos após a administração do hormônio.Assim, as cinéticas de ativação da insulina e leptina são diferentes, com a leptina induzindo uma ativação mais precoce e permanente da MAPK e transitória da via IRSs/PI 3-quinase comparada à insulina. O efeito da administração simultânea dos hormônios nessas vias de sinalização não foi aditivo, inibitório ou sinérgico.

Surpreendentemente, a inter-relação entre a insulina e leptina em direção à ativação da via JAK/STAT foi singular. Assim, a insulina isoladamente induz a fosforilação da JAK2. A JAK2 ativada fosforila o OBR. O recrutamento desta via leva à associação do OBR ao STAT3, não ocorrendo ativação do STAT3. Por outro lado, a leptina fosforila e ativa a JAK2, que fosforila o OBR e recruta o STAT3 ativando-o. O estímulo simultâneo com insulina e leptina gera um sinal mais potente aumentando a associação OBR/STAT3 e a ligação do STAT3 ao núcleo.

Várias manifestações clínicas observadas em alguns modelos animais e em humanos podem ser atribuídas a uma ruptura da transmissão normal do sinal da insulina e leptina no

hipotálamo. Camundongos que não têm IR no SNC (NIRKO) (BRUNING *et al.*, 2000) apresentam obesidade, apesar dos elevados níveis circulantes de leptina. Isso sugere que a resistência específica à insulina no SNC leva à resistência à leptina. Por outro lado, a falta de leptina no camundongo *ob/ob* leva à obesidade acompanhada de hiperinsulinemia, a qual por si só não é capaz de reverter o fenótipo obeso. Finalmente, em humanos com diabetes do tipo 1, os estados de hipoinsulinemia e hipoleptinemia podem contribuir para a hiperfagia. Assim, resistência à insulina, bem como ausência total de insulina podem impedir algumas das respostas da leptina. Nossos resultados indicam um mecanismo para modulação da via de sinalização da leptina pela insulina. A insulina aumenta rapidamente a fosforilação do OBR, e incrementa a fosforilação do STAT3 mediada por leptina, com um conseqüente aumento na atividade do STAT3. Esse mecanismo pode funcionar normalmente para potencializar a atividade dessas vias, aumentando o estímulo fisiológico em processos como a regulação do peso corporal e da ingestão alimentar. O potencial desta interação como um novo sítio para desenvolvimento de novas drogas no tratamento da resistência à leptina e para aumento de nosso entendimento da sinalização da leptina e das vias das famílias das tirosina quinase, merecem investigação *a posteriori*.

Concluindo, nossos dados demonstram efeitos rápidos e diretos da leptina e insulina em hipotálamo de ratos sugerindo que os mecanismos centrais controladores do apetite estão sob o controle combinado da insulina e leptina e envolvem convergência e sinergismo entre as vias de sinalização desses hormônios.

2. Redução da sinalização da insulina em hipotálamo de ratos Zucker obesos (Trabalho 3)

Para demonstrar que a interdependência entre as vias de sinalização da insulina e leptina é essencial para o adequado controle de peso corporal, investigamos a sinalização da insulina em hipotálamo de ratos Zucker magros e obesos. Os ratos Zucker obesos têm resistência periférica à insulina, dislipidemia e intolerância à glicose, características semelhantes à obesidade relacionada à resistência à insulina e ao diabetes do tipo 2 (BRAY, 1977). Mutações do receptor de leptina são algumas das causas descritas para obesidade em roedores. A mutação *fa* que é responsável pela obesidade nos ratos Zucker é uma mutação do tipo *missense* (269 gln→pro) na porção extracelular do receptor de leptina. Essa mutação diminui a expressão na superfície celular, além de diminuir a afinidade da ligação da leptina ao receptor. Além disso, foi demonstrado que o OBR $^{269\ gln\rightarrow pro}$ tem defeito na sinalização da via JAK/STAT (DA SILVA *et al.*, 1998). Nossos resultados demonstraram que a administração de insulina icv reduziu marcadamente a ingestão alimentar em ratos magros tratados anteriormente com veículo, mas não detectamos o efeito anorético da insulina em ratos anteriormente tratados com um inibidor da PI 3-quinase. Por outro lado, de acordo com um relato prévio, a administração central de insulina falhou em suprimir o apetite de ratos Zucker obesos (*fa/fa*) em contraste com o efeito adequado desse hormônio nos controles magros. Assim, a resistência à ação insulínica no cérebro pode contribuir diretamente para a patogênese da obesidade desses animais. Observamos que a capacidade da insulina em fosforilar IR, IRS-1/2, induzir as associações entre IRSs/PI 3-quinase e fosforilar em serina a Akt apresenta-se significativamente reduzida nos ratos Zucker obesos, embora os níveis de fosforilação no estado basal da Akt fossem significativamente maiores nos animais obesos comparados com os controles magros. Notavelmente, a

fosforilação em tirosina induzida por insulina da ERK1/2 MAPK é semelhante em hipotálamos dos ratos magros e obesos.

A resistência à sinalização da insulina em hipotálamo de ratos Zucker obesos provavelmente acontece no IR e nas etapas iniciais da sinalização. Observamos redução na fosforilação em tirosina do IR e semelhança na quantidade total do receptor em hipotálamo de ratos Zucker obesos comparados ao ratos magros, de forma análoga ao observado nas células vasculares desses animais (JIANG *et al.*, 1999).

Como observado em fígado (ANAI *et al.*, 1998), no hipotálamo de ratos obesos houve uma redução moderada na expressão de IRS-1 e IRS-2. Em paralelo, a fosforilação do IRS-1 e IRS-2 também estavam diminuídas, refletindo a redução dos níveis protéicos assim como uma diminuição da estequiometria dos níveis de fosforilação. Essas reduções foram acompanhadas de decréscimo da associação entre o IRS-1 e IRS-2 com a PI 3-quinase e a fosforilação da Akt. Apesar da fosforilação basal da Akt ser significativamente maior no hipotálamo dos animais obesos, efeito provavelmente decorrente da hiperinsulinemia desses animais, apenas um pequeno incremento da fosforialção pode ser observado após a infusão de insulina.

Existem vários possíveis mecanismos que podem causar a redução da sinalização insulínica em hipotálamo de ratos obesos. Primeiro, aumentos na fosforilação em serina e treonina do IR podem reduzir a atividade quinase do receptor e conseqüentemente a autofosforilação do mesmo. Há evidências de que a ativação da PKC induz fosforilação em serina do IR, a qual pode inibir a sua atividade tirosina quinase e levar à redução da atividade da PI 3-quinase induzida por insulina (CHIN *et al.*, 1993; PILLAY *et al.*, 1996). A ativação das proteinas quinases C-α, -β, -ε, e -δ foi demonstrada em músculos de ratos

Zucker e no rato diabético Goto-Kakizaki (AVIGNON *et al.*, 1996). Segundo, o fator de necrose tumoral -α (TNF –α) é considerado um importante mediador de resistência à insulina em animais obesos (HOTAMISLIGIL *et al.*, 1994). O TNF-α induz fosforilação em serina do IRS-1, resultando em diminuição da atividade quinase do IR e resistência à insulina (HOTAMISLIGIL *et al.*, 1996). Em roedores, anticorpos anti-TNF-α melhoram significativamente a resistência à insulina (VENTRE *et al.*, 1997). Por fim, a alteração da atividade de fosfatases poderiam levar à redução da fosforilação em tirosina do IR e das proteínas IRS (AHMAD *et al.*, 1997; KUSARI *et al.*, 1994; WORM *et al.*, 1996).

A diminuição da sinalização da insulina na via da PI 3-quinase no hipotálamo dos ratos obesos pode ser de relevância fisiopatológica para o desenvolvimento da obesidade. Um estudo recente (NISWENDER *et al.*, 2001) demonstrou que a ativação da via da PI 3-quinase estava envolvida nos efeitos anoréticos da leptina. Em associação com os dados apresentados nesse estudo em situação de resistência à insulina, este resultado pode indicar que as ações antiobesidade da insulina podem estar reduzidas devido a uma inibição parcial da via da PI 3-quinase. Se o mecanismo usado pela insulina para reduzir o apetite seja dependente da PI 3-quinase como nossos dados sugerem, o defeito na ativação da PI 3-quinase nos neurônios hipotalâmicos podem reduzir a capacidade da insulina em promover a redução do peso corporal nos ratos Zucker obeso.

A resistência à insulina na via de sinalização da PI 3-quinase contrasta marcadamente com a capacidade da insulina em estimular a via da MAPK em hipotálamo de ratos obesos. A hiperinsulinemia aumenta a fosforilação da ERK1/2 de forma semelhante nos controles magros e nos ratos Zucker obesos, isto é, a resistência à insulina não afeta a via de sinalização da MAPK no hipotálamo dos animais obesos. A resistência à

insulina seletiva observada é semelhante àquela observada nos vasos desses animais (JIANG *et al.*, 1999). Duas possíveis razões para essa diferença são vias de sinalização alternativas e amplificação diferenciada da transmissão do sinal. Em consideração à última hipótese, a via da MAPK também pode ser ativada através da interação entre o Grb2/Sos com a proteína Shc. Uma vez que a fosforilação do IRS-1 e IRS-2 estão bastante diminuídas nos ratos obesos, é possível que a ativação da via da MAPK pela insulina in vivo ocorra principalmente através da proteína Shc. Algumas evidências de nosso laboratório e de outros amparam esta hipótese (PAEZ-ESPINOSA *et al.*, 1999; SASAOKA *et al.*, 1994).

A manutenção do estímulo da MAPK na presença de resistência à sinalização insulínica na via da PI 3-quinase pode ser importante no desenvolvimento ou manutenção da resistência à insulina. As ERKs podem fosforilar o IRS-1 em serina (DE FEA & ROTH, 1997) e como visto acima a fosforilação do IRS-1 e do próprio receptor nestes resíduos têm sido relacionados à dessensibilização da via de sinalização da insulina (DUNAIF *et al.*, 1995). A atividade da ERK mantida em situação que a foforilação do IRS-1 já está diminuída pode levar à piora da resistência à insulina. Assim, ratos obesos podem ter níveis inapropriadamente elevados de atividade da MAPK no hipotálamo.

Em resumo, nossos dados demonstram que a insulina pode ativar tanto a PI 3-quinase como a via da MAPK no hipotálamo e que a capacidade da insulina em reduzir a ingestão alimentar é dependente da via da PI 3-quinase. Em estado de resistência à insulina a via da PI 3-quinase parece estar seletivamente prejudicada, quando comparada a via da MAPK. Esses resultados amparam a hipótese que a insulina pode ter suas ações antiobesidade mediadas pelas vias da PI 3-quinase e que a diminuição da sinalização na via

da PI 3-quinase no hipotálamo pode levar ao desenvolvimento da obesidade em situações de resistência à insulina.

3. A Leptina Modula a Sensibilidade à Insulina em Tecido Hepático (Trabalho 4)

Inicialmente, achava-se que o hipotálamo era o único tecido a expressar a forma longa do receptor de leptina. Entretanto, evidências posteriores demonstraram que vários tecidos periféricos também expressam a forma longa do receptor de leptina, como o fígado e o pâncreas (COHEN *et al.*, 1996; EMILSSON *et al.*, 1997; SIEGRIST-KAISER *et al.*, 1997; YU *et al.*, 1997). Assim, a etapa seguinte deste estudo constituiu-se na avaliação da possibilidade de interações diretas entre as vias de sinalização de insulina e leptina em fígado de ratos.

Nossos resultados no tecido hepático demonstram que a leptina após estimular a fosforilação em tirosina da JAK2, induz a co-imunoprecipitação da JAK2 com o STAT3, STAT5b, IRS-1 e IRS-2. Este fenômeno acontece em paralelo à fosforilação do STAT3, STAT5b, IRS-1 e IRS-2. A insulina induz um discreto aumento na fosforilação em tirosina da JAK2, STAT3 e STAT5b no fígado. A leptina é bem menos efetiva que a insulina em estimular a via IRS/PI 3-quinase. A fosforilação da Akt não modifica com leptina, mas aumenta significativamente após estímulo com insulina. O estímulo simultâneo com insulina e leptina não induziu modificações na fosforilação máxima do IRS-1, IRS-2, Akt, e STAT3, mas resultou em aumento expressivo da fosforilação da JAK2 e do STAT5b quando comparado com a administração isolada de insulina ou leptina. Assim, existe uma

inter-relação direta entre as vias de sinalização da insulina e leptina na ativação da JAK2 e do STAT5b, de forma que a administração simultânea de ambos os hormônios modula o sinal que é enviado através de elementos comuns dessas vias.

O mecanismo molecular pelo qual o OBR causa a fosforilação em tirosina no fígado é desconhecido. Neste estudo, a fosforilação e associação da JAK2 com o IRS-1, IRS-2, STAT3 e STAT5b, sugerem que seja formado um grande complexo de sinalização em torno do OBR após o tratamento com leptina. A co-imunoprecipitação da JAK2 com o IRS-1, IRS-2, STAT3 e STAT5b pode ser decorrente da associação direta da JAK2, IRSs, STAT3 e STAT5b com o OBR, ou a uma associação indireta entre a JAK2 e as proteínas IRS-1, IRS-2, STAT3 e STAT5b. Existem vários mecanismos que podem explicar como a JAK2, IRSs e STATs podem se associar ao OBR. Uma possibilidade é que a JAK2 inicialmente se associe ao OBR e leve ao recrutamento dos IRSs, STAT3 e STAT5b. Uma segunda possibilidade é que o OBR recrute os IRSs e/ou STATs, que sirvam de moléculas adaptadoras para a ligação da JAK2. Finalmente, a JAK2, IRSs e STATs podem estar constitutivamente associadas ao OBR, e após a ligação da leptina, a JAK2 seria ativado e induziria a fosforilação dos IRSs e STATs.

Anteriormente, havíamos constatado que no hipotálamo o STAT3 é a única molécula da família STAT que é ativada após estímulo com leptina. Entretanto, experimentos envolvendo células transfectadas demonstraram a ativação de outras isoformas da família STAT dependendo do modelo celular utilizado (BAUMANN *et al.*, 1996; GHILARDI *et al.*, 1996; VAISSE *et al.*, 1996; WHITE *et al.*, 1997). Assim, as isoformas necessárias para a adequada sinalização da leptina ainda são motivo de controvérsia e podem depender do contexto celular e da presença concomitante de outros

estímulos *in vivo*. Nesse estudo, demonstramos não apenas a fosforilação do STAT3, mas também que o STAT5b é fortemente ativado no tecido hepático após estímulo com leptina. Estes resultados estão de acordo com um estudo recente que mostra que a leptina induz a ativação do STAT5b em hepatócitos (BRISCOE *et al.*, 2001). Esta é a primeira demonstração da ativação do STAT5b *in vivo*. Vários estudos analisaram as alterações da sinalização da leptina induzida pela mutação *fa* do OBR, demonstrando uma ativação defeituosa do STAT5b pelo receptor mutante (WHITE *et al.*, 1997). Assim, nossos dados mostrando que a leptina induz a fosforilação do STAT5b em fígado de ratos controle e uma leve fosforilação no fígado de ratos Zucker obesos, fortalece a hipótese de que hepatócitos expressam a forma longa do receptor como sugerido previamente (KIM *et al.*, 2000)

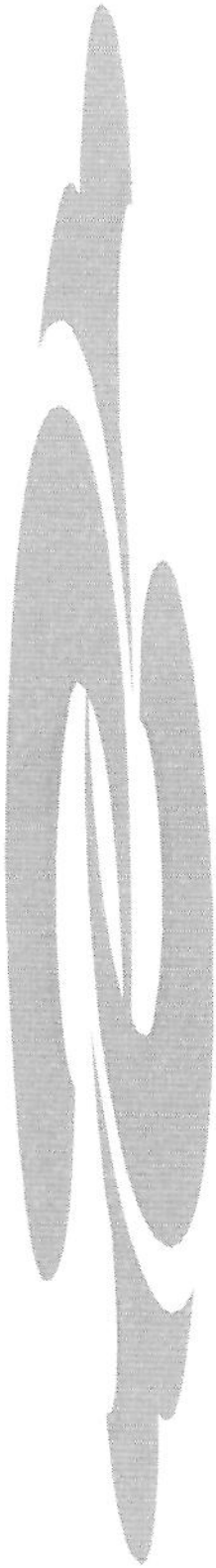
As inter-relações entre as diferentes vias de sinalização servem como mecanismos para aumentar ou contra-regular a ação hormonal. No caso da insulina, a inter-relação com as vias de sinalização da leptina resulta em interações diretas na fosforilação da JAK2 e do STAT5b. O estímulo simultâneo com insulina e leptina aumentou a fosforilação em tirosina da JAK2 e do STAT5b, enquanto não se observaram efeitos na fosforilação do STAT3, quando comparado com a administração isolada de insulina ou leptina. Uma boa razão para essa diferença é a ampliação diferenciada do sinal. O STAT5b pode ser ativado tanto por insulina através do IR de forma independente do JAK2 (SAWKA-VERHELLE *et al.*, 1997; SAWKA-VERHELLE *et al.*, 2000) como pela leptina através do OBR-JAK2 (BRISCOE *et al.*, 2001). Em contraste, o papel da insulina na ativação do STAT3 é controversa (CARVALHEIRA *et al.*, 2001; CERESA & PESSIN, 1996; KIM *et al.*, 2000) e pode ser tecido-específica. Neste estudo detectamos um leve aumento da fosforilação do STAT3, mas esse efeito não foi aditivo com a leptina.

Apesar dos efeitos da leptina em melhorar a sensibilidade à insulina, o que se torna evidente pela rápida redução da glicemia e dos níveis de insulina nos camundongos ob/ob (deficentes em leptina e com resistência à insulina) após administração de leptina (PELLEYMOUNTER *et al.*, 1995) e pelo aumento da captação de glicose em ratos normais após a infusão de leptina (SIVITZ *et al.*, 1997), nesse estudo demonstramos que a insulina e a leptina induzem a ativação de vias de sinalização distintas, mas que se interrelacionam na direção da via PI 3-quinase. Notoriamente, a administração de leptina resultou na fosforilação da JAK2 e em fosforilação discreta do IRS-1 e IRS-2 além de um aumento proporcional da associação desses substratos com a PI 3-quinase, quando comparado com o potente efeito da insulina nessa via de sinalização. No fígado, a Akt teve aumento de sua fosforilação em serina após estímulo com insulina, o que não foi observado após a administração de leptina. O tratamento combinado com os dois hormônios não foi sinérgico nem antagônico.

Em resumo, demonstramos efeitos rápidos e diretos da leptina em fígado de ratos com uma convergência do sinal da insulina e leptina sem sinergismo ou antagonismo na via PI 3-quinase/Akt. Interessantemente, demonstramos uma inter-relação direta e positiva entre insulina e leptina na ativação do STAT5b. Assim, esse mecanismo pode potencializar a atividade de ambos hormônios e aumentar o estímulo fisiológico em processos como a regulação do metabolismo de carboidratos e lipídeos que estão sob o controle combinado da insulina e leptina.

Concluindo, estes estudos caracterizaram as vias de transmissão do sinal de leptina em hipotálamo e fígado, evidenciando mecanismos para a integração da sinalização de

insulina e leptina bem como a modulação destas vias em modelo animal de resistência à insulina e leptina nesses tecidos.



6. SUMMARY

Insulin and leptin have overlapping effects in the control of energy homeostasis and glucose metabolism, but the molecular basis of this synergism is unknown. Insulin signals through a receptor tyrosine kinase that phosphorylates and activates the docking proteins IRSs (insulin receptor substrates), whereas the leptin receptor and its associated protein tyrosine kinase JAK2 (Janus kinase 2) mediate phosphorylation and activation of the transcription factor STAT3 (signal transducer and activator of transcription).

To investigate whether insulin and leptin share common intracellular signal transduction pathways and to determine whether these hormonal signaling systems modulate each other's action in rat hypothalamus male Wistar rats were studied after chronic implantation of an intracerebroventricular (i.c.v.) catheter into the third ventricle. Immunoprecipitation, immunoblotting and EMSA assays were used to examine the activation of insulin and leptin signaling molecules in the rat hypothalamus.

Acute i.c.v. administration of insulin resulted in a time-dependent increase in tyrosine phosphorylation of the insulin receptor (IR), insulin receptor substrate 1 (IRS-1), insulin receptor substrate 2 (IRS-2) and MAPK (mitogen activated proein kinase), PI 3 kinase docking and serine phosphorylation of Akt. The i.c.v. administration of leptin resulted in tyrosine phosphorylation of JAK2, IRS-1, IRS-2 and MAPK and PI 3 kinase docking but no phosphorylation of Akt was observed. Simultaneous stimulation with both hormones did not increment tyrosine phosphorylation of IRS-1, IRS-2, Akt and MAPK when compared with isolated administration of insulin. In addition insulin induced JAK2 tyrosine phosphorylation and leptin receptor phosphorylation, which in the presence of leptin, augmented the interaction between STAT3 and this receptor. Insulin also increased the leptin-induced phosphorylation of STAT3 and its activation.

Leptin rapidly activates classically insulin signaling pathways directly at the level of hypothalamus, and these pathways overlap with, but are distinct from, those engaged by insulin. On the other side, insulin modulates the leptin signal transduction pathway, and may provide a molecular basis for the coordinated effects of insulin and leptin in feeding behavior and weight control.

To demonstrate the interdependence between insulin and leptin signaling in rat hypothalamus in vivo insulin signaling through the phosphatidylinositol 3-kinase (PI 3-kinase), mitogen-activated protein (MAP) kinase were compared in the hypothalamic tissues of lean (*Fa/?*) and obese Zucker *(fa/fa)* rats (animal model of insulin resistance, which has a defect in the leptin receptor). Icv insulin infusion reduced food intake in lean rats but no effect was observed in obese Zucker rats. Pretreatment with PI 3-kinase inhibitors prevents insulin-induced anorexia in lean rats. Insulin-stimulated tyrosine phosphorylation of insulin receptor (IR), IR substrates (IRS-1, IRS-2), the associations of p85 subunit of PI 3-kinase to the IRS proteins and serine phosphorylation of Akt in the hypothalamus of obese rats were significantly decreased compared with the lean rats. In contrast, insulin stimulated tyrosine phosphorylation of MAP kinase was similar in lean and obese rats. This segment of the study provides direct measurements of insulin signaling in hypothalamic tissues, and document selective resistance to insulin signaling in the hypothalamus of obese Zucker rats at the molecular level. These findings have provided support for the hypothesis that insulin may have anti-obesity actions mediated by the PI 3-kinase pathway and that the impaired PI 3-kinase signal pathway in the hypothalamus may lead to the development of obesity in insulin resistance and diabetic patients.

Originally, it was thought that the hypothalamus was the only tissue expressing OBRb. However, recent evidence at the level of messenger RNA expression and cellular

function suggest that peripheral organs including liver also express OBRb. Therefore the second part of the study was designed to investigate the rapid and potentially direct effects of leptin on signal transduction in liver and to determine whether insulin and leptin share common intracellular signal transduction pathways.

Chronic leptin treatment markedly enhances the action of insulin on hepatic glucose production out of proportion to the body weight loss and increased insulin sensitivity. In the present experiments the cross-talk between insulin and leptin was evaluated in rat liver. Leptin, upon stimulation of JAK2 tyrosine phosphorylation, induced JAK2 co-immunoprecipitation with STAT3, STAT5b, IRS-1 and IRS-2. This phenomenon parallels the leptin-induced tyrosine phosphorylation of STAT3, STAT5b, IRS-1 and IRS-2. Acutely injected insulin stimulated a mild increase in tyrosine phosphorylation of JAK2, STAT3 and STAT5b. Leptin was less effective than insulin at stimulating IRSs phosphorylation and their associations with PI 3-kinase. Simultaneous treatment with both hormones promoted no change in maximal phosphorylation of STAT3, IRS-1, IRS-2 and Akt, but led to marked increase in tyrosine phosphorylation of JAK2 and STAT5b when compared with isolated administration of insulin or leptin. Thus, there is a positive cross-talk between insulin and leptin signaling pathways at the level of JAK2 and STAT5b in rat liver.

We have characterized the leptin signaling pathway in hypothalamus and liver and demonstrate various mechanisms for the integration between insulin and leptin in these tissues. We have also showed that the cross-talk between these hormones is tissue dependent and that the effects of this interaction affects the energetic homeostasis and glucose metabolism in the various steps.

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