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CAMPINAS
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Dissertação de Mestrado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas, para obtenção do título de Mestre em Fisiopatologia Médica, área de concentração: Medicina Experimental.

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CAMPINAS
2010

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DA FACULDADE DE CIÊNCIAS MÉDICAS DA UNICAMP

Bibliotecário: Sandra Lúcia Pereira – CRB-8ª / 6044

R582e

Rocha, Guilherme Zweig
Efeito do Paclitaxel na via IRS/P13K/Akt/mTOR em linhagem de adenocarcinoma de mama e pulmão / Guilherme Zweig Rocha. Campinas, SP : [s.n.], 2010.

Orientador: José Barreto Campello Cavalheira
Dissertação (Mestrado) Universidade Estadual de Campinas. Faculdade de Ciências Médicas.

1. Paclitaxel. I. Cavalheira, José Barreto Campello. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III. Título.

Título em inglês : Paclitaxel effect on the IRS/P13-kinase/Akt/mTOR pathway in breast cancer adenocarcinoma and lung cancer carcinoma

Keywords: • Paclitaxel

Titulação: Mestre em Fisiopatologia Médica
Área de concentração: Medicina Experimental

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Data da defesa: 27-08-2010

Banca examinadora de Dissertação de Mestrado

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Data: 27/08/2010

DEDICATÓRIA

Aos meus pais, Artur e Glacy, a toda a minha Família e à minha querida Marília por me darem apoio para a realização deste trabalho.

AGRADECIMENTOS

Agradeço inicialmente às primeiras pessoas que me apresentaram ao universo da pesquisa e foram responsáveis pela minha formação.

Ao meu amor, Marília, sempre presente nos momentos bons e ruins, sempre melhorando a minha vida. É a grande responsável por minha felicidade. Sem você, nada teria acontecido!

Ao professor e meu orientador José Barreto Campello Carvalheira, que me recebeu em seu laboratório, me deu a oportunidade de realizar este trabalho, e, principalmente, por ter confiado em mim.

Aos meus amigos, de todos os lugares por onde passei. Carlos Koga, Rafael Camargo, Diogo Siqueira, Fernando Campos, vocês me mostraram a real amizade. Aos amigos do IB, em quem sempre me espelhei para tentar ser melhor. Vocês têm participação decisiva em todos os trabalhos por mim realizados.

Ao grupo de pesquisa, onde algumas pessoas realmente se destacaram por terem me ensinado algo além do laboratório.

Ao Eduardo, que me ajudou muito, seja de maneira profissional ou então tornando a convivência mais harmoniosa.

Ao Felipe, pela grande inteligência, amizade, humildade, paciência, senso de humor e por entender o que é trabalhar em equipe.

Ao Gerson, que ao longo dos anos tornou-se um grande amigo, e por ser um profissional exemplar.

Ao Bruno e ao Tiago, por terem contribuído com enriquecimento cultural e por terem dado apoio.

À Dioze e ao Alexandre, pelas dificuldades partilhadas e, acima de tudo, pela amizade que ficou.

Ao Rodrigo, à Carol e ao Carlos, por terem auxiliado de diversas maneiras, mostrando o que é trabalho de equipe.

Aos outros membros do laboratório, pela convivência diária.

Ao Prof. Mario Saad, que permitiu a utilização de seu laboratório e pelo conhecimento transmitido.

Aos meus pais, Artur e Glacy, que me deram suporte psicológico, pela dedicação, paciência e incentivo, mostrando que tudo é possível e belo desde que você olhe pelo lado bom. Com eles aprendi, mais do que tudo, a ter caráter. Vocês são meus grandes exemplos.

À FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), pela bolsa de estudo concedida.

SUMÁRIO

PÁG.

6-MP	6-mercaptopurina
AMP	Monofosfato de adenosina
AMPK	Proteína quinase dependente de AMP
AKT/PKB	Proteína quinase B
ATP	Trifosfato de Adenosina
CDK	Cyclin Dependent Kinase (Proteína quinase dependente de ciclina)
DNA	Ácido desoxirribonucleico
DTT	Ditiotreitol
EDTA	Ácido etilenodiaminotetracético
EGFR	*Epidermal Growth Factor Receptor* (Receptor do Fator de Crescimento Epidérmico)
ERK	*Extra-celular Regulated Kinase* (Proteína Quinase regulada por sinal extracelular)
GTP	Guanosina trifosfato
IKK	*Inhibitor of IκB Kinase* (Complexo de quinases que ativam o NFκB)
IκB	Inbidor do NFκB no citoplasma
IRS-1	Substrato 1 do receptor de insulina
JNK	Quinase da c-jun
Ki-67	Anticorpo mouse monoclonal – clone MIB-1
LKB1	*Liver Kinase B1* (Proteína Quinase B1 do fígado)
MOPP	Regime quimioterápico composto por: vincristina, procarbazine e predinisona
mTOR	*Mammalian Target of Rapamycin*
NFκB	Fator de transcrição *nuclear factor kappa B*
PDK	*Phosphatidilinositol Dependent Kinase* (Proteína Quinase Dependente de fosfatidilinositol)

PDGFRβ	Platelet Derived Growth Factor Receptor β (Receptor β do fator de crescimento derivado de plaquetas)
PI	fosfatidilinositol
PIP	fosfatidilinositol fosfato
PIP3	fosfatidilinositol3,4,5-trifosfato
PI3K	proteína fosfatidilinositol 3-quinase
PMSF	Fluoreto de fenilmetil sulfonila
POMP	Regime quimioterápico composto por: metotrexato, vincristina, 6-MP e predinisona
PTEN	*Phosphatase and Tensin Homolog* (Fosfatase e homólogo tensina)
p85	Subunidade da PI3-quinase
Raf	Proteína da cascata de ativação da MAP quinase
RNA	Ácido ribonucléico
SCID	*Severe combined immunodeficiency* (Imunodeficiência severa combinada)
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
Tris	Tri (hidroximetil)-aminometano
TUNEL	Terminal deoxynucleotidyl Transferase Biotin–d UTP Nick End Labeling

RESUMO

A elucidação das vias de crescimento celular e a observação de que essas vias estão alteradas no câncer humano incentivou a procura de inibidores específicos. Proteínas adaptadoras, que se ligam a múltiplos elementos de uma cascata de sinalização e coordenam a sinalização celular, bem como quinases intracelulares podem ser candidatos ideais a alvos para o bloqueio da sinalização celular. Nesse sentido, a via de sinalização IRS/PI3K/Akt/mTOR e a AMPK aparecem como alvos para o bloqueio de crescimento e indução de apoptose de células tumorais. Por outro lado, a descoberta dos taxanos, ésteres de alcalóides complexos, compostos por um sistema de anéis e com diversas ramificações laterais que são essenciais para a ação única contra os microtúbulos, é provavelmente a mais importante adição ao arsenal quimioterápico do final do século vinte. Entretanto, os efeitos da associação entre taxanos e moduladores da atividade da AMPK e de bloqueadores da mTOR em linhagens de câncer de mama e de pulmão são apenas parcialmente conhecidos. Assim, o objetivo do projeto foi avaliar o efeito do tratamento concomitante entre taxanos e moduladores da atividade da AMPK em diferentes linhagens de câncer de mama e de pulmão. As células de adenocarcinoma de mama (MCF-7) e de carcinoma de pulmão (A549) foram tratadas com metformina, ativador da AMPK, e com paclitaxel; e animais SCID foram inoculados com células de câncer de pulmão A549 e tratados com metformina ou paclitaxel ou então uma combinação das duas drogas. Os resultados obtidos demonstram que AMPK é ativada por metformina e que mTOR, p70S6K e 4EBP-1 são inibidas pelo tratamento com metformina de maneira dependente do tempo e da dose a que foram submetidos nas células MCF-7 e A549. Além disso, observamos que o tratamento com paclitaxel ativou não apenas a AMPK, mas também p53 e sestrina 2, e inibiu as proteínas mTOR, p70S6K e 4EBP-1 de maneira dependente do tempo e da

dose nas duas linhagens de células utilizadas. Assim, na associação de 2-DG com paclitaxel e de metformina com paclitaxel, verificamos que as proteína ativadas pela associação de drogas eram p53, sestrina 2 e AMPK, enquanto mTOR, p70S6K e 4EBP-1 encontravam-se inibidas nas células MCF-7 e A549. Também observamos que o tratamento de metformina com paclitaxel resulta em aumento no número de células com parada na fase G2/M do ciclo celular e diminui o crescimento tumoral em animais com diminuição da proliferação e aumento da apoptose, em relação aos tratamentos isolados e ao grupo controle. Assim, podemos sugerir que a associação de um ativador da AMPK, que leva a uma diminuição da atividade de vias de crescimento, proliferação e diferenciação celular pode ser uma alternativa mais eficiente que o tratamento com paclitaxel isolado.

ABSTRACT

Metformin is a widely-used antidiabetic drug whose anti-cancer effects, mediated by the activation of AMPK and reduction of mTOR signaling, have become noteworthy. Chemotherapy produces genotoxic stress and induces p53 activity, which can cross-talk with AMPK/mTOR pathway. Herein, we investigate whether the combination of metformin and paclitaxel has an effect in cancer cell lines. Human tumors were xenografted into SCID mice and the cancer cell lines were treated only with paclitaxel or metformin, or a combination of both drugs. Western Blotting, flow cytometry and immunohistochemistry were then used to characterize the effects of the different treatments. The results presented herein, demonstrate that the addition of metformin to paclitaxel leads to quantitative potentialization of molecular signaling through AMPK and a subsequent potent inhibition of the mTOR signaling pathway. Treatment with metformin and paclitaxel resulted in an increase in the number of cells arrested in the G2/M phase of the cell cycle, decreased tumor growth and increased apoptosis in tumor-bearing mice, when compared to individual drug treatments. We have provided evidence for a convergence of metformin and paclitaxel induced signaling at the level of AMPK. This mechanism illustrates how different drugs may cooperate to augment anti-growth signals, and suggests that target activation of AMPK by metformin may be a compelling ally in cancer treatment.

INTRODUÇÃO

Em 2005, o câncer foi responsável por 13% de todas as mortes ocorridas no mundo. Os principais tipos de câncer com maior mortalidade foram: de pulmão (1,3 milhão); de estômago (cerca de 1 milhão); de fígado (662 mil); de cólon (655 mil); e de mama (502 mil). Do total de óbitos por câncer ocorridos em 2005, mais de 70% ocorreram em países de média ou baixa renda (World Health Organization (WHO), 2006). Estima-se que, em 2020, o número de casos novos anuais seja da ordem de 15 milhões, sendo que cerca de 60% desses novos casos ocorrerão em países em desenvolvimento. Sabe-se ainda que pelo menos um terço dos casos novos de câncer que ocorrem anualmente no mundo poderiam ser prevenidos. Ferlay e colaboradores (Ferlay et al 2010) viram que, no ano de 2008, o número de casos novos de câncer em todo o mundo foi de aproximadamente 13 milhões. Os tumores de pulmão (1,61 milhão de novos casos), de mama (1,31 de milhão novos casos) e de cólon e reto (1,23 milhão de novos casos) foram os tipos de câncer mais freqüentes no mundo.

No Brasil, as estimativas para o ano de 2008, válidas também para o ano de 2009, apontam que ocorreram 466.730 casos novos de câncer. Os tipos mais incidentes, à exceção do câncer de pele do tipo não melanoma, foram os cânceres de próstata e de pulmão, no sexo masculino, e os cânceres de mama e de colo do útero, no sexo feminino, acompanhando o mesmo perfil da magnitude observada no mundo (Instituto Nacional do Câncer (INCA) 2007).

Assim, a busca de terapias capazes de controlar a doença é cada vez mais urgente e inspiram a comunidade científica há mais de um século para a descoberta de mecanismos que possam diminuir os índices de crescimento dos casos de incidência de câncer e dos casos de morte por câncer.

Histórico da quimioterapia

Dentre os muitos desafios da medicina, nenhum teve início mais controverso do que o tratamento do câncer. Embora os processos neoplásicos sejam reconhecidos há séculos, pouco se sabia a respeito dos mecanismos biológicos de transformação e progressão tumoral até os adventos da medicina molecular na metade do século vinte (Chabner and Roberts 2005).

O início da era moderna da quimioterapia pode ser vinculado diretamente à descoberta do gás mostarda. Em maio de 1942, Louis Goodman e Alfred Gilman, ambos farmacologistas, convenceram seus colaboradores a tratar um paciente portador de linfoma não-Hodgkin com o gás mostarda (Gilman 1963). Propuseram que este reagente deveria destruir o tumor, baseado em achados da autópsia de soldados que morreram na I Guerra Mundial ao entrarem em contato com o gás mostarda. Estas vítimas tinham uma profunda hipoplasia medular e mielossupressão, razões pelas quais as doses de um reagente similar deveriam causar a regressão de linfomas e leucemias. O tratamento de fato resultou em regressão tumoral, entretanto, em poucas semanas, a doença voltou a progredir, mas o paradigma de que as drogas poderiam ser administradas sistemicamente para induzir a regressão tumoral estava estabelecido (Chabner and Roberts 2005).

Nos estudos seguintes, os mesmos cientistas definiram a ação molecular do componente do gás mostarda, demonstrando a formação de um agente alquilante intermediário, o anel etileimonium, que reagia com os sítios doadores de elétrons nas proteínas e ácidos nucléicos. Os resultados destas pesquisas permitiram que novo princípio fosse estabelecido: os tumores são mais suscetíveis às toxinas do que os tecidos normais. A descoberta de que o reagente formava uma ligação covalente com o

DNA foi feita através de estudos posteriores que demonstraram os sítios específicos de alquilação nas bases purínicas, levando a um "*crosslinking*" de extremidades e induzindo apoptose. Outros agentes alquilantes foram desenvolvidos nos 20 anos que se seguiram. Ciclofosfamida, Clorambucil e outros se tornaram drogas "*standard*" para o tratamento de pacientes com linfomas e leucemias. Infelizmente, Goodman e seus colaboradores notaram nestes primeiros experimentos que os tumores tornavam-se rapidamente resistentes a estas drogas, uma observação que antecipou a experiência clínica com o uso da monoterapia (Chabner and Roberts 2005).

Uma segunda abordagem para o tratamento do câncer teve início logo após a segunda guerra mundial, quando Sydney Farber, um patologista da escola de medicina da Harvard, investigou o efeito do ácido fólico em pacientes com leucemia. Esta vitamina parecia estimular a proliferação de células de leucemia linfoblástica aguda (LLA) quando administrada a crianças com este câncer (Wills, Clutterbuck et al. 1937). Este fato levou a síntese de agentes análogos do ácido fólico, aminopterina e depois o metotrexato, que foram administrados a crianças com LLA. As remissões foram de curta duração, mas o princípio era claro: antifolatos podiam suprimir a proliferação de células malignas e restabelecer o funcionamento normal da medula óssea.

Início da quimioterapia moderna

A partir de 1950, outras drogas antileucêmicas, como o análogo purínico 6-mercaptopurina (6-MP), foram estudadas nos trabalhos de George Hitchings e Gertrude Elion (Hitchings and Elion 1954; Johnson, Armstrong et al. 1963; Skipper, Schabel et al. 1964), que demonstraram que uma pequena mudança em um componente necessário para as células poderia inibir o crescimento das células tumorais através da inibição dos

passos que precediam à síntese de RNA e DNA. Paralelamente foram descobertos os alcalóides da vinca, originalmente descritos como agentes antidiabetes, mas que também eram capazes de bloquear a proliferação das células tumorais (Johnson, Armstrong et al. 1963).

O efeito antitumoral dos alcalóides da vinca decorre da habilidade dessas drogas em inibir a polimerização do microtúbulo e, portanto, a divisão celular. Finalmente, em 1965, a combinação do metotrexato (um antifolato), da vincristina (alcalóide da vinca), da 6-MP (6-mercaptopurina) e da predinisona – conjuntamente referidos como "regime POMP" (Frei, Karon et al. 1965) – provou ser mais efetiva na prevenção da resistência das células tumorais às drogas.

Em 1960, Frank Schabel e Howard Skipper analisaram a cinética do crescimento tumoral e criaram ensaios *in vivo* para quantificação da citotoxidade (Skipper and Griswold 1984). Eles mostraram que diferentes doses da droga anticâncer destruíam uma fração de células do tumor e que, dependendo da droga, a célula deveria ser exposta ao quimioterápico durante um período particular do ciclo celular. Assim, inibidores da síntese de DNA e o metotrexato foram mais efetivos durante a divisão celular, enquanto drogas que danificavam fisicamente o DNA, como os agentes alquilantes, matavam as células em todas as fases do ciclo celular. Estas pesquisas também demonstraram que a citotoxidade é dose-dependente e que a combinação das terapias é eficaz na prevenção da resistência às drogas. Finalmente, Schabel e Skipper foram os primeiros a sugerir que altas doses de quimioterápicos poderiam ser usadas para curar pacientes com tumores refratários.

Assim, ficou claro para os pesquisadores da época que a combinação de drogas era mais eficaz para a destruição das células tumorais do que o tratamento com drogas

isoladas, gerando a busca por agentes que pudessem agir de forma a potencializar os efeitos pró-apoptóticos ou antiproliferativos.

Combinação de drogas em quimioterapia

Clinicamente, a combinação quimioterápica começou a ser usada no tratamento de crianças com LLA. Esta abordagem foi estendida para o tratamento dos linfomas na década de 60 quando o gás mostarda, a vincristina, o procarbazine e a prednisona – regime quimioterápico conhecido como "regime MOPP" – foram utilizados conjuntamente e com êxito no tratamento de linfoma de Hodgkin e não-Hodgkin (Moxley, De Vita et al. 1967; Devita, Serpick et al. 1970).

De acordo com os resultados obtidos em modelos animais, os agentes quimioterápicos são mais efetivos se usados combinados em pacientes com tumores de pequeno volume, de forma que mesmo drogas com atividade mais modesta como o 5-fluorouracil, um inibidor da síntese de DNA, pode diminuir a taxa de recidiva se usada como adjuvante ao tratamento de pacientes com câncer de cólon (Moertel, Fleming et al. 1990). Dados semelhantes foram obtidos no tratamento de tumores de outros sítios como no caso do câncer de mama (Bonadonna, Brusamolino et al. 1976). Em geral, a combinação quimioterápica das drogas provou ser mais eficiente do que a monoterapia tanto no tratamento do câncer metastático quanto no tratamento adjuvante.

Os produtos naturais

Em 1956, C. Gordon Zubrod estabeleceu um amplo programa nos Estados Unidos para a coleta e teste da atividade anticâncer de plantas e algas marinhas. Este

programa resultou na descoberta dos taxanos (em 1964) e das camptotecinas (em 1966), drogas que apresentaram grandes dificuldades para o seu desenvolvimento. O paclitaxel, um dos principais taxanos usados atualmente e cuja promoção de morte celular decorre de seus efeitos em microtúbulos, inicialmente era difícil de ser sintetizado e virtualmente insolúvel, só tendo sido possível seu uso clínico a partir de 1991.

A necessidade de mudança das estratégias

Na década de 80, o progresso no desenvolvimento do tratamento quimioterápico tornou-se mais lento. Os estudos clínicos realizados com as drogas já existentes mostravam apenas ganhos marginais, e, além disso, os modelos animais de leucemia e tumores sólidos, que eram essenciais para o *screening* de drogas na época, não eram bons preditores das respostas clínicas. Em 1985, começou-se a produzir grupos monotônicos de antimetabólitos, alquilantes, antimitóticos e inibidores de topoisomerase. Análogos destas drogas determinaram aumento na eficácia dos tratamentos, gerando entusiasmo para clínicos e pacientes, atentos para novos tipos de agentes.

Em resposta a esse período, os testes de *screening* de drogas passaram a ser feitos com 60 diferentes linhagens de tumores humanos. Componentes químicos de plantas, organismos marinhos e agentes descobertos na natureza tiveram seus extratos testados nessas linhagens tumorais. Houve sucesso em parte deles, a maioria agentes antimitóticos e inibidores da topoisomerase, resultando em aumento do número de agentes anticâncer.

Terapia alvo – Revolução no tratamento quimioterápico

Enquanto as atenções estavam voltadas para a descoberta dos agentes citotóxicos, no final de 1980, conhecimentos sobre a genética e a biologia molecular propiciaram entendimento sobre as vias de sinalização e atividades celulares reguladas, tais como proliferação e sobrevivência. Muitas destas vias foram encontradas radicalmente alteradas em células de câncer. Pesquisas para reparar esses defeitos moleculares em células tumorais deram início à era da terapia alvo, que incluía novos alvos como fatores de crescimento, moléculas de sinalização, proteínas do ciclo celular, moduladores de apoptose e moléculas que promoviam a angiogênese (Hanahan and Weinberg 2000).

Inovações na tecnologia aumentaram o sucesso dos inibidores para alvos específicos. A química combinatória gerou milhares de estruturas para *screening* de inibidores *in vitro*, além disso, as características das drogas anticâncer tornaram-se mais bem delineadas de forma que um agente quimioterápico deveria ser metabolicamente estável, ter meia vida longa em modelo animal e em humanos e ter uma baixa taxa de depuração por enzimas da família do citocromo P450. As moléculas candidatas deveriam ser bem absorvidas por via oral, o que não era uma característica típica dos quimioterápicos descobertos entre 1970 e 1980.

Um dos eventos que marcaram a era da terapia alvo foi o desenvolvimento do mesilato de imatinib (Glivec®). O Imatinib é um inibidor potente da quinase BCR-ABL, que está envolvida na patogênese da leucemia mielóide crônica. O Imatinib também inibe a tirosina quinase c-KIT e o receptor fator de crescimento derivado de plaquetas (PDGFRβ). Estas características do Imatinib permitiram seu uso no tratamento da leucemia mielóide crônica e de tumores do estroma gastrointestinal (Hughes, Kaeda et al. 2003).

Uma segunda classe de drogas são aquelas que inibem o receptor do fator de crescimento epidérmico (EGFR), que apesar de apresentarem uma menor atividade antitumoral que o Imatinib fazem parte do arsenal de quimioterapias. O Geftinib (Iressa®) está em testes no tratamento do câncer de pulmão (Kris, Natale et al. 2003) e o Cetuximab (Erbitux®) indicado na terapia combinada do câncer de cólon (Cunningham, Humblet et al. 2004). Além dessas classes, os inibidores da angiogênese estão em franco desenvolvimento na terapia clínica: o Bevacizumab (Avastin®) atualmente é usado na terapia combinada para câncer de cólon; o SU-11248 (Sutent®) usado no tratamento do câncer renal e o Bayer 43-9006 (Sorafenib ®) usado no tratamento do câncer renal e hepatocarcinoma (Gnarra, Tory et al. 1994).

Há mais de 60 anos, clínicos dependem da classificação histológica dos tumores para ditar as escolhas terapêuticas. A tendência atual é de que o tratamento seja específico, isto é, os pacientes são cada vez mais selecionados para determinado tipo de tratamento baseado nas características moleculares do tumor. Esta abordagem confere vantagens enormes na eficiência e reduzem os custos do tratamento (Roberts and Chabner 2004).

Quimiorresistência

A resistência dos tumores à quimioterapia é um problema clínico comum em cânceres humanos (Fisher 1994). A resistência a quimioterápicos pode já existir antes do início da terapia ou ser adquirida. A efetividade do tratamento quimioterápico nos diferentes tipos de câncer é bastante distinta, sendo a quimiorresistência um dos principais responsáveis. Dessa forma, os diferentes tipos de câncer são subdivididos de

acordo com os padrões das respostas aos tratamentos e sensibilidade tumoral, conforme descrito abaixo:

1- Tumores intrinsecamente sensíveis às drogas. Exemplo: leucemia linfoblástica, linfoma de Hodgkin e câncer testicular.

2- Tumores que respondem inicialmente aos tratamentos, mas que depois se tornam refratários à terapia. Exemplo: câncer de mama, câncer de pequenas células de pulmão e carcinoma de ovário.

3- Tumores intrinsecamente resistentes à maioria dos agentes quimioterápicos. Para este grupo, o número de agentes antineoplásicos ativos é pequeno e as respostas quimioterápicas significantes são afetadas na minoria dos casos. Exemplo: melanoma maligno e câncer de cólon.

A seleção experimental de resistência às drogas por repetidas exposições a um único agente antineoplásico geralmente resulta em resistência cruzada a outros agentes quimioterápicos. Este fenômeno é explicado com base nos transportadores da droga, vias de metabolismo da droga e alvos citotóxicos intracelulares. Assim, agentes antineoplásicos de diferentes classes freqüentemente dividem as mesmas vias metabólicas, sistemas de transporte de efluxo ou sítios de ação citotóxica.

Diminuição do acúmulo de quimioterápicos

A diminuição dos níveis intracelulares de agentes citotóxicos é um dos mecanismos mais comuns de resistência às drogas. Drogas solúveis em água não podem penetrar a bicamada lipídica da membrana celular e requerem mecanismos específicos para entrar na célula. Como exemplo, podemos citar a diminuição do influxo intracelular causado pela alta afinidade da droga ao sistema de transporte (Antony, Kane

et al. 1985), bem como a redução do transporte (Dixon, Lanpher et al. 1994), mecanismos que causam resistência ao metotrexato (Hill, Bailey et al. 1979; Sirotnak, Moccio et al. 1981). Um sistema de transporte deficiente pela membrana tem sido identificado em células resistentes ao gás mostarda (Goldenberg, Vanstone et al. 1970). Drogas apolares podem facilmente atravessar a membrana celular, entretanto a concentração intracelular destas drogas pode ser reduzida com o aumento da atividade do efluxo da droga. O aumento da glicoproteína P (codificada pelo gene MDR1 associado à resistência a múltiplas drogas) constitui um dos principais mecanismos responsáveis pelo efluxo da droga, sendo importante exemplo deste mecanismo de resistência (Endicott and Ling 1989; Gottesman and Pastan 1993). Assim, a resistência associada às antraciclinas, antibióticos e agentes antimicrotúbulos, são freqüentemente associadas ao aumento da expressão de glicoproteína P.

Tolerância celular e reparo aumentado aos danos induzidos pelos quimioterápicos

As células contêm um sistema complexo múltiplo envolvido no reparo de danos causados à membrana celular e ao ácido desoxirribonucléico (DNA). Como estes danos podem decorrer da ação direta ou secundária das drogas citotóxicas, mecanismos de reparo intrínsecos alterados podem influenciar a sensibilidade ao quimioterápico (Perez 1998).

Apesar das drogas anticâncer terem suas citotoxidades mediadas através de uma infinidade de alvos moleculares, elas terminam por afetar as vias de morte celular associadas com a morte celular programada, ou apoptose (Hickman 1996; Zunino, Perego et al. 1997). Os quimioterápicos podem levar a várias respostas celulares alternativas, incluindo atrasos no ciclo celular e ativação de processos de reparo ou

ativação de células suicidas por apoptose. Mutações ou níveis de expressões alterados dos genes que regulam estas respostas alternativas ao estresse induzido pela droga estão freqüentemente associados à quimiorresistência. Por exemplo, a expressão de genes p53, p21 e genes da família bcl-2, por influenciar profundamente a apoptose, modifica a sensibilidade celular ou a resistência às drogas anticâncer.

Alvos intracelulares modificados

O mecanismo de vários agentes antineoplásicos para destruir as células envolve interações entre as drogas e as enzimas intracelulares. Estas interações resultam em alteração ou inibição das funções normalmente exercida pelas enzimas. Mudanças quantitativas ou alternativas nestas enzimas alvos das drogas antineoplásicas podem comprometer a eficácia das drogas. Estas mudanças têm sido demonstradas em várias enzimas associadas com células resistentes às drogas, incluindo as topoisomerases (Vassetzky, Alghisi et al. 1995).

Expressão alterada dos genes

Os mecanismos celulares de resistência às drogas citados anteriormente dependem dos níveis alterados ou da função dos produtos dos genes alvos. Estas alterações podem ser resultado de mudanças que ocorrem em qualquer ponto ao longo da via de expressão gênica e de regulação. Processos moleculares múltiplos estão envolvidos na resistência às drogas, incluindo mutações no DNA, deleção ou amplificação e controles transcricionais dos níveis de RNA. As prevalências destas

mudanças refletem nas instabilidades genéticas e fenotípicas das células de câncer, possíveis pressões mutagênicas de toxinas xenobióticas e exposição às drogas.

Em resumo, as células tumorais têm uma necessidade premente de sobreviver, de forma que qualquer alteração genética que favoreça a sobrevida em condições adversas será selecionada. A conseqüência, certamente, será a sobrevivência de alguns tumores ao mais potente agente terapêutico. Entretanto, o crescimento do conhecimento das vias envolvidas na sobrevivência trouxe a esperança de que o bloqueio de moléculas específicas aumente a sensibilidade das células tumorais aos diferentes quimioterápicos, uma abordagem combinatória que sobrepuje a quimiorresistência (McCormick 2004).

As vias de sinalização intracelular

A complexidade do desenvolvimento e crescimento de organismos pode, em parte, ser atribuído às interações dinâmicas e diversas entre hormônios, fatores de crescimento, contatos entre as células e outros estímulos externos que coordenam o destino de cada célula através de seus receptores de membrana. A explosão da pesquisa em transmissão do sinal intracelular nos últimos 10 anos vem decifrando os mecanismos básicos de sinalização intracelular de um grande número desses receptores de membrana (Gough and Ray 2002). Entretanto, embora um grande progresso tenha sido realizado para identificar e descrever algumas das funções de moléculas intermediárias da sinalização celular, estamos distantes de um entendimento completo de muitas dessas vias de sinalização. O sequenciamento do genoma humano permitiu a identificação de diferentes membros de várias vias de sinalização. Nesse sentido, 11,2% dos genes que podem ter sua função predita estão diretamente envolvidos na transmissão do sinal (Venter, Adams et al. 2001). Este é um número subestimado do

total de genes envolvidos, já que 41,7% dos genes no genoma humano não têm sua função predita e muitos genes são importantes em múltiplas funções, tais como ancoramento celular e participação na matriz extracelular, os quais não foram classificados na categoria de genes envolvidos na sinalização celular (Venter 2000).

Um dos mais interessantes aspectos da transmissão do sinal intracelular descoberto nos últimos cinco anos é a constatação de que muitas vias de sinalização com diversas ações podem interagir em múltiplos níveis – isto é freqüentemente chamado de *cross-talk*. Uma procura pelo termo "*cross-talk*" no Medline mostra que ele aparece pela primeira vez em 1991 e tem sido usado em mais de 3500 artigos desde então (Carvalheira, Calegari et al. 2003; Carvalheira, Ribeiro et al. 2003; Carvalho, Carvalheira et al. 2003). É cada vez mais claro que grande parte das vias de sinalização são intricadas e complexas de forma que, para entender uma única via de sinalização, é necessário que se entenda globalmente a rede de interação entre elas (Arkin 2001; Levchenko 2001).

A elucidação das vias de crescimento celular e a observação de que essas vias estão alteradas no câncer humano levou a procura de inibidores específicos dessas vias (Zwick, Bange et al. 2001). Um dos principais desafios para descoberta de inibidores de sinalização é que vias distintas têm *cross-talk* em múltiplos níveis e, além disso, existe redundância de vias de sinalização usadas com o mesmo objetivo. Embora múltiplos inibidores de sinalização estejam atualmente em desenvolvimento ou já em ensaios clínicos, apenas alguns mostraram eficácia. Uma razão óbvia para isso é que vias distintas apresentam *cross-talk*. Assim, uma via compensatória pode emergir ou ganhar força após o bloqueio de uma via específica. Diferentes vias de sinalização podem controlar ou afetar a mesma função celular, e uma única via de sinalização pode regular diferentes funções celulares. Esta rede de sinalização celular redundante parece refletir

um mecanismo de ajuste fino para as células responderem e se ajustarem a efeitos combinados, a estímulos seqüenciais de muitos sinalizadores extracelulares ou sinais internos e controlar a duração e a intensidade de cada sinal.

Embora a rede de sinalização seja de alta complexidade e redundante, existem obstáculos a essas vias, isto é, existem proteínas onde o sinal converge e que ao serem bloqueadas podem impedir diversos processos celulares ao mesmo tempo, correspondendo às diversas capacidades da célula cancerosa como: auto-suficiência de fatores de crescimento, defeito de apoptose, insensibilidade a sinais que inibem o crescimento e metástase (Cui and Lee 2004).

Proteínas adaptadoras, que se ligam a múltiplos elementos de uma cascata de sinalização e coordenam a sinalização celular, bem como quinases intracelulares podem ser candidatos ideais a alvos para o bloqueio da sinalização celular. Nesse sentido, a via de sinalização IRS/PI3K/Akt/mTOR e a AMPK aparecem como alvos para o bloqueio de crescimento e indução de apoptose de células tumorais.

A via IRS/PI3K/Akt/mTOR

Recentemente foi descrito que a ativação constitutiva das proteínas IRS é um fenômeno comum em vários tumores humanos (Chang, Li et al. 2002) e que os IRSs são também importantes mediadores da angiogênese tumoral em células de câncer de pâncreas (Neid, Datta et al. 2004). Além disso, a inter-relação entre a ativação do IRS-1 com oncoproteínas já foi estabelecida. As oncoproteínas BCR-ABL (Traina, Carvalheira et al. 2003), TRK-T1 (Miranda, Greco et al. 2001) e ETV6-NTRK3 (Lannon, Martin et al. 2004) se ligam ao IRS-1 e estão associados com o aumento da

fosforilação em tirosina do IRS-1. Assim, a ativação das proteínas IRS pode ser um mecanismo mais geral da mediação da transformação tumoral mediada por oncogenes.

Uma das principais moléculas ativada pelas proteínas IRS é a PI 3-quinase, uma importante enzima na regulação da mitogênese, diferenciação celular e transporte de glicose estimulado pela insulina (Folli, Saad et al. 1992; Saad, Folli et al. 1993; Shepherd, Nave et al. 1995). A PI-3 quinase foi originalmente identificada como um dímero composto de uma subunidade catalítica (p110) e uma subunidade regulatória (p85). A ligação dos sítios YMXM e YXXM (onde Y = tirosina, M = metionina e X = qualquer aminoácido) fosforilados das proteínas IRS ao domínio SH2 da subunidade p85 da PI3–quinase ativa o domínio catalítico associado (Backer, Myers et al. 1992). A enzima catalisa a fosforilação dos fosfoinositídeos na posição 3 do anel de inositol produzindo fosfatidilinositol 3 fosfato, fosfatidilinositol 3,4 difosfato e fosfatidilinositol 3,4,5 trifosfato (Lietzke, Bose et al. 2000).

A produção do fosfatidinositol 3,4,5 trifosfato (PIP3) pela PI3-quinase recruta as serinas treoninas quinases PDK1 e Akt para a membrana plasmática, onde a Akt é fosforilada e ativada pela PDK1 (Lawlor and Alessi 2001). A via de sinalização iniciada após a ativação da PI3-quinase também é importante para a prevenção de apoptose, e a Akt destaca-se como uma das principais proteínas alvo da PI3-quinase para esse efeito (Carvalheira, Ribeiro et al. 2003; Thirone, Carvalheira et al. 2004). Esforços para delinear a base molecular dos efeitos antiapoptose da Akt tomaram várias direções (Vivanco and Sawyers 2002) (figura 1). Como uma quinase, a tendência natural da Akt é adicionar um grupamento fosfato em substratos, assim, Bad e caspase 9, proteínas que levam a morte celular, foram os primeiros alvos fosforilados pela Akt descritos (Basu, Totty et al. 2003). Em seguida vieram outros reguladores da morte celular como o IKKα, a família dos fatores de transcrição tipo forkhead, o Mdm2 e YAP (Basu, Totty

et al. 2003). Assim, bloqueando a Akt seria esperada a indução de morte celular em vários níveis (figura 1). Entretanto, se algum desses candidatos pode ser responsabilizado pela a ação antiapoptose da Akt na sobrevida de células cancerosas ainda não é claro.

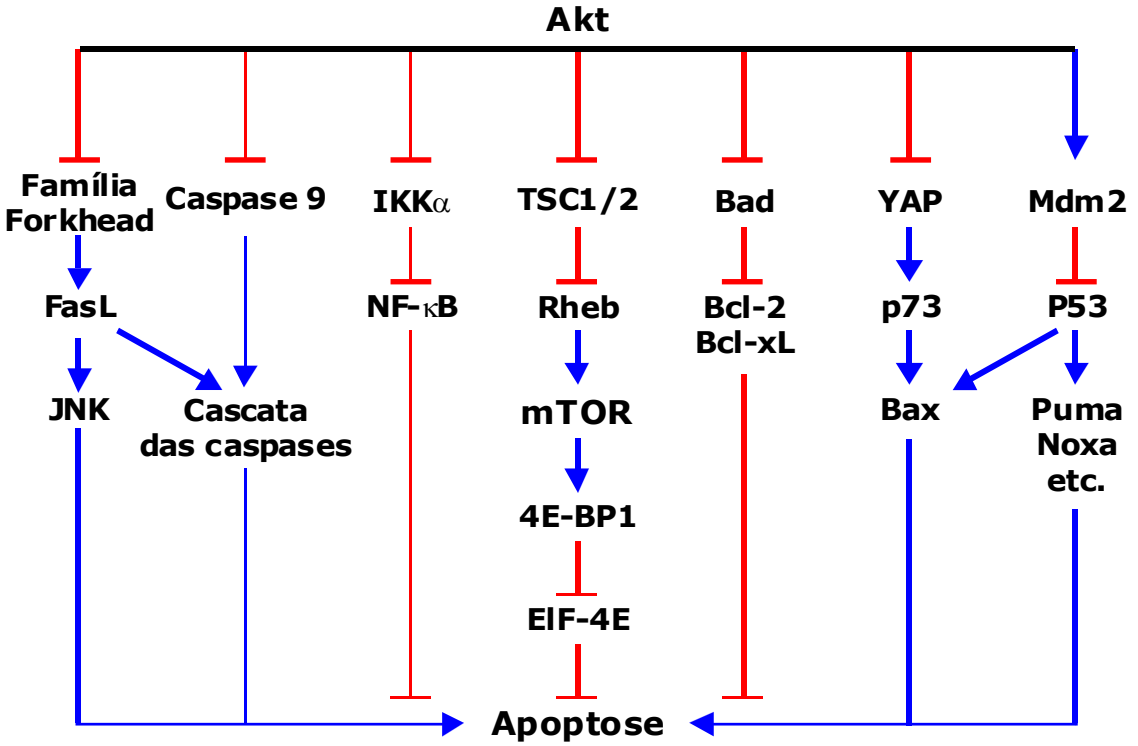

Figura 1. A complexidade da via de antiapoptose emergindo da AKT.

Mais recentemente, a atenção foi direcionada para um diferente ramo da via IRS/PI3K/Akt, na qual figura a proteína mTOR. A mTOR é regulada positivamente pela proteína Rheb, que por sua vez é controlada pelos supressores tumorais TSC1 e TSC2 (Manning and Cantley 2003). A Akt é responsável por fosforilar e inativar a proteína TSC2 e, desta forma, a mTOR pode ser fosforilada pela Rheb. Quando ativada, a mTOR fosforila e inativa a proteína 4E-BP1 (*eIF4E binding protein 1*), liberando o fator traducional eIF4E (*eukaryotic translation initiation factor 4E*) para interagir com outras proteínas, restabelecendo a tradução cap-dependente de RNAm. A mTOR fosforila, ainda, a p70S6K, ativando a maquinaria de síntese protéica da célula

(Sarbassov, Guertin et al. 2005; Tee and Blenis 2005). Wendel e colaboradores (Wendel, De Stanchina et al. 2004), usando um modelo animal de linfoma de células B para explorar as consequências da inibição da Akt, estabeleceram a sinalização da Akt através da mTOR e do eIF4E como um importante mecanismo de oncogênese e resistência à drogas quimioterápicas convencionais.

Atualmente, descobriu-se que a mTOR atua em conjunto com outras proteínas, formando complexos protéicos distintos. O primeiro complexo, sensível à droga rapamicina, e composto pelas proteínas mTOR, GβL e raptor (*regulatory-associated protein of mTOR*). O segundo complexo, insensível à rapamicina, é constituído pelas proteínas mTOR, GβL e rictor (*rapamycin-insensitive companion of mTOR*) e é responsável pela fosforilação do resíduo de serina 473 da Akt, participando da ativação da proteína e permitindo a posterior fosforilação da treonina 308 (Sarbassov, Ali et al. 2005).

Mecanismos de contra-regulação da via IRS/PI3K/Akt/mTOR

Muitos mecanismos podem contribuir para contra-regular a via da PI3–quinase, incluindo as fosfatases e a fosforilação em serina das proteínas IRS pelas proteínas serina quinases, como a c-jun-N-terminal kinase (JNK) (Lee, Giraud et al. 2003; Barreiro, Prattali et al. 2004; Prada, Zecchin et al. 2005), a mTOR e a AMPK.

A via da AMPK

A proteína quinase ativada por AMP (AMPK) é um heterotrímero composto de uma subunidade catalítica α e duas subunidades regulatórias β e γ. Os genes que

codificam as subunidades α, β e γ da AMPK são altamente conservados em todas as espécies eucarióticas que tiveram seus genomas sequenciados, incluindo vertebrados e invertebrados, plantas, fungos e protozoários (Hardie, Scott et al. 2003). O sistema da AMPK parece estar envolvido em resposta a uma variedade de estresses metabólicos que causam distúrbio na homeostasia da energia celular e corporal (Towler and Hardie 2007).

A AMPK é ativada por 5'-AMP de três maneiras distintas, todas antagonizadas por altas concentrações de ATP (Hardie 2004). Primeiramente, a ligação do AMP causa ativação alostérica da AMPK (Hardie 2004), aumentando cerca de cinco vezes a sua atividade. Segundo, a ligação com o AMP torna a AMPK um substrato melhor para a quinase que esta a montante na via, a supressora tumoral LKB1, que ativa a AMPK através da fosforilação da subunidade α no resíduo de treonina 172 (Thr 172) (Hawley, Davison et al. 1996; Hardie and Carling 1997) e aumenta a sua atividade de 50 a 100 vezes. Por fim, a ligação do AMP com a AMPK também inibe a desfosforilação do resíduo Thr 172 por proteínas fosfatases (Davies, Helps et al. 1995).

A AMPK é classicamente ativada por estresses que esgotam o ATP intracelular, como estresse oxidativo, hipóxia e falta de nutrientes que interferem na produção de ATP (Winder and Hardie 1996). Além disso, a AMPK também pode ser ativada pelo uso de certas drogas, como 2-deoxiglucose (2-DG), que esgota o ATP sendo transformado em 2-deoxiglucose-6-fosfato (uma substância não metabolizável), também pode ser ativada pelo ribosídeo de 5-aminoimidazole-4-carboxamide (AICAR), que mimetiza o efeito da hipóxia e também é ativada pela metformina, uma biguanida comumente usada no tratamento de diabetes tipo 2 como agente hipoglicemiante, que ativa LKB1 (Towler and Hardie 2007).

Em geral, a ativação da AMPK diminui a atividade de vias metabólicas biossintéticas e ativa vias catabólicas que geram ATP, como oxidação de ácidos graxos captação de glicose e glicólise. Esse efeito é atingido não apenas por fosforilação direta de enzimas metabólicas, mas também pelo efeito na expressão de genes (Hardie 2004).

Uma via biossintética que tem sua atividade diminuída pela AMPK é a tradução, que pode representar até 20% do gasto energético em células em crescimento e é sensível a diminuição da síntese de ATP (Buttgereit and Brand 1995). A inibição ocorre por duas maneiras distintas: a primeira, com a fosforilação e ativação da quinase do fator 2 de elongação (Horman, Browne et al. 2002; Browne, Finn et al. 2004) que pára a fase de elongação durante a tradução; e a segunda, que inibe a via da mTOR (Bolster, Crozier et al. 2002; Krause, Bertrand et al. 2002; Kimura, Tokunaga et al. 2003), responsável pelo estímulo para a síntese de proteínas e crescimento celular (Figura 2).

A via da mTOR é ativada por fatores de crescimento e aminoácidos e acredita-se que estimula a tradução e o crescimento celular por dois mecanismos: a ativação da proteína ribossomal S6 quinase (S6K1) e o aumento da fosforilação da proteína 1 ligadora do fator de elongação 4E (4EBP1), que estimula o passo inicial da tradução (Carrera 2004).

Estudos recentes sugerem que a inibição da via da mTOR pela AMPK pode ocorrer pela fosforilação do TSC2 (Inoki, Zhu et al. 2003) e que o complexo TSC1-TSC2 regula negativamente o crescimento celular agindo a montante da mTOR para causar esta inibição (Gao, Zhang et al. 2002).

A via de sinalização da insulina é ativada quando há disponibilidade de nutrientes e a via da AMPK é ativada quando as células sofrem com a falta de fontes de carbono (Towler and Hardie 2007). Em mamíferos, a insulina promove a síntese de lipídeos, proteínas e glicogênio e a AMPK inibe estas vias biossintéticas. O efeito da

insulina na síntese de proteínas é mediado em parte pela ativação da via da mTOR via fosforilação de TSC2 (Figura 2). Em contrapartida, a ativação de AMPK causa fosforilação de TSC2 em sítios diferentes dos fosforilados pela Akt e inibem a mTOR (Inoki, Li et al. 2002; Inoki, Zhu et al. 2003). Entretanto, também parece existir uma conexão direta entre estas duas vias. Em alguns tecidos, como o músculo cardíaco, a insulina antagoniza a ativação de AMPK (Gamble and Lopaschuk 1997; Beauloye, Marsin et al. 2001) aparentemente através da ativação de Akt (Kovacic, Soltys et al. 2003). Aparentemente isso ocorre através da fosforilação pela Akt de resíduos de serina 485 ou 491 na subunidade α1 ou α2, respectivamente, da AMPK, que antagoniza a ativação da AMPK via fosforilação de Thr 172 por LKB1 (Horman, Vertommen et al. 2006). Ainda não é estabelecido se este mecanismo também ocorre em outros tecidos (Towler and Hardie 2007).

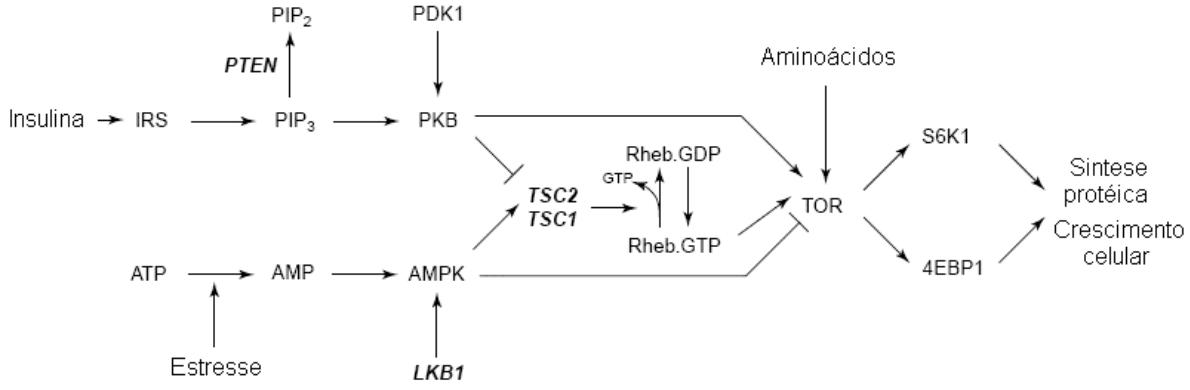

Figura 2: inter-relação entre as vias IRS/PI3-quinase/Akt/mTOR e a via da AMPK

Paclitaxel

Os taxanos são provavelmente os mais importantes reforços ao arsenal quimioterápico no final do século vinte. O paclitaxel foi descoberto como parte de um programa do Instituto Nacional do Câncer dos Estados Unidos, em que extratos de milhares de plantas foram avaliados para atividades anticâncer (Rowinsky and Donehower 1995). Em 1963, um extrato bruto com atividade antitumoral foi isolado a partir da casca do tronco de *Taxus brevifolia*, uma planta de demorado crescimento encontrada no Noroeste dos Estados Unidos, e o paclitaxel foi identificado como o constituinte ativo do extrato por Wall e Wani em 1971 (Wani, Taylor et al. 1971). Os interesses no agente aumentaram em 1979 após a descrição de seu mecanismo de ação em microtúbulos. Hoje em dia, o paclitaxel é obtido através da semi-síntese do produto originado das folhas de *Taxus baccata*, o precursor de paclitaxel e docetaxel, 10-deacetilbaccatin III.

Os taxanos são ésteres de alcalóides complexos, compostos por um sistema de anéis e com diversas ramificações laterais que são essenciais para a ação única contra os microtúbulos (Figura 3) (Lataste, Senilh et al. 1984; Gueritte-Voegelein, Guenard et al. 1991; Rao, Krauss et al. 1994). A mais impressionante atividade clínica dos taxanos ocorreu em pacientes com câncer de ovário e mama (Cortes and Pazdur 1995; Rao, Orr et al. 1995; McGuire, Hoskins et al. 1996; Katsumata 2003; Nowak, Wilcken et al. 2004).

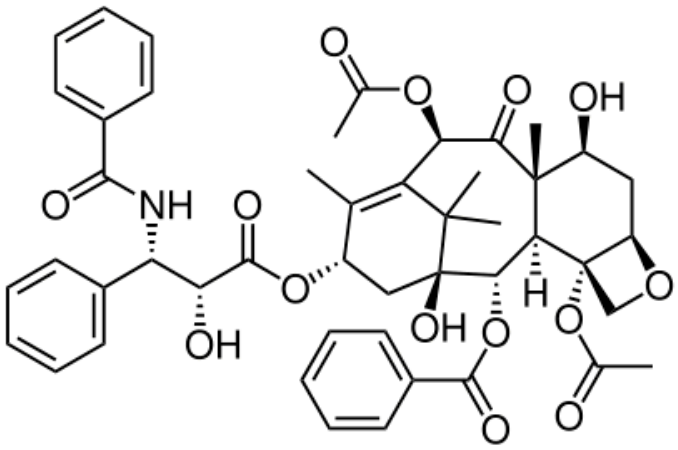

Figura 3: Estrutura química do Paclitaxel

Os taxanos ligam-se fracamente à tubulina solúvel e se ligam diretamente e com grande afinidade à tubulina encontrada ao longo do microtúbulo. O sítio de ligação é diferente do local de troca de GTP, do sítio de ligação da colchicina, da podofilotoxina e dos alcalóides da vinca, e os taxanos não impedem a ligação destes agentes com seus respectivos sítios de ligação. Tanto o paclitaxel quanto o docetaxel ligam-se à superfície interior do lúmen do microtúbulo (Jordan, Hadfield et al. 1998; Nogales, Wolf et al. 1998).

Os alcalóides da vinca e os taxanos, no entanto, parecem produzir efeitos nocivos semelhantes nas fibras do fuso. A ligação dos taxanos ao seu sítio de ligação no interior do microtúbulo estabiliza sua estrutura e aumenta a polimerização possivelmente por induzir uma mudança conformacional na tubulina que, por um mecanismo desconhecido, aumenta sua afinidade com moléculas de tubulina próximas a ela (Jordan and Wilson 2004). Em essência, estas ações alteram profundamente a constante da taxa de dissociação da tubulina nas extremidades do microtúbulo sem afetar a constante da taxa de associação, suprimindo, deste modo, a renovação e a instabilidade dinâmica. Além disso, microtúbulos tratados com taxanos são altamente estáveis, resistindo à despolimerização por frio, íons de cálcio, diluição e agentes

despolimerizantes. Esta estabilidade inibe a reorganização dinâmica da rede de microtúbulos que é essencial para muitas funções vitais da célula na mitose e interfase.

Os taxanos atrasam ou bloqueiam a mitose no limite da metáfase/anáfase semelhantemente aos alcalóides da vinca. Em baixas concentrações (menores que 10 nmol/L), a mitose é bloqueada sem aumento concomitante da massa de microtúbulos. Alterações na organização das fibras do fuso também são semelhantes àquelas induzidas pelos alcalóides da vinca, sugerindo que a parada na mitose deve-se principalmente à perturbações na dinâmica do microtúbulos. Em concentrações maiores, (maior que 100 nmol/L), a massa de microtúbulo é aumentada, a mitose é bloqueada e as fibras do fuso densas, largas e com grandes quantidades de microtúbulos são formadas (Schiff and Horwitz 1980). Similarmente aos alcalóides da vinca, mesmo concentrações subestquiométricas de taxanos, que são suficientes para induzir a parada da mitose sem aumentar a massa de microtúbulos, podem induzir apoptose (Bhalla, Ibrado et al. 1993; Jordan, Wendell et al. 1996; Strobel, Swanson et al. 1996; Hoff, Valero et al. 1998; Moos and Fitzpatrick 1998; Poruchynsky, Wang et al. 1998; Scatena, Stewart et al. 1998; Torres and Horwitz 1998; Dumontet and Sikic 1999; Wang, Liu et al. 1999; Zhang, Yang et al. 1999; Ferlini, Raspaglio et al. 2003).

Apesar de os mecanismos específicos pelos quais o distúrbio dos microtúbulos levam à apoptose não serem identificados, os taxanos interagem com diversas substâncias e moléculas regulatórias. A alteração na formação dos microtúbulos induz o gene supressor tumoral p53 e inibidores de quinase dependentes de ciclina (p21/Waf-1) e modula muitas proteínas quinases (Schulze, Asai et al. 1987; Bhalla, Ibrado et al. 1993; Jordan, Wendell et al. 1996; Strobel, Swanson et al. 1996; Moos and Fitzpatrick 1998; Poruchynsky, Wang et al. 1998; Scatena, Stewart et al. 1998; Torres and Horwitz 1998; Dumontet and Sikic 1999; Wang, Liu et al. 1999; Zhang, Yang et al. 1999;

Ferlini, Raspaglio et al. 2003). Como conseqüência, as células permanecem entre G2/M, podendo sofrer apoptose ou então atravessar esta fase e se dividir (Ganansia-Leymarie, Bischoff et al. 2003). Muitos mecanismos que conectam a parada da mitose induzida pelos taxanos e outros agentes antimicrotúbulos ao evento inicial da via intrínseca da apoptose foram caracterizados. Estes eventos iniciais incluem a ativação das moléculas pró-apoptóticas Bax e Bad e a inativação dos reguladores antiapoptóticos Bcl-2 e BclxL (Blagosklonny, Schulte et al. 1995; Blagosklonny 2001; Konishi, Lehtinen et al. 2002).. Muitas quinases foram envolvidas na fosforilação de Bcl-2 induzida por taxanos e outros agentes antimicrotúbulos, incluindo Jun N-terminal quinase (JNK) e seus efetores pró-apoptóticos Bim, c-Raf, quinase regulada por sinal extracelular (ERK) 1/2 , quinase dependente de ciclina (CDK)-1, proteína quinase A dependente de cAMP e proteína quinase Cα (Blagosklonny 2001; Bhalla 2003). A fosforilação (inativação) dos membros da família de Bcl-2 e a fosforilação de moléculas pró-apoptóticas (ativação) estimulam a via intrínseca da apoptose e suas caspases efetoras (Moos and Fitzpatrick 1998; Ganansia-Leymarie, Bischoff et al. 2003). Apesar do mecanismo preciso pelo qual Bcl-2 é inativado após o tratamento com as drogas não ter sido elucidado, mostrou-se que o paclitaxel se liga ao domínio de Bcl-2, apesar de não parecer que a fosforilação de Bcl-2 seja necessária para induzir apoptose em todos os tipos de câncer (Rodi, Janes et al. 1999). O efeito antimitótico dos taxanos e outros agentes antimicrotúbulos podem estar relacionados a apoptose através de outros eventos regulatórios como a fosforilação da proteína pró-apoptótica Bad pela ativação de CDK1 (Konishi, Lehtinen et al. 2002).

Os taxanos também perturbam microtúbulos na intérfase em células não proliferativas e o paclitaxel também induz fatores de transcrição e enzimas que medeiam a proliferação, a apoptose e a inflamação (Moos and Fitzpatrick 1998; Dumontet and Sikic 1999; Rodi, Janes et al. 1999; Konishi, Lehtinen et al. 2002). Os

taxanos aumentam os efeitos da radiação ionizante *in vitro* em condições atingidas em tratamentos clínicos (menor que 50 nmol/L) e *in vivo* que podem estar relacionadas com a inibição da progressão do ciclo celular na fase G2, fase do ciclo celular mais sensível à radiação (Rowinsky, Donehower et al. 1988; Tishler, Geard et al. 1992; Burkhart, Berman et al. 1994; Fetell, Grossman et al. 1997; Mason, Hunter et al. 1997; Creane, Seymour et al. 1999; Niero, Emiliani et al. 1999).

Os efeitos da interação entre taxanos e diversas moléculas envolvidas na manutenção da integridade do DNA, como o supressor tumoral p53, bem como os efeitos da associação entre taxanos e moduladores da atividade da AMPK são parcialmente conhecidos.

Estresse Genotóxico

O genoma humano é exposto a eventos potencialmente nocivos durante cada ciclo de divisão celular. Esta fonte endógena de danos ao DNA resulta do metabolismo celular ou de erros rotineiros na replicação e recombinação do DNA. Além disso, a exposição celular a agentes genotóxicos como raios X, luz ultravioleta, estresse oxidativo, quimioterápicos que causam dano ao DNA, inibidores da síntese de DNA e agentes que prejudicam os componentes dos microtúbulos do citoesqueleto, originam uma variedade de alterações de nucleotídeos e de quebras nas fitas do DNA (Lindahl 1993). Para contornar os danos sobre o genoma, a célula possui um sistema de resposta que induz parada no ciclo celular para que haja tempo suficiente para reparar os danos sofridos. O sistema de resposta a estresse genotóxico também ativa a via apropriada de reparo ao DNA, ou, no caso de dano irreparável, induz à apoptose (Hoeijmakers 2001) .

Os pontos de checagem do ciclo celular e de dano ao DNA agem de modo a impedir a progressão do ciclo celular frente a um estresse genotóxico endógeno ou exógeno. Muitas proteínas que participam nos pontos de checagem também atuam durante cada divisão do ciclo celular garantindo fidelidade e servem como importantes barreiras à progressão do ciclo celular em condições de estresse até que o dano ao DNA seja reparado. Nesse sentido, a principal proteína responsável por parar o ciclo celular e dar início ao mecanismo de resposta de dano ao DNA é a proteína p53 (Yamaizumi and Sugano 1994).

A proteína p53, o principal supressor tumoral celular, é um fator transcricional ativado por estresse que pode inibir a proliferação celular ou levar à morte celular por apoptose (Levine 1997). As diferentes funções de p53 são mediadas por diversos genes alvo como, por exemplo, por p21, que atua como inibidor do ciclo celular (Levine 1997). Além de impedir a proliferação celular, a p53 também inibe o crescimento celular, uma função importante para prevenir gasto energético com a montagem desnecessária de proteínas e outros componentes celulares durante períodos de estresse (Vousden and Lane 2007). O crescimento celular, por sua vez, é regulado principalmente pela mTOR.

O complexo 1 da mTOR (mTORC1), é o responsável por controlar a síntese protéica e o crescimento celular. Sua atividade é regulada positivamente por fatores de crescimento através da via da IRS/PI3K/Akt, da via Wnt/GSK3 e da ERK/RSK (Wullschleger, Loewith et al. 2006). A atividade de mTORC1 é inibida por condições metabólicas e ambientais adversas como limitação nutricional, hipóxia e dano ao DNA (Corradetti and Guan 2006).

O estresse genotóxico inibe mTOR através de regulação positiva dependente de p53 de seus reguladores negativos PTEN, TSC2 e AMPKβ1 (Feng, Hu et al. 2007). A

p53 também aumenta a fosforilação da subunidade AMPKα, levando a sua ativação (Feng, Zhang et al. 2005). Dois genes que têm sua expressão induzida por dano ao DNA e são alvos de p53 são Sestrin 1 (SESN1) e Sestrin 2 (SESN2). Recentemente foi demonstrado que SESN1 e SESN2 também atuam como reguladores negativos da sinalização da mTOR, atuando através da ativação de AMPK e fosforilação de TSC2 (Budanov and Karin 2008). Assim, as sestrinas 1 e 2 conectam estresse genotóxico, p53 e sinalização da mTOR.

OBJETIVOS

1. Objetivo geral

Investigar os efeitos de taxanos e moduladores de atividade da AMPK em linhagens de células de adenocarcinoma de mama e carcinoma de pulmão

2. Objetivos específicos

· Caracterizar a via de sinalização da mTOR em linhagem de células tumorais de adenocarcinoma de mama (MCF-7)

· Caracterizar a via de sinalização da mTOR em linhagem de células tumorais de carcinoma de pulmão (A549)

· Determinar se a ativação de AMPK modula a via da mTOR em linhagem de células tumorais de adenocarcinoma de mama (MCF-7)

· Determinar se a ativação de AMPK modula a via da mTOR em linhagem de células tumorais de carcinoma de pulmão (A549)

· Investigar os efeitos da administração de Paclitaxel na via da mTOR, na via da AMPK e no estresse genotóxico, através da ativação de p53 e sestrina 2, em linhagem de células tumorais de adenocarcinoma de mama (MCF-7)

· Investigar os efeitos da administração de Paclitaxel na via da mTOR, na via da AMPK e no estresse genotóxico, através da ativação de p53 e sestrina 2, em linhagem de células tumorais de carcinoma de pulmão (A549)

· Investigar os efeitos da administração de Paclitaxel e de ativadores da AMPK na via da mTOR e no estresse genotóxico, através da ativação de p53 e sestrina 2, em linhagem de células tumorais de adenocarcinoma de mama (MCF-7)

· Investigar os efeitos da administração de Paclitaxel e de ativadores da AMPK na via da mTOR e no estresse genotóxico, através da ativação de p53 e sestrina 2, em linhagem de células tumorais de carcinoma de pulmão (A549)

· Avaliar o crescimento tumoral, a proliferação e a morte celular em modelo pré-clínico de câncer de pulmão submetidos ao tratamento combinado de Paclitaxel e ativador da AMPK

CAPÍTULO

METFORMIN AMPLIFIES CHEMOTHERAPY-INDUCED AMPK ACTIVATION AND ANTITUMORAL GROWTH

Running Title: Metformin Sensitizes Cancer Cells to Paclitaxel

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ABSTRACT

Purpose: Metformin is a widely-used antidiabetic drug whose anti-cancer effects, mediated by the activation of AMPK and reduction of mTOR signaling, have become noteworthy. Chemotherapy produces genotoxic stress and induces p53 activity, which can cross-talk with AMPK/mTOR pathway. Herein, we investigate whether the combination of metformin and paclitaxel has an effect in cancer cell lines.

Experimental design: Human tumors were xenografted into SCID mice and the cancer cell lines were treated only with paclitaxel or metformin, or a combination of both drugs. Western Blotting, flow cytometry and immunohistochemistry were then used to characterize the effects of the different treatments.

Results: The results presented herein, demonstrate that the addition of metformin to paclitaxel leads to quantitative potentialization of molecular signaling through AMPK and a subsequent potent inhibition of the mTOR signaling pathway. Treatment with metformin and paclitaxel resulted in an increase in the number of cells arrested in the G2/M phase of the cell cycle, decreased tumor growth and increased apoptosis in tumor-bearing mice, when compared to individual drug treatments.

Conclusion: We have provided evidence for a convergence of metformin and paclitaxel induced signaling at the level of AMPK. This mechanism illustrates how different drugs may cooperate to augment anti-growth signals, and suggests that target activation of AMPK by metformin may be a compelling ally in cancer treatment.

Metformin is an oral hypoglycemiant agent used as first-line therapy for type 2 diabetes, which is now prescribed to almost 120 million people in the world. There are a large number of epidemiological studies indicating that diabetics have an increased risk of cancer and cancer mortality [1-2]. Increasing evidence also supports a decreased risk of cancer mortality associated with metformin use in patients with type 2 diabetes [3-6]. Furthermore, metformin has been shown to inhibit the growth of cancer cells *in vitro* and *in vivo* [7-12] and, whilst there are still no randomized control trials of metformin as a therapy for cancer, there is intriguing evidence that metformin may enhance chemotherapy for established tumors [13-14].

Metformin has been found to activate AMP-activated protein kinase (AMPK) signaling [15], and this has become an important focus of interest in carcinogenesis, since AMPK has been implicated in the regulation of mammalian target of rapamycin (mTOR) activity, which is frequently activated in cancer [16-20]. AMPK is the downstream component of the tumor suppressor, LKB1, which acts as a sensor of cellular energy charge, being activated by increasing AMP, coupled with falling ATP [21]. The AMP/LKB1-dependent activation of AMPK results from pathological stresses such as heat shock, hypoxia, glucose deprivation and metformin administration [15, 21]. AMPK is also activated through Ca^{+2}/calmodulin (CaM)-dependent protein kinase kinase (CaMKK), which in contrast to that mediated by AMP/LKB1, is mediated by calcium increases and functions independently of AMP [22-23]. Once activated, AMPK phosphorylates acetyl-CoA carboxylase (ACC) and switches on energy-producing pathways at the expense of energy-depleting processes [24].

Another direct consequence of AMPK activation is the inhibition of the mTOR kinase signaling pathway. mTOR catalytic activity is halted by AMPK activation of the TSC1:TSC2 complex, which inactivates the Rheb GTPase [25-26]. In addition, mTOR activity is positively regulated by growth factors, as well as nutrients (amino acids). PI3K/Akt signaling regulates mTOR through phosphorylation/inactivation of mTOR's negative regulator, TSC2 [17, 27]. mTOR activation results in the phosphorylation of the serine/threonine kinase p70S6K and the translational repressor eukaryotic initiation factor (eIF) 4E binding protein (4E-BP1), which have an essential role in regulating cell growth and proliferation by controlling mRNA translation and ribosome biogenesis [28].

To achieve normal cell growth and proliferation, it is critical for cells to have robust anti-growth signaling systems. AMPK has a major role as an anti-growth signal, since it is activated by p53, a sensor of DNA damage stress [29]. Recently, the genotoxic stress effect was further evaluated and it has been suggested that the inhibition of mTOR activity occurs through the p53-dependent upregulation of sestrins 1 and 2 and consequent activation of AMPK [30]. These observations indicate that metformin acts synergistically with chemotherapeutic drugs that increase genotoxic stress through a convergent signaling of metformin-mediated LKB-1/AMPK activation and chemotherapeutic drug activation of sestrins, culminating in an increased AMPK activation and mTOR inhibition. Thus, the present study was designed to investigate whether metformin potentiates paclitaxel antitumor effects, a well known chemotherapeutic drug, as well as to observe whether these drugs share common intracellular signal transduction pathways and to determine whether these signaling systems modulate each other’s actions in different cancer cell lineages and in xenografted tumor cells in mice.

Methods

Antibodies, Chemicals and Buffers

The reagents and apparatus for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, dithiothreitol (DTT) and nitrocellulose membrane (0.45μm) were from Bio-Rad (Richmond, CA, USA). Tris-[hydroxymethyl]amino-methane (Tris), phenylmethylsulfonylfluoride (PMSF), Triton X-100, Tween 20, glycerol, and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was from Calbiochem (La Jolla, CA, USA). Aprotinin was from Amresco (Solon, OH, USA). Protein A-Sepharose 6 MB was from Amersham (Buckinghamshire, UK). Sodium thiopental was from Cristália (Itapira, SP, Brazil), 2-Deoxy-D-glucose and Metformin (1,1-Dimethylbiguanide hydrochloride) were from Sigma-Aldrich (Taufkirchen, Bavaria, Germany), Paclitaxel was from Laboratório Químico Farmacêutico Bergamo Ltda. (São Paulo, SP, Brazil). Anti-phospho-mTOR, anti-mTOR, anti-phospho-p70S6K, anti-p70S6K, anti-phospho-4E-BP1, anti-4E-BP1, anti-phospho-AMPKα, anti-AMPKα, anti-β-actin and anti-acetyl-lys379-p53 antibodies for immunoblotting were from Cell Signaling Technology (Beverly, MA, USA) and anti-p53 and anti-SESN2 antibodies for immunoblotting were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture

The human breast cancer cell lines MCF-7 and A549 were obtained from ATCC, (Philadelphia, PA, USA). MCF-7 and A549 cells were cultured in DMEM containing 10% fetal bovine serum without addition of antibiotics or fungicides. Both cell lines were maintained at 37°C in a humid atmosphere and 5% CO_2.

MTT Assay

Cells were seeded at a density of 2 x 10^4 cells/well in 24-well plates containing 1mL of complete medium in triplicate. Cells were allowed to attach overnight, before treating with the indicated dose of metformin and paclitaxel for 24 hours. Subsequently, cells were treated with 0.3mg/mL of [3-[4,5-dimethylthazol-2-yl]-2,5-diphenyl tetrazolium-Bromide] (MTT) for 4 hours and MTT-formazan conversion was analyzed by spectrophotometry at 570 nm after culture medium was removed and ethanol was added.

Cell cycle analysis

Cells were trypsinized, washed in PBS, centrifuged and pellets were fixed in 200μL of 70% ethanol and stored at -20°C until use. Cells were centrifuged and pellets ressuspended in 200μL of PBS and 10μg/mL of RNAse A was incubated for 1 hour at 37°C. Subsequently, cells were ressuspended in propidium iodide solution (0.1% sodium citrate, 0.1% Triton X-100 and 50μg/mL propidium iodide). Cell cycle analysis was performed by flow cytometry (FACScalibur). Data were analyzed using ModFit LT software.

Human tumor xenograft models

SCID mice were provided by the State University of Campinas - Central Breeding Center (Campinas, SP, Brazil). Male SCID mice, approximately 4 weeks old and with a body weight of approximately 20 grams, were randomly selected and divided into four groups; Control, Metformin, Paclitaxel and Metformin + Paclitaxel. Animals were inoculated in the dorsal region, subcutaneously, with 1 x 10^6 cells of the lung

cancer cell line, A549. The mice had *ad libitum* access to food and water. Body weight was measured weekly and mice were monitored daily to check for the presence of palpable tumors. Once tumors became palpable, tumor volume (V) was calculated daily by measuring the length (L) and width (W) of the tumor with calipers and using the formula V= {W x L x [(W+L)/2]}x 0.52. Each group contained at 10 animals.

Treatments began when tumors reached 50-100mm^3. Metformin was given daily by gavage at 500mg/kg body weight. Paclitaxel was given once a week by intraperitoneal injection of 10mg/kg body weight. At the end of experment, the mice were weighed and sacrificed, and their tumors were excised. Treatment-related toxicity was evaluated by means of serial weight measurements. All experiments were approved by the Ethics Committee of the State University of Campinas.

Tissue extracts

Mice were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.), and were used 10–15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. Tumors were removed, minced coarsely and homogenized immediately in extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/ml) at 4°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 s. The extracts were centrifuged at 11,000 rpm and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA) for 30 min to remove insoluble material, and the supernatants of these tissues were used.

Protein analysis by immunobloting

Whole tissue extracts were treated with Laemmli sample buffer [31] containing 100 mM DTT and heated in a boiling water bath for 4 min, after which they were subjected to SDS-PAGE (6.5-15% bis-acrylamide) in a Bio-Rad miniature slab gel apparatus (Mini-Protean, Bio-Rad Laboratories, Inc., Richmond, CA, USA). For total extracts, similar-sized aliquots (50 μg protein) were subjected to SDS-PAGE.

Electrotransfer of proteins from the gel to nitrocellulose was performed for 45 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean), as described by Towbin et al. [32], 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 two hours at room temperature 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. The blots were subsequently incubated with $0.8x10^{-4}$g/L of anti-rabbit or anti-mouse Horseraddish peroxidase (HRP) (Thermo Scientific Rockford, IL, USA) in 10 ml of blocking buffer for 2 h at room temperature and then washed again for 30 min, as described above. The membranes were then incubated with 2mL Luminol (Thermo Scientific Rockford, IL, USA) for 5 minutes. Antibodies were detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens. Band intensities were quantified by optical densitometry of developed autoradiographs using Scion Image software (Scion Corporation, Frederick, MD, USA).

Immunohistochemistry

To detect Ki-67, microwave postfixation was carried out using a domestic oven (Panasonic Junior) at 700 W, which was delivered to slides immersed in 0.01 mol/l citrate buffer, pH 6.0, in two 7-min doses separated by a 2-min break, which allowed for buffer replenishment. The slides were allowed to cool to room temperature before being removed from the oven. Sections were then incubated at room temperature for 1 h with primary monoclonal mouse anti-human Ki-67 clone MIB-1 from Dako (Glostrup, Denmark) (diluted 1:100). The antibodies for Ki-67 were applied overnight. The slides were then incubated with avidin-biotin complex LSAB+ Kit from Dako Cytomation (Carpinteria, CA, USA) for 30 min followed by the addition of diaminobenzidine tetrahydrochloride (DAB) as a substrate-chromogen solution. After hematoxylin counterstaining and dehydration, the slides were mounted in Entellan from Merck (Darmstadt, Germany). The experiments were performed at least in triplicate for each mouse.

TUNEL assay

Terminal deoxynucleotidyl Transferase Biotin–d UTP Nick End Labeling (TUNEL) staining was performed using a commercial apoptosis detection kit (Roche), according to the recommendations of the manufacturer.

Statistical analysis

Data are presented as means ± SEM of at least three independent experiments. All groups were studied in parallel and differences between groups were analyzed using ANOVA, as appropriate. The level of significance adopted was $P<0.05$.

Results

Metformin activates AMPK and inhibits mTOR in MCF-7 breast cancer cells and A549 lung cancer cells.

To examine the effect of metformin on cancer cell growth, MCF-7 breast cancer and A549 lung cancer cell lines were treated with various concentrations of metformin (1-50mM) for different periods of time (0-8 hours). Metformin treatment resulted in the activation of AMPK, with increased phosphorylation of AMPKα at Thr-172 in a time and dose-dependent manner. Activation of AMPK is associated with decreased activation of mTOR and p70S6K, a critical translational pathway for protein synthesis [10]. Metformin treatment resulted in attenuated activation of mTOR, as shown by the decreased phosphorylation of mTOR, p70S6K and 4E-BP1, in a time and dose-dependent manner in treated cancer cells, compared to untreated cells (Figure 1A-D).

We also observed the effect of 2-deoxy-D-glucose (2-DG), another AMPK activator, in both cell lines at various concentrations, and for different periods of time. As observed for the metformin treatment, 2-DG led to activation of AMPK and inactivation of the mTOR signaling pathway (Supplementary Figure 1).

Paclitaxel activates AMPK and inhibits mTOR in MCF-7 breast cancer cells and A549 lung cancer cells.

In order to investigate the mechanisms underlying the anti-proliferative effects of paclitaxel, we characterized the effects of paclitaxel on AMPK and the mTOR pathway. As recently reported, genotoxic stress increases the amount of sestrins, and this effect leads to AMPK activation [30]. Our results show that paclitaxel treatment increased the acetylation of p53 at Lys-379, as well as the amount of SESN2 in a time

and dose-dependent manner in both cell lines. Paclitaxel treatment also resulted in increased phosphorylation of AMPKα at Thr-172, in a time and dose-dependent manner. The increased activation of AMPK led to inactivation of mTOR as evidenced by diminished phosphorylation of mTOR, p70S6K and 4E-BP1 also in a time and dose-dependent manner (Figure 1E-H).

Effect of combined treatment of AMPK activators and paclitaxel on cancer cell lines

Since treatments with AMPK activators alone and paclitaxel alone were found to activate AMPK and inhibit mTOR, we next sought to determine the effects of the combined treatment of AMPK activators with paclitaxel on MCF-7 and A549 cells. These experiments were performed using the best doses and time for each drug, as established previously (all treatments were performed for 6 hours with corresponding doses of 10mM for metformin, 10mM for 2-DG and of 1μM for paclitaxel). In MCF-7 cells, as shown in Figure 2A, paclitaxel treatment, but not metformin or 2-DG treatment, led to an increase in acetyl-Lys379 p-53, as well as an increase in the amount of sestrin 2 (Figure 2B). This increase in sestrin 2 in paclitaxel-treated cells was followed by an increase in the phosphorylation of Thr-172 of AMPK (Figure 2C) and inhibition of mTOR (Figure 2D), and its downstream targets, p70S6K (Figure 2E) and 4E-BP1 (Figure 2F), when compared to metformin or 2-DG treatments alone. In A549 cells, we also observed an increase in acetyl-Lys379 p-53 and in the amount of sestrin 2 only in the paclitaxel-treated cells (Figure 2G and 2H), and this was correlated with an increase in the Thr-172 phosphorylation of AMPK (Figure 2I) and decrease in the activation of mTOR (Figure 2J) and its downstream targets, p70S6K (Figure 2K) and

4E-BP1 (Figure 2L), when compared to control and metformin and 2-DG treatments alone.

Metformin and paclitaxel inhibit cell viability

To examine the effects of metformin and paclitaxel on cancer cell growth, we treated MCF-7 and A549 cell lines with metformin or paclitaxel alone or in combination and cell viability was determined by MTT assay. As shown in Figure 3 (A and B), both metformin and paclitaxel inhibited cell viability, as related to control, and the combined treatment were more effective than either treatment alone. MCF-7 breast cancer cells, when treated with metformin demontrated a 23% reduction in cell viability and a 37% reduction when treated with paclitaxel. In contrast, the combination of both drugs led to a 44% reduction in cell viability. A549 lung cancer cells presented similar results, with a 40% reduction when treated with metformin, a 35% reduction after paclitaxel treatment and a 50% reduction with the combined treatment.

Effect of metformin and paclitaxel on cell cycle

To evaluate the mechanism of proliferation inhibition by metformin and paclitaxel, the cell cycle profile was analyzed by flow cytometry after treatment with metformin or paclitaxel alone, or the combination of the drugs. Control treatment presented the majority of cells in the G1-phase of the cell cycle (MCF-7 72.7%, A549 73.2%), a very small part in the G2/M-phase (MCF-7 4.7%, A549 5.5%) and the rest of the cells were found to be in the S-phase (MCF-7 22.5%, A549 21.2%). Metformin treatment resulted in an increase in the number of cells in the G1-phase (MCF-7 83%, A549 81.2%) with a slight increase in the number of cells in the G2/M-phase (MCF-7 8.6% A549 6.8%) and a reduction in the number of cells in the S-phase in both cell lines (MCF-7 8%, A549 12%). Paclitaxel treatment, as expected, caused an increase in

the number of cells in the G2/M-phase (MCF-7 15.4%, A549 16%) with a reduction in the number of cells in the G1-phase (MCF-7 69.6%, A549 67.6%) and in the S-Phase (MCF-7 15%, A549 16.4%) in both cell lines (Figure 3C and D).

The combined treatment of metformin and paclitaxel resulted in a synergistic effect of G2/M cell cycle arrest. Cells treated with metformin and paclitaxel demonstrated a reduction in G1-phase, compared to either treatment alone (MCF-7 40.1%, A549 39.2%). Combined treatment also resulted in an increased number of cells in the S-phase compared to metformin treatment alone (MCF-7 16.4%, A549 15.4%). Finally, when we analyzed the G2/M-phase, we observed a significant increase in the number of cells in these phase in the metformin combined with paclitaxel treatment, as compared to either treatment alone (MCF-7 43.5%, A549 45.4%). Thus, Figure 3 C and D show an increase in cell cycle arrest in the G2/M-phase, during the combined treatment of metformin and paclitaxel and a decrease in the G1-phase, indicating that cells submitted to the combined treatment were no longer undergoing division.

The effect of metformin and paclitaxel on A549 tumors grown in SCID mice

Xenografted SCID mice were treated with control vehicle, metformin, paclitaxel, or metformin+paclitaxel. Treatments began when the tumors presented an average size of 50 mm^3 and tumor growth rate was measured daily after the beginning of the treatment. Fig. 4A shows that metformin+paclitaxel is clearly more effective in reducing tumor growth, as compared with either paclitaxel alone, metformin alone, or the control. For the entire experiment, the animals treated with metformin+paclitaxel presented almost no tumor growth, with the final tumor volume of 64mm^3 being very close to the tumor volume at the beginning of the treatment, as compared with the final volumes of

the control (363mm^3), metformin (189mm^3) and paclitaxel (139mm^3) as shown in Figure 4B.

The reduced tumor growth following metformin+paclitaxel treatment is due to the reduced proliferation of tumor cells, as demonstrated by Ki67 quantification (Figure 4C and D) and increased apoptosis, as quantified by TUNEL staining (Figure 4E and F). In the control group, Ki67 positive cells were 25.5% (± 2.8) of the total, in metformin these cells were 13.4% (± 1.1), while in paclitaxel these cells were 10.8% (± 1.2) and metformin+paclitaxel presented 7.2% (± 0.4) Ki67 positive cells. The results of the TUNEL staining experiments show that the control group presented a 9.5% (± 1.1) apoptosis, while metformin apoptosis was 16.7% (± 4.5), and with paclitaxel apoptosis was 31.8% (± 1.8) and metformin+paclitaxel presented a 35.9% (± 4.8) apoptosis. These data are consistent in demonstrating that there is a significant advantage in the use of combination treatment with metformin+paclitaxel, as compared with treatment with either agent alone.

Effect of metformin, paclitaxel and metformin+paclitaxel treatment on AMPK and the mTOR pathway in A549 xenografts

As treatment with metformin+paclitaxel inhibited tumor growth, we sought to determine AMPK and mTOR pathway activation status in the tumor tissue of animals treated with metformin, paclitaxel and metformin+paclitaxel. Figure 5A shows that both treatments with paclitaxel resulted in a higher acetylation of Lysine 379 of p-53 and increased quantity of SESN2, as compared to control or metformin (Figure 5B). The phosphorylation of AMPK at Thr172 was also higher in the metformin+paclitaxel treatment, when compared to paclitaxel alone, metformin alone or control (Figure 5C).

Both treatments with paclitaxel and metformin also activated AMPK, as compared to the control. Similarly, phosphorylation of mTOR (Figure 5D), and its direct substrates p70S6K (Figure 5E) and 4E-BP1 (Figure 5F), were reduced following metformin+paclitaxel treatments.

DISCUSSION

In the present study, we show that the combination of metformin and paclitaxel has a major antitumor effect *in vivo* and induces massive cell cycle arrest *in vitro*. These effects are correlated with a potent activation of AMPK. Our results show that metformin, which induces a moderate decrease in ATP levels [33], is able to produce molecular activation of AMPK and inactivation of mTOR signaling in breast and lung cancer cells, whilst paclitaxel, through activation of p53 and sestrins, yields similar effects to metformin. Combined treatment with metformin and paclitaxel leads to a quantitative increase in AMPK activation and a drastic reduction of molecular signaling through the mTOR pathway. Likewise, the combination of paclitaxel with 2DG, which like metformin, leads to intracellular ATP depletion [34-35], severely inhibited the mTOR signaling pathway.

It was initially demonstrated that meformin was capable of reducing proliferation in different types of cancer including, prostate, colon and breast cancer cell lines. Subsequently, *in vivo* experiments with metformin resulted in tumor growth inhibition of up to 55% [12, 36]. In accordance with these data, we herein show that metformin treatment resulted in a reduction of A549 and MCF-7 cell viability and a decreased tumor volume of A549 tumor when xenografted in SCID mice, of approximately 50%. These effects were paralleled by a decrease in the central regulator of cell growth and survival, mTOR signaling pathway, as measured by p70S6K and 4EBP-1 phosphorylation.

The mechanisms by which cells protect their genetic material during genotoxic stress include the alert of checkpoint proteins and arrest of cell growth and proliferation [37-38]. The major cellular stress-sensing molecule is p53, which halts cell growth and

proliferation by increasing sestrins, thus leading to activation of AMPK and inhibition of mTOR [29-30]. Here we show that paclitaxel induces p53 acetylation in the cancer cells and activates AMPK. AMPK activation resulted in decreased mTOR pathway activity; this effect may be related to the reduction of cell metabolism that is observed during prolonged mitosis, induced by paclitaxel.

Intracellular interactions between different signaling systems may function as mechanisms for enhancing or counter-regulating signaling pathways. In the case of metfomin, the cross-talk with paclitaxel-induced signaling pathways resulted in direct interactions between these drug-induced signaling systems at the level of AMPK. Simultaneous treatment with both drugs led to increased phosphorylation of AMPK and a drastic reduction of mTOR signaling pathway. These results suggest that the positive cross-talk between metformin and paclitaxel-induced signaling was due to additive effects on AMPK activation.

The mTOR pathway is a crucial pathway, downstream of several growth factor receptors including EGF, PDGF, KIT and IGF1R, which coordinate tumor growth [39-40]. The deregulated mTOR pathway is very frequent in human cancer. These alterations include mutational activation of the p110α subunit of PI3K, loss of PTEN function, overexpression of PI3K, Akt, eIF4E and p70S6K as well as inactivation of tuberous sclerosis 1 or 2 [39-40]. More recently, it was also established that the mTOR pathway can be inactivated by AMPK, which acts through a PI3K-independent mechanism.

The susceptibility of cancer cells to PI3K inhibitors is highly determined by the presence of mutations in components of the PI3K/Akt/mTOR pathway [41]. In contrast, our results showed that a drastic reduction of the mTOR pathway, elicited by the

combination of metformin and paclitaxel, yields decreased cell viability in both MCF-7, which has a mutational activation of the PI3K catalytic subunit, and A549 cells, which does not harbor genetic alterations in the PI3K/Akt/mTOR pathway. Since the mTOR pathway is essential to cell metabolism and growth, and delayed mitosis induced by paclitaxel is associated with a reduction in gene transcription, our data suggest that the cancer cells may be "pathway addicted", independently of harboring a mutation in the PI3K/Akt/mTOR pathway during paclitaxel-induced cell cycle arrest.

Toxicity elicited by paclitaxel has been linked to irreversible tubulin polymerization, a cell cycle block at the metaphase–anaphase transition, and cell death [42-44]. On the other hand, in addition to the metabolic activity of AMPK, there is growing evidence that AMPK has a crucial role in the establishment of cell division, and it has been suggested that AMPK may be essential in the coordination between the sensing of energy resources and genome division [45-47]. Our results show that the combination of metformin and paclitaxel has an additive effect on cell viability and, in accordance with a previous study that combined two activators of AMPK, metformin and 2-DG, we observed a compelling accumulation of cells in G2/M [33]. Since gene transcription is silenced during mitosis and paclitaxel plus metformin induced a more prolonged division, our results suggest that this event leads to a greater decrease in cell viability.

In conclusion, we observed a convergence of paclitaxel- and metformin- induced signaling at the level of AMPK. This mechanism illustrates how different drugs may cooperate to augment anti-growth signals, and suggests that target activation of AMPK by metformin may be a compelling ally in cancer treatment.

ACKNOWLEDGMENTS

These studies were supported by grants from Fundacão de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de desenvolvimento científico e tecnológico (CNPq).

We thank Dr. Nicola Conran for English language editing. We also thank Mr. Luiz Janeri, Jósimo Pinheiro and, Gerson Ferraz for technical assistance.

DISCLOSURE

The authors have nothing to disclose.

REFERENCES

1. Barone, B.B., et al., *Long-term all-cause mortality in cancer patients with preexisting diabetes mellitus: a systematic review and meta-analysis.* Jama, 2008. **300**(23): p. 2754-64.
2. Vigneri, P., et al., *Diabetes and cancer.* Endocr Relat Cancer, 2009. **16**(4): p. 1103-23.
3. *Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group.* Lancet, 1998. **352**(9131): p. 854-65.
4. Evans, J.M., et al., *Metformin and reduced risk of cancer in diabetic patients.* Bmj, 2005. **330**(7503): p. 1304-5.
5. Li, D., et al., *Antidiabetic therapies affect risk of pancreatic cancer.* Gastroenterology, 2009. **137**(2): p. 482-8.
6. Libby, G., et al., *New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes.* Diabetes Care, 2009. **32**(9): p. 1620-5.
7. Anisimov, V.N., et al., *Effect of metformin on life span and on the development of spontaneous mammary tumors in HER-2/neu transgenic mice.* Exp Gerontol, 2005. **40**(8-9): p. 685-93.
8. Ben Sahra, I., et al., *The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level.* Oncogene, 2008. **27**(25): p. 3576-86.
9. Buzzai, M., et al., *Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth.* Cancer Res, 2007. **67**(14): p. 6745-52.
10. Dowling, R.J., et al., *Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells.* Cancer Res, 2007. **67**(22): p. 10804-12.
11. Huang, X., et al., *Important role of the LKB1-AMPK pathway in suppressing tumorigenesis in PTEN-deficient mice.* Biochem J, 2008. **412**(2): p. 211-21.
12. Zakikhani, M., et al., *Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells.* Cancer Res, 2006. **66**(21): p. 10269-73.
13. Jiralerspong, S., et al., *Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer.* J Clin Oncol, 2009. **27**(20): p. 3297-302.
14. Hirsch, H.A., et al., *Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission.* Cancer Res, 2009. **69**(19): p. 7507-11.
15. Shaw, R.J., et al., *The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin.* Science, 2005. **310**(5754): p. 1642-6.
16. Meric-Bernstam, F. and A.M. Gonzalez-Angulo, *Targeting the mTOR signaling network for cancer therapy.* J Clin Oncol, 2009. **27**(13): p. 2278-87.
17. Inoki, K., et al., *TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling.* Nat Cell Biol, 2002. **4**(9): p. 648-57.
18. Kimura, N., et al., *A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway.* Genes Cells, 2003. **8**(1): p. 65-79.
19. Shaw, R.J., et al., *The LKB1 tumor suppressor negatively regulates mTOR signaling.* Cancer Cell, 2004. **6**(1): p. 91-9.

20. Ben Sahra, I., et al., *Metformin in cancer therapy: a new perspective for an old antidiabetic drug?* Mol Cancer Ther. **9**(5): p. 1092-9.
21. Shaw, R.J., et al., *The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3329-35.
22. Hawley, S.A., et al., *Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase.* Cell Metab, 2005. **2**(1): p. 9-19.
23. Hurley, R.L., et al., *The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases.* J Biol Chem, 2005. **280**(32): p. 29060-6.
24. Kahn, B.B., et al., *AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism.* Cell Metab, 2005. **1**(1): p. 15-25.
25. Inoki, K., T. Zhu, and K.L. Guan, *TSC2 mediates cellular energy response to control cell growth and survival.* Cell, 2003. **115**(5): p. 577-90.
26. Saucedo, L.J., et al., *Rheb promotes cell growth as a component of the insulin/TOR signalling network.* Nat Cell Biol, 2003. **5**(6): p. 566-71.
27. Manning, B.D., et al., *Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway.* Mol Cell, 2002. **10**(1): p. 151-62.
28. Brunn, G.J., et al., *Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin.* Science, 1997. **277**(5322): p. 99-101.
29. Feng, Z., et al., *The coordinate regulation of the p53 and mTOR pathways in cells.* Proc Natl Acad Sci U S A, 2005. **102**(23): p. 8204-9.
30. Budanov, A.V. and M. Karin, *p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling.* Cell, 2008. **134**(3): p. 451-60.
31. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
32. Towbin, H., T. Staehelin, and J. Gordon, *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.* Proc Natl Acad Sci U S A, 1979. **76**(9): p. 4350-4.
33. Ben Sahra, I., et al., *Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells.* Cancer Res. **70**(6): p. 2465-75.
34. Brown, J., *Effects of 2-deoxyglucose on carbohydrate metablism: review of the literature and studies in the rat.* Metabolism, 1962. **11**: p. 1098-112.
35. McComb, R.B. and W.D. Yushok, *Metabolism of Ascites Tumor Cells. Iv. Enzymatic Reactions Involved in Adenosinetriphosphate Degradation Induced by 2-Deoxyglucose.* Cancer Res, 1964. **24**: p. 198-205.
36. Liu, B., et al., *Metformin induces unique biological and molecular responses in triple negative breast cancer cells.* Cell Cycle, 2009. **8**(13): p. 2031-40.
37. Levine, A.J., *p53, the cellular gatekeeper for growth and division.* Cell, 1997. **88**(3): p. 323-31.
38. Vogelstein, B., D. Lane, and A.J. Levine, *Surfing the p53 network.* Nature, 2000. **408**(6810): p. 307-10.
39. Faivre, S., G. Kroemer, and E. Raymond, *Current development of mTOR inhibitors as anticancer agents.* Nat Rev Drug Discov, 2006. **5**(8): p. 671-88.
40. Guertin, D.A. and D.M. Sabatini, *An expanding role for mTOR in cancer.* Trends Mol Med, 2005. **11**(8): p. 353-61.

41. Yu, K., et al., *Response and determinants of cancer cell susceptibility to PI3K inhibitors: combined targeting of PI3K and Mek1 as an effective anticancer strategy.* Cancer Biol Ther, 2008. **7**(2): p. 307-15.
42. Jordan, M.A. and L. Wilson, *Microtubules as a target for anticancer drugs.* Nat Rev Cancer, 2004. **4**(4): p. 253-65.
43. Rieder, C.L. and H. Maiato, *Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint.* Dev Cell, 2004. **7**(5): p. 637-51.
44. Schiff, P.B. and S.B. Horwitz, *Taxol stabilizes microtubules in mouse fibroblast cells.* Proc Natl Acad Sci U S A, 1980. **77**(3): p. 1561-5.
45. Nakano, A., et al., *AMPK controls the speed of microtubule polymerization and directional cell migration through CLIP-170 phosphorylation.* Nat Cell Biol. **12**(6): p. 583-90.
46. Vazquez-Martin, A., C. Oliveras-Ferraros, and J.A. Menendez, *The active form of the metabolic sensor: AMP-activated protein kinase (AMPK) directly binds the mitotic apparatus and travels from centrosomes to the spindle midzone during mitosis and cytokinesis.* Cell Cycle, 2009. **8**(15): p. 2385-98.
47. Williams, T. and J.E. Brenman, *LKB1 and AMPK in cell polarity and division.* Trends Cell Biol, 2008. **18**(4): p. 193-8.

Figure Legends

Figure 1

Metformin and paclitaxel activate AMPK and inhibit mTOR in MCF-7 and A549 cells. A MCF-7 cells were treated with 10mM metformin for the indicated time and **B** for 6 hours with the indicated doses, the lysates were immunoblotted with the indicated antibodies. **C** A549 cells were treated with 10mM metformin for the indicated time and **D** for 6 hours with the indicated doses, the lysates were immunoblotted with the indicated antibodies. **E** MCF-7 cells were treated with 1uM paclitaxel for the indicated time and **F** for 6 hours with the indicated doses, the lysates were immunoblotted with the indicated antibodies. **G** A549 cells were treated with 1uM paclitaxel for the indicated time and **H** for 6 hours with the indicated doses, the lysates were immunoblotted with the indicated antibodies. Data are representative of at least 3 experiments.

Figure 2

Effect of combined treatment with 2-DG, metformin and paclitaxel on MCF-7 and A549 cells. Cells were treated with 10mM metformin, 10mM 2-DG and 1uM paclitaxel for 6 hours. After 6 hours, cells were prepared as described in Methods. The lysates of MCF-7 cells were immunoblotted (IB) with **A** Acetyl-Lys-379 p53 and p53. **B** SESN2 and β-actin. **C** pAMPKα and AMPKα. **D** pmTOR and mTOR. **E** pp70S6K and p70S6K. **F** p4E-BP1 and 4E-BP1. The lysates of A549 cells were immunoblotted (IB) with **G** Acetyl-Lys-379 p53 and p53. **H** SESN2 and β-actin. **I** pAMPKα and AMPKα. **J** pmTOR and mTOR. **K** pp70S6K and p70S6K. **L** p4E-BP1and 4E-BP1. Data (mean ±

SEM; *n* = 3 experiments in triplicate) are presented as relative to control (* p ≤ 0,05 vs control; # p ≤ 0,05 vs paclitaxel; ‡ p ≤ 0,05 vs metformin)

Figure 3

Metformin and paclitaxel inhibit cell viability. A Cell viability of MCF-7 cells treated with metformin (10mM) alone, paclitaxel (1uM) alone, or metformin and paclitaxel, as measured by MTT assay. Cells were treated for 24hours with respective drugs. Data (mean ± SEM; *n* = 3 experiments in triplicate) are presented as relative to control (* p ≤ 0.05 vs control; # p ≤ 0.05 vs paclitaxel; ‡ p ≤ 0.05 vs metformin). **B** Cell viability of A549 cells treated with metformin alone (10mM), paclitaxel alone (1uM) or metformin and paclitaxel, as measured by MTT assay. Cells were treated for 24hours with respective drugs. Data (mean ± SEM; *n* = 3 experiments in triplicate) are presented as relative to control (* p ≤ 0.05 vs control; # p ≤ 0.05 vs paclitaxel; ‡ p ≤ 0.05 vs metformin). **C** Cell cycle analysis of MCF-7 cells treated with metformin (10mM) alone, paclitaxel alone (1uM) or metformin and paclitaxel, as measured by flow cytometry (FACScalibur). Cells were treated for 24hours with respective drugs. **D** Cell cycle analysis of A549 cells treated with metformin (10mM) alone, paclitaxel (1uM) alone or metformin and paclitaxel, as measured by flow cytometry (FACScalibur). Cells were treated for 24hours with respective drugs.

Figure 4

Metformin and paclitaxel synergize in vivo to reduce A549 tumor growth. 1.0 x 10^6 A549 cells were injected subcutaneously into the flank of SCID mice. Once the tumor reached 50-100 mm^3, treatments were initiated, as indicated in Methods, n=10 mice per

group. Data are presented as means ± SEM. **A** Tumor growth was measured daily after beginning treatment. **B** Tumor volume after 3 weeks of treatment. **C** Representative microphotograph of Ki-67 staining on tumor sections (arrows indicate positive Ki-67 staining). **D** Graph of % Ki-67-positive cells per field, 4 fields per tumor section, mean ± SEM. **E** Representative microphotograph of TUNEL staining on tumor sections. **F** Graph of %TUNEL-positive nuclei of cells per field, 4 fields per tumor section, mean ± SEM. (* $p \leq 0.05$ vs control; # $p \leq 0.05$ vs paclitaxel; ‡ $p \leq 0.05$ vs metformin).

Figure 5

Metformin and paclitaxel activate AMPK and inhibit mTOR in vivo. Mice bearing A549 xenografts were treated with metformin or paclitaxel alone or in combination, as described in Methods. The A549 tumor lysates were immunoblotted (IB) with **A** Acetyl-Lys-379 p53 and p53. **B** SESN2 and β-actin. **C** pAMPKα and AMPKα. **D** pmTOR and mTOR. **E** pp70S6K and p70S6K. **F** p4E-BP1 and 4E-BP1. Data (mean ± SEM; $n = 8$) are presented as relative to control (* $p \leq 0.05$ vs control; # $p \leq 0.05$ vs paclitaxel; ‡ $p \leq 0.05$ vs metformin).

Supplemental Figure1

2-DG activates AMPK and inhibits mTOR in MCF-7 and A549 cells. A MCF-7 cells were treated with 10mM 2-DG for the indicated time and **B** for 6 hours with the indicated doses, the lysates were immunoblotted with the indicated antibodies. **C** A549 cells were treated with 10mM 2-DG for the indicated time and **D** for 6 hours with the indicated doses, the lysates were immunoblotted with the indicated antibodies. Data are representative of at least 3 experiments.

Figures

Figure 1

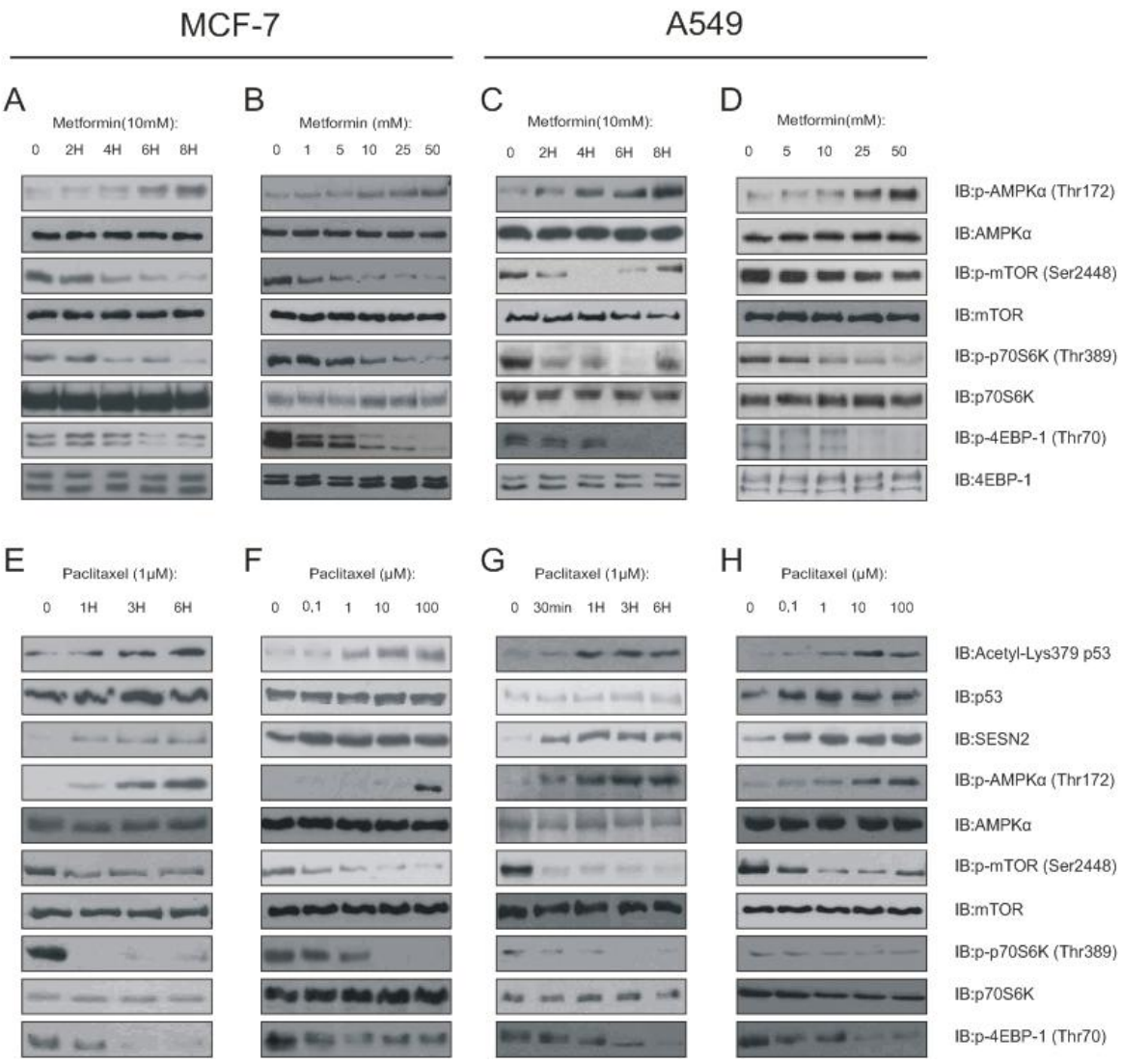
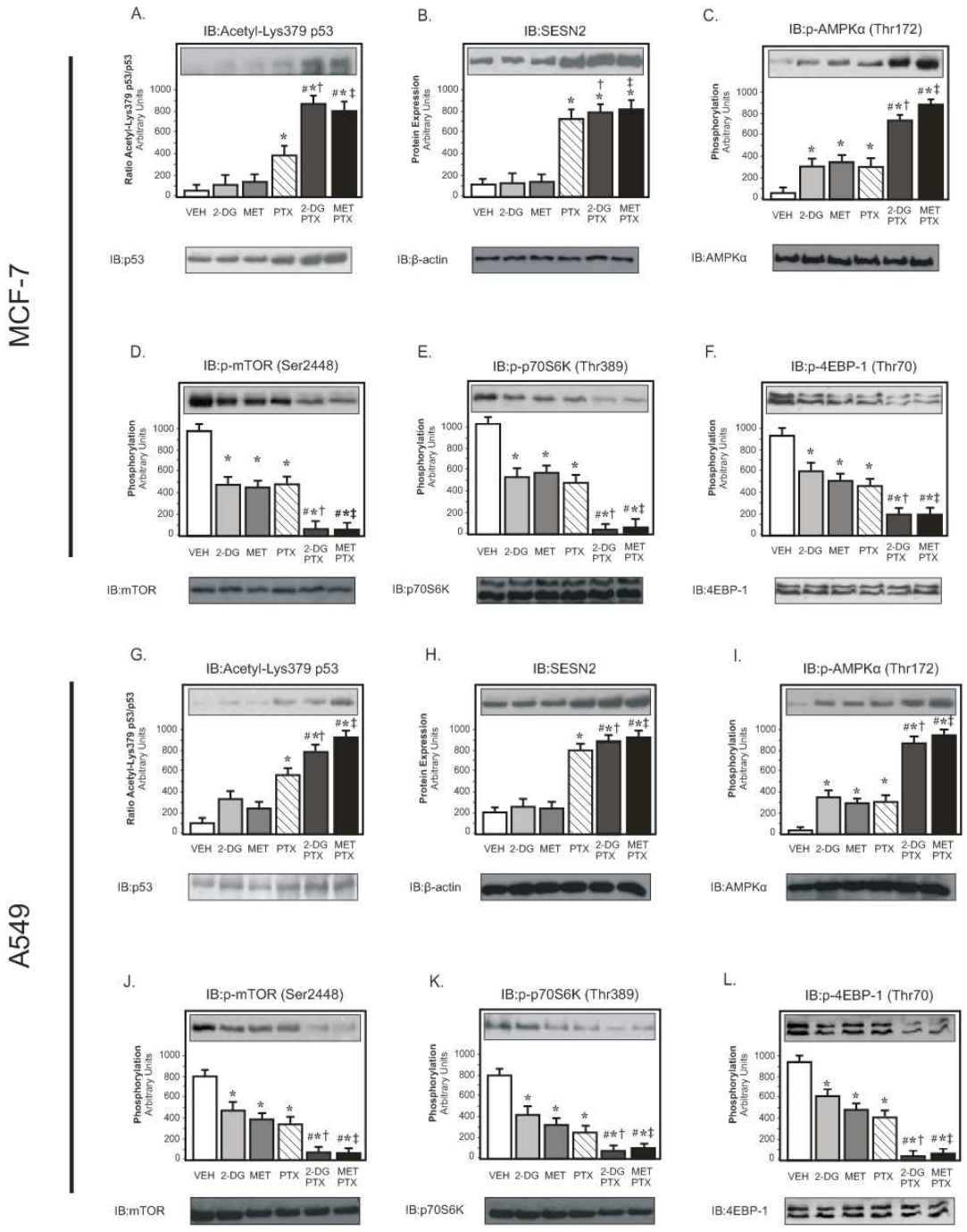


Figure 2


MCF-7
A549
A.
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Ratio Acetyl-Lys379 p53/p53
Arbitrary Units
IB:p53
B.
IB:SESN2
Protein Expression
Arbitrary Units
IB:β-actin
C.
IB:p-AMPKα (Thr172)
Phosphorylation
Arbitrary Units
IB:AMPKα
D.
IB:p-mTOR (Ser2448)
IB:mTOR
E.
IB:p-p70S6K (Thr389)
IB:p70S6K
F.
IB:p-4EBP-1 (Thr70)
IB:4EBP-1
G.
IB:Acetyl-Lys379 p53
IB:p53
H.
IB:SESN2
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I.
IB:p-AMPKα (Thr172)
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J.
IB:p-mTOR (Ser2448)
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K.
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2-DG
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PTX
2-DG PTX
MET PTX

Figure 3

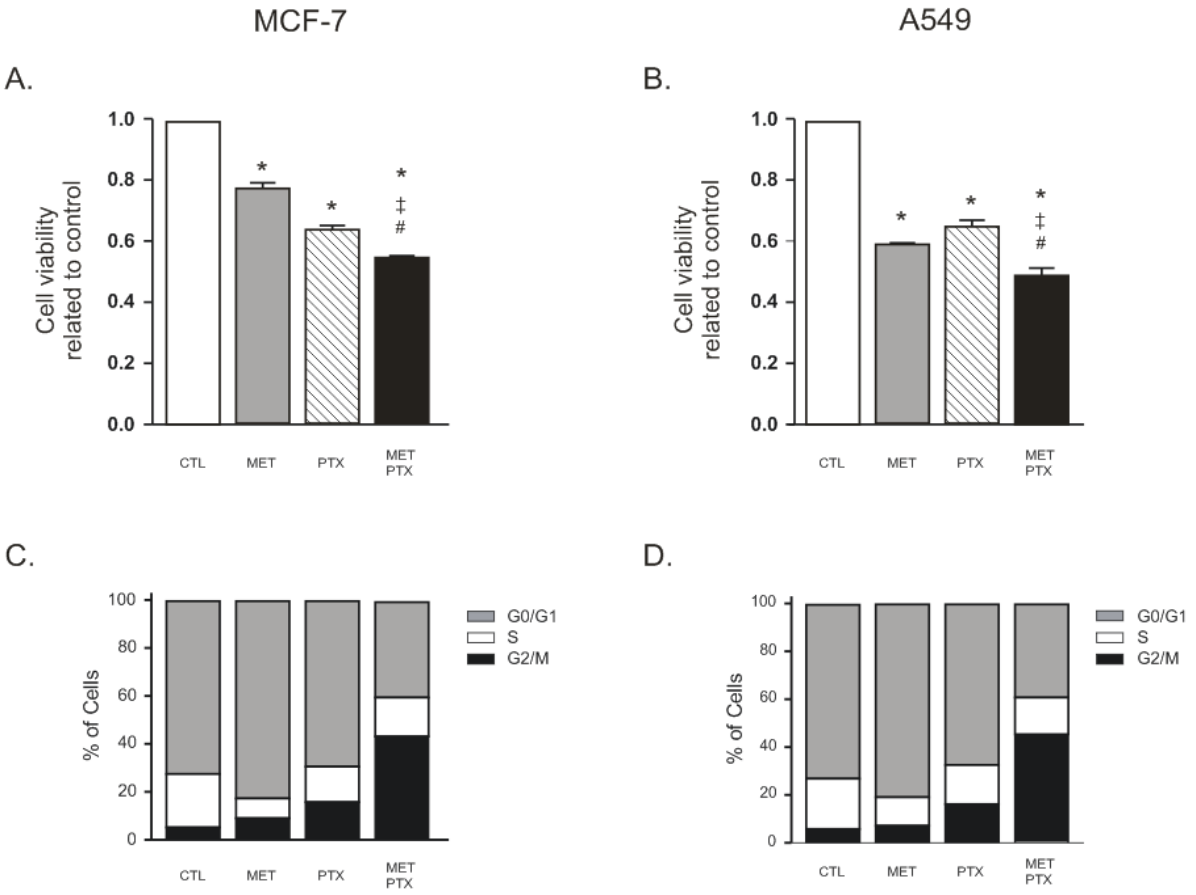

MCF-7
A549
A.
B.
Cell viability related to control
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
‡
#
CTL
MET
PTX
MET PTX
C.
D.
% of Cells
100
80
60
40
20
0
G0/G1
S
G2/M

Figure 4

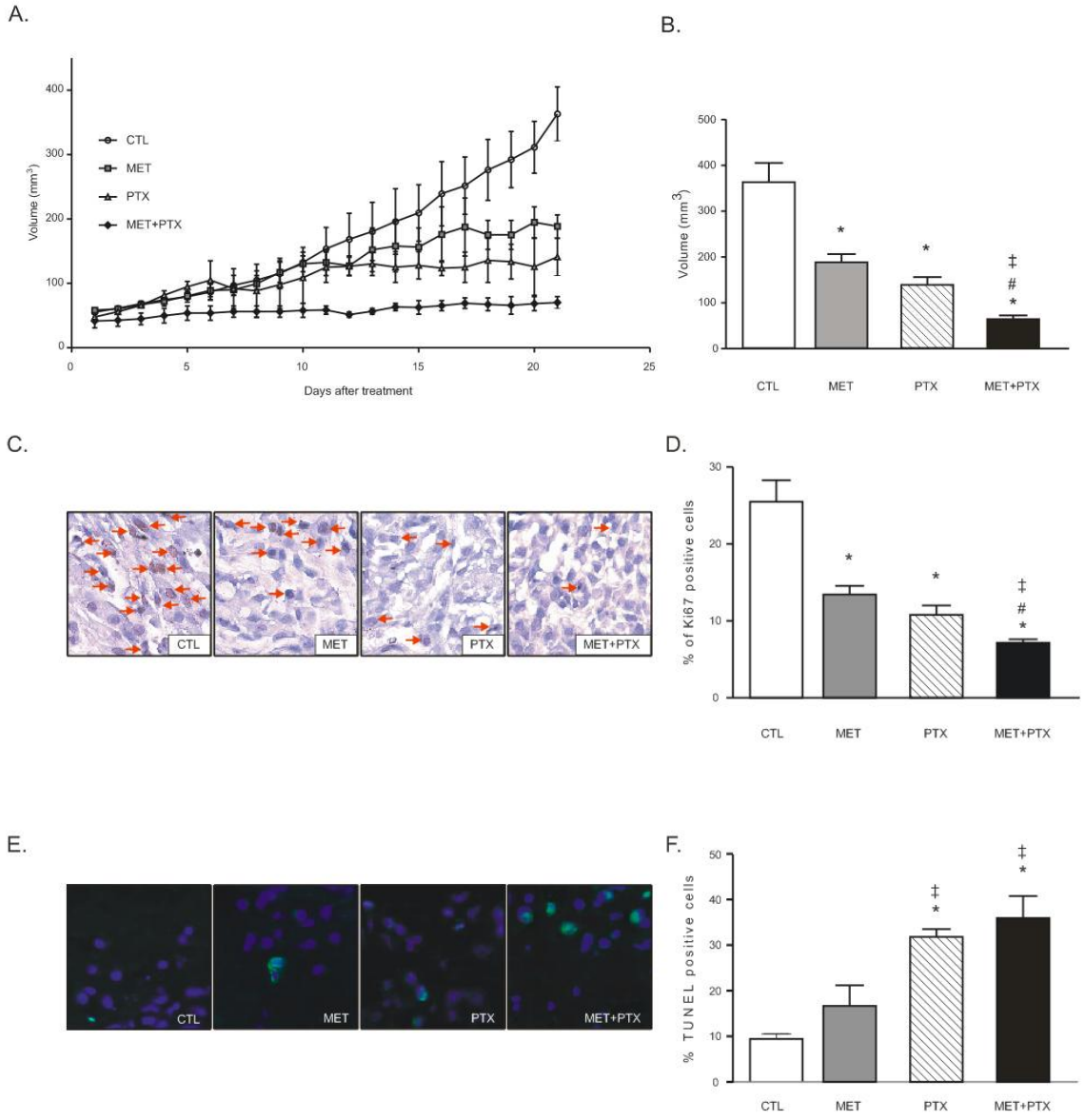
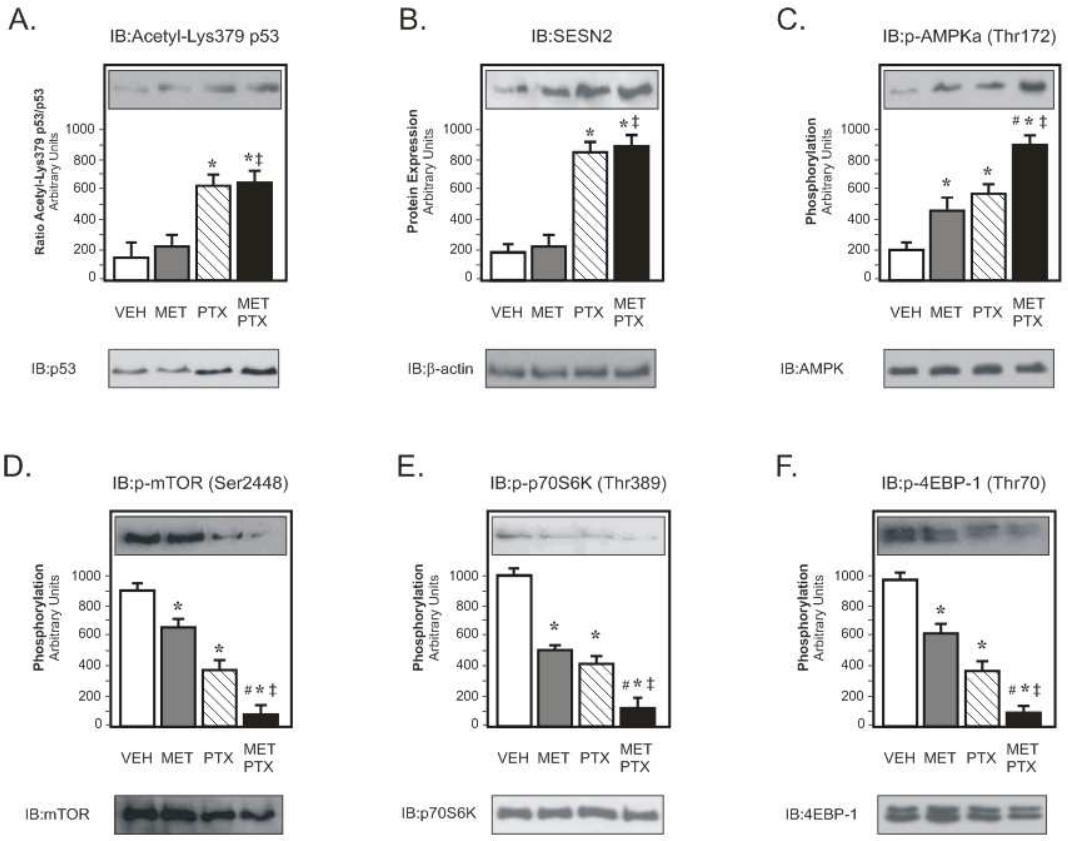

A.
B.
C.
D.
E.
F.
CTL
MET
PTX
MET+PTX
Volume (mm3)
Days after treatment
% of Ki67 positive cells
% TUNEL positive cells

Figure 5


A.
IB:Acetyl-Lys379 p53
Ratio Acetyl-Lys379 p53/p53
Arbitrary Units
VEH MET PTX MET PTX
IB:p53
B.
IB:SESN2
Protein Expression
Arbitrary Units
VEH MET PTX MET PTX
IB:β-actin
C.
IB:p-AMPKa (Thr172)
Phosphorylation
Arbitrary Units
VEH MET PTX MET PTX
IB:AMPK
D.
IB:p-mTOR (Ser2448)
Phosphorylation
Arbitrary Units
VEH MET PTX MET PTX
IB:mTOR
E.
IB:p-p70S6K (Thr389)
Phosphorylation
Arbitrary Units
VEH MET PTX MET PTX
IB:p70S6K
F.
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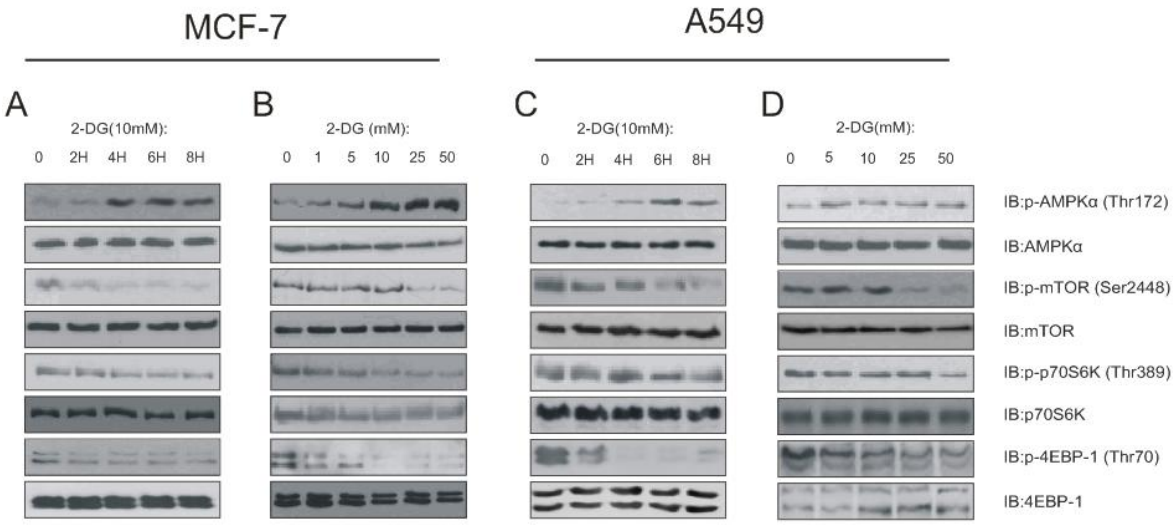
Phosphorylation
Arbitrary Units
VEH MET PTX MET PTX
IB:4EBP-1

Figure Supplementar 1



CONCLUSÕES

CONCLUSÕES

A ativação de AMPK modula negativamente a via da mTOR em linhagens adenocarcinoma de mama e de carcinoma de pulmão.

O paclitaxel ativa a AMPK e modula negativamente a via da mTOR, através de ativação de p53 e de aumento na quantidade de sestrina 2 em linhagens de adenocarcinoma de mama e carcinoma de pulmão.

A associação entre paclitaxel e ativadores da AMPK causa parada no ciclo celular na fase G2/M e possui efeito aditivo na ativação de AMPK e na inibição da via da mTOR. A ativação da AMPK e inibição da via da mTOR ocorre através do estresse genotóxico, com ativação de p53 e aumento na quantidade de sestrina 2, em linhagem de adenocarcinoma de mama e carcinoma de pulmão.

O crescimento tumoral de células de carcinoma de pulmão em animais tratados com paclitaxel e metformina foi significativamente menor do que os tratamentos isolados. Essa redução no crescimento tumoral deve-se principalmente à menor proliferação das células e também ao maior índice de apoptose observado no tratamento combinado.

CONCLUSÃO GERAL

No presente trabalho nós apresentamos evidências de ativação da AMPK induzida através da convergência de sinalização entre metformina e paclitaxel. Esse mecanismo ilustra como diferentes drogas podem cooperar para aumentar os sinais inibitórios de crescimento, e sugerem que a ativação da AMPK pela metformina como alvo principal pode ser um potente aliado no tratamento do câncer.

Antony, A. C., M. A. Kane, et al. (1985). "Studies of the role of a particulate folate-binding protein in the uptake of 5-methyltetrahydrofolate by cultured human KB cells." J Biol Chem **260**(28): 14911-14917.

Arkin, A. P. (2001). "Synthetic cell biology." Curr Opin Biotechnol **12**(6): 638-644.

Backer, J. M., M. G. Myers, Jr., et al. (1992). "Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation." EMBO J **11**(9): 3469-3479.

Barreiro, G. C., R. R. Prattali, et al. (2004). "Aspirin inhibits serine phosphorylation of IRS-1 in muscle and adipose tissue of septic rats." Biochem Biophys Res Commun **320**(3): 992-997.

Basu, S., N. F. Totty, et al. (2003). "Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis." Mol Cell **11**(1): 11-23.

Beauloye, C., A. S. Marsin, et al. (2001). "Insulin antagonizes AMP-activated protein kinase activation by ischemia or anoxia in rat hearts, without affecting total adenine nucleotides." FEBS Lett **505**(3): 348-352.

Bhalla, K., A. M. Ibrado, et al. (1993). "Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells." Leukemia **7**(4): 563-568.

Bhalla, K. N. (2003). "Microtubule-targeted anticancer agents and apoptosis." Oncogene **22**(56): 9075-9086.

Blagosklonny, M. V. (2001). "Unwinding the loop of Bcl-2 phosphorylation." Leukemia **15**(6): 869-874.

Blagosklonny, M. V., T. W. Schulte, et al. (1995). "Taxol induction of p21WAF1 and p53 requires c-raf-1." Cancer Res **55**(20): 4623-4626.

Bolster, D. R., S. J. Crozier, et al. (2002). "AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling." J Biol Chem **277**(27): 23977-23980.

Bonadonna, G., E. Brusamolino, et al. (1976). "Combination chemotherapy as an adjuvant treatment in operable breast cancer." N Engl J Med **294**(8): 405-410.

Browne, G. J., S. G. Finn, et al. (2004). "Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398." J Biol Chem **279**(13): 12220-12231.

Budanov, A. V. and M. Karin (2008). "p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling." Cell **134**(3): 451-460.

Burkhart, C. A., J. W. Berman, et al. (1994). "Relationship between the structure of taxol and other taxanes on induction of tumor necrosis factor-alpha gene expression and cytotoxicity." Cancer Res **54**(22): 5779-5782.

Buttgereit, F. and M. D. Brand (1995). "A hierarchy of ATP-consuming processes in mammalian cells." Biochem J **312 (Pt 1)**: 163-167.

Carrera, A. C. (2004). "TOR signaling in mammals." J Cell Sci **117**(Pt 20): 4615-4616.

Carvalheira, J. B., V. C. Calegari, et al. (2003). "The cross-talk between angiotensin and insulin differentially affects phosphatidylinositol 3-kinase- and mitogen-activated protein kinase-mediated signaling in rat heart: implications for insulin resistance." Endocrinology **144**(12): 5604-5614.

Carvalheira, J. B., E. B. Ribeiro, et al. (2003). "Selective impairment of insulin signalling in the hypothalamus of obese Zucker rats." Diabetologia **46**(12): 1629-1640.

Carvalheira, J. B., E. B. Ribeiro, et al. (2003). "Interaction between leptin and insulin signaling pathways differentially affects JAK-STAT and PI 3-kinase-mediated signaling in rat liver." Biol Chem **384**(1): 151-159.

Carvalho, C. R., J. B. Carvalheira, et al. (2003). "Novel signal transduction pathway for luteinizing hormone and its interaction with insulin: activation of Janus kinase/signal transducer and activator of transcription and phosphoinositol 3-kinase/Akt pathways." Endocrinology **144**(2): 638-647.

Chabner, B. A. and T. G. Roberts, Jr. (2005). "Timeline: Chemotherapy and the war on cancer." Nat Rev Cancer **5**(1): 65-72.

Chang, Q., Y. Li, et al. (2002). "Constitutive activation of insulin receptor substrate 1 is a frequent event in human tumors: therapeutic implications." Cancer Res **62**(21): 6035-6038.

Corradetti, M. N. and K. L. Guan (2006). "Upstream of the mammalian target of rapamycin: do all roads pass through mTOR?" Oncogene **25**(48): 6347-6360.

Cortes, J. E. and R. Pazdur (1995). "Docetaxel." J Clin Oncol **13**(10): 2643-2655.

Creane, M., C. B. Seymour, et al. (1999). "Radiobiological effects of docetaxel (Taxotere): a potential radiation sensitizer." Int J Radiat Biol **75**(6): 731-737.

Cui, X. and A. V. Lee (2004). "Regulatory nodes that integrate and coordinate signaling as potential targets for breast cancer therapy." Clin Cancer Res **10**(1 Pt 2): 396S-401S.

Cunningham, D., Y. Humblet, et al. (2004). "Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer." N Engl J Med **351**(4): 337-345.

Davies, S. P., N. R. Helps, et al. (1995). "5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC." FEBS Lett **377**(3): 421-425.

Devita, V. T., Jr., A. A. Serpick, et al. (1970). "Combination chemotherapy in the treatment of advanced Hodgkin's disease." Ann Intern Med **73**(6): 881-895.

Dixon, K. H., B. C. Lanpher, et al. (1994). "A novel cDNA restores reduced folate carrier activity and methotrexate sensitivity to transport deficient cells." J Biol Chem **269**(1): 17-20.

Dumontet, C. and B. I. Sikic (1999). "Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death." J Clin Oncol **17**(3): 1061-1070.

Endicott, J. A. and V. Ling (1989). "The biochemistry of P-glycoprotein-mediated multidrug resistance." Annu Rev Biochem **58**: 137-171.

Feng, Z., W. Hu, et al. (2007). "The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways." Cancer Res **67**(7): 3043-3053.
T
Feng, Z., H. Zhang, et al. (2005). "The coordinate regulation of the p53 and mTOR pathways in cells." Proc Natl Acad Sci U S A **102**(23): 8204-8209.

Ferlay, J., H. R. Shin, et al. (2010). "Estimates of worlwide burden of cancer in 2008: GLOBOCAN 2008".Int J Cancer 2010

Ferlini, C., G. Raspaglio, et al. (2003). "Bcl-2 down-regulation is a novel mechanism of paclitaxel resistance." Mol Pharmacol **64**(1): 51-58.

Fetell, M. R., S. A. Grossman, et al. (1997). "Preirradiation paclitaxel in glioblastoma multiforme: efficacy, pharmacology, and drug interactions. New Approaches to Brain Tumor Therapy Central Nervous System Consortium." J Clin Oncol **15**(9): 3121-3128.

Fisher, D. E. (1994). "Apoptosis in cancer therapy: crossing the threshold." Cell **78**(4): 539-542.

Folli, F., M. J. Saad, et al. (1992). "Insulin stimulation of phosphatidylinositol 3-kinase activity and association with insulin receptor substrate 1 in liver and muscle of the intact rat." J Biol Chem **267**(31): 22171-22177.

Frei, E., 3rd, M. Karon, et al. (1965). "The effectiveness of combinations of antileukemic agents in inducing and maintaining remission in children with acute leukemia." Blood **26**(5): 642-656.

Gamble, J. and G. D. Lopaschuk (1997). "Insulin inhibition of 5' adenosine monophosphate-activated protein kinase in the heart results in activation of acetyl

coenzyme A carboxylase and inhibition of fatty acid oxidation." Metabolism **46**(11): 1270-1274.

Ganansia-Leymarie, V., P. Bischoff, et al. (2003). "Signal transduction pathways of taxanes-induced apoptosis." Curr Med Chem Anticancer Agents **3**(4): 291-306.

Gao, X., Y. Zhang, et al. (2002). "Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling." Nat Cell Biol **4**(9): 699-704.

Gilman, A. (1963). "The initial clinical trial of nitrogen mustard." Am J Surg **105**: 574-578.

Gnarra, J. R., K. Tory, et al. (1994). "Mutations of the VHL tumour suppressor gene in renal carcinoma." Nat Genet **7**(1): 85-90.

Goldenberg, G. J., C. L. Vanstone, et al. (1970). "Evidence for a transport carrier of nitrogen mustard in nitrogen mustard-sensitive and -resistant L5178Y lymphoblasts." Cancer Res **30**(9): 2285-2291.

Gottesman, M. M. and I. Pastan (1993). "Biochemistry of multidrug resistance mediated by the multidrug transporter." Annu Rev Biochem **62**: 385-427.

Gough, N. R. and L. B. Ray (2002). "Mapping cellular signaling." Sci STKE **2002**(135): EG8.

Gueritte-Voegelein, F., D. Guenard, et al. (1991). "Relationships between the structure of taxol analogues and their antimitotic activity." J Med Chem **34**(3): 992-998.

Guertin, D. A. and D. M. Sabatini (2005). "An expanding role for mTOR in cancer." Trends Mol Med **11**(8): 353-361.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hardie, D. G. (2004). "The AMP-activated protein kinase pathway--new players upstream and downstream." J Cell Sci **117**(Pt 23): 5479-5487.

Hardie, D. G. and D. Carling (1997). "The AMP-activated protein kinase--fuel gauge of the mammalian cell?" Eur J Biochem **246**(2): 259-273.

Hardie, D. G., J. W. Scott, et al. (2003). "Management of cellular energy by the AMP-activated protein kinase system." FEBS Lett **546**(1): 113-120.

Hawley, S. A., M. Davison, et al. (1996). "Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase." J Biol Chem **271**(44): 27879-27887.

Hickman, J. A. (1996). "Apoptosis and chemotherapy resistance." Eur J Cancer **32A**(6): 921-926.

Hill, B. T., B. D. Bailey, et al. (1979). "Characteristics of transport of 4-amino antifolates and folate compounds by two lines of L5178Y lymphoblasts, one with impaired transport of methotrexate." Cancer Res **39**(7 Pt 1): 2440-2446.

Hitchings, G. H. and G. B. Elion (1954). "The chemistry and biochemistry of purine analogs." Ann N Y Acad Sci **60**(2): 195-199.

Hoeijmakers, J. H. (2001). "Genome maintenance mechanisms for preventing cancer." Nature **411**(6835): 366-374.

.

Hoff, P. M., V. Valero, et al. (1998). "Hand-foot syndrome following prolonged infusion of high doses of vinorelbine." Cancer **82**(5): 965-969.

Horman, S., G. Browne, et al. (2002). "Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis." Curr Biol **12**(16): 1419-1423.

Horman, S., D. Vertommen, et al. (2006). "Insulin antagonizes ischemia-induced Thr172 phosphorylation of AMP-activated protein kinase alpha-subunits in heart via hierarchical phosphorylation of Ser485/491." J Biol Chem **281**(9): 5335-5340.

Hughes, T. P., J. Kaeda, et al. (2003). "Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia." N Engl J Med **349**(15): 1423-1432.

Inoki, K., Y. Li, et al. (2002). "TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling." Nat Cell Biol **4**(9): 648-657.

Inoki, K., T. Zhu, et al. (2003). "TSC2 mediates cellular energy response to control cell growth and survival." Cell **115**(5): 577-590.

Johnson, I. S., J. G. Armstrong, et al. (1963). "The Vinca Alkaloids: A New Class Of Oncolytic Agents." Cancer Res **23**: 1390-1427.

Jordan, A., J. A. Hadfield, et al. (1998). "Tubulin as a target for anticancer drugs: agents which interact with the mitotic spindle." Med Res Rev **18**(4): 259-296.

Jordan, M. A., K. Wendell, et al. (1996). "Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death." Cancer Res **56**(4): 816-825.

Jordan, M. A. and L. Wilson (2004). "Microtubules as a target for anticancer drugs." Nat Rev Cancer **4**(4): 253-265.

Katsumata, N. (2003). "Docetaxel: an alternative taxane in ovarian cancer." Br J Cancer **89 Suppl 3**: S9-S15.

Kimura, N., C. Tokunaga, et al. (2003). "A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway." Genes Cells **8**(1): 65-79.

Konishi, Y., M. Lehtinen, et al. (2002). "Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery." Mol Cell **9**(5): 1005-1016.

Kovacic, S., C. L. Soltys, et al. (2003). "Akt activity negatively regulates phosphorylation of AMP-activated protein kinase in the heart." J Biol Chem **278**(41): 39422-39427.

Krause, U., L. Bertrand, et al. (2002). "Control of p70 ribosomal protein S6 kinase and acetyl-CoA carboxylase by AMP-activated protein kinase and protein phosphatases in isolated hepatocytes." Eur J Biochem **269**(15): 3751-3759.

Kris, M. G., R. B. Natale, et al. (2003). "Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial." JAMA **290**(16): 2149-2158.

Lannon, C. L., M. J. Martin, et al. (2004). "A highly conserved NTRK3 C-terminal sequence in the ETV6-NTRK3 oncoprotein binds the phosphotyrosine binding domain of insulin receptor substrate-1: an essential interaction for transformation." J Biol Chem **279**(8): 6225-6234.

Lataste, H., V. Senilh, et al. (1984). "Relationships between the structures of taxol and baccatine III derivatives and their in vitro action on the disassembly of mammalian brain and Physarum amoebal microtubules." Proc Natl Acad Sci U S A **81**(13): 4090-4094.

Lawlor, M. A. and D. R. Alessi (2001). "PKB/Akt: a key mediator of cell proliferation, survival and insulin responses?" J Cell Sci **114**(Pt 16): 2903-2910.

Lee, Y. H., J. Giraud, et al. (2003). "c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade." J Biol Chem **278**(5): 2896-2902.

Levchenko, A. (2001). "Computational cell biology in the post-genomic era." Mol Biol Rep **28**(2): 83-89.

Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell **88**(3): 323-331.

Lietzke, S. E., S. Bose, et al. (2000). "Structural basis of 3-phosphoinositide recognition by pleckstrin homology domains." Mol Cell **6**(2): 385-394.

Lindahl, T. (1993). "Instability and decay of the primary structure of DNA." Nature **362**(6422): 709-715.

Manning, B. D. and L. C. Cantley (2003). "Rheb fills a GAP between TSC and TOR." Trends Biochem Sci **28**(11): 573-576.

Mason, K. A., N. R. Hunter, et al. (1997). "Docetaxel enhances tumor radioresponse in vivo." Clin Cancer Res **3**(12 Pt 1): 2431-2438.

McCormick, F. (2004). "Cancer: survival pathways meet their end." Nature **428**(6980): 267-269.

McGuire, W. P., W. J. Hoskins, et al. (1996). "Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer." N Engl J Med **334**(1): 1-6.

Miranda, C., A. Greco, et al. (2001). "IRS-1 and IRS-2 are recruited by TrkA receptor and oncogenic TRK-T1." J Cell Physiol **186**(1): 35-46.

Moertel, C. G., T. R. Fleming, et al. (1990). "Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma." N Engl J Med **322**(6): 352-358.

Moos, P. J. and F. A. Fitzpatrick (1998). "Taxane-mediated gene induction is independent of microtubule stabilization: induction of transcription regulators and enzymes that modulate inflammation and apoptosis." Proc Natl Acad Sci U S A **95**(7): 3896-3901.

Moxley, J. H., 3rd, V. T. De Vita, et al. (1967). "Intensive combination chemotherapy and X-irradiation in Hodgkin's disease." Cancer Res **27**(7): 1258-1263.

Neid, M., K. Datta, et al. (2004). "Role of insulin receptor substrates and protein kinase C-zeta in vascular permeability factor/vascular endothelial growth factor expression in pancreatic cancer cells." J Biol Chem **279**(6): 3941-3948.

Niero, A., E. Emiliani, et al. (1999). "Paclitaxel and radiotherapy: sequence-dependent efficacy--a preclinical model." Clin Cancer Res **5**(8): 2213-2222.

Nogales, E., S. G. Wolf, et al. (1998). "Structure of the alpha beta tubulin dimer by electron crystallography." Nature **391**(6663): 199-203.

Nowak, A. K., N. R. Wilcken, et al. (2004). "Systematic review of taxane-containing versus non-taxane-containing regimens for adjuvant and neoadjuvant treatment of early breast cancer." Lancet Oncol **5**(6): 372-380.

Perez, R. P. (1998). "Cellular and molecular determinants of cisplatin resistance." Eur J Cancer **34**(10): 1535-1542.

Poruchynsky, M. S., E. E. Wang, et al. (1998). "Bcl-xL is phosphorylated in malignant cells following microtubule disruption." Cancer Res **58**(15): 3331-3338.

Prada, P. O., H. G. Zecchin, et al. (2005). "Western diet modulates insulin signaling, c-Jun N-terminal kinase activity, and insulin receptor substrate-1ser307 phosphorylation in a tissue-specific fashion." Endocrinology **146**(3): 1576-1587.

Rao, S., N. E. Krauss, et al. (1994). "3'-(p-azidobenzamido)taxol photolabels the N-terminal 31 amino acids of beta-tubulin." J Biol Chem **269**(5): 3132-3134.

Rao, S., G. A. Orr, et al. (1995). "Characterization of the taxol binding site on the microtubule. 2-(m-Azidobenzoyl)taxol photolabels a peptide (amino acids 217-231) of beta-tubulin." J Biol Chem **270**(35): 20235-20238.

and H. Maiato (2004). "Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint." Dev Cell **7**(5): 637-651.

Roberts, T. G., Jr. and B. A. Chabner (2004). "Beyond fast track for drug approvals." N Engl J Med **351**(5): 501-505.

Rodi, D. J., R. W. Janes, et al. (1999). "Screening of a library of phage-displayed peptides identifies human bcl-2 as a taxol-binding protein." J Mol Biol **285**(1): 197-203.

Rowinsky, E. K. and R. C. Donehower (1995). "Paclitaxel (taxol)." N Engl J Med **332**(15): 1004-1014.

Rowinsky, E. K., R. C. Donehower, et al. (1988). "Microtubule changes and cytotoxicity in leukemic cell lines treated with taxol." Cancer Res **48**(14): 4093-4100.

Saad, M. J., F. Folli, et al. (1993). "Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats." J Clin Invest **92**(4): 2065-2072.

Sarbassov, D. D., S. M. Ali, et al. (2005). "Growing roles for the mTOR pathway." Curr Opin Cell Biol **17**(6): 596-603.

Sarbassov, D. D., D. A. Guertin, et al. (2005). "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." Science **307**(5712): 1098-1101.

L. J., X. Gao, et al. (2003). "Rheb promotes cell growth as a component of the insulin/TOR signalling network." Nat Cell Biol **5**(6): 566-571.

Scatena, C. D., Z. A. Stewart, et al. (1998). "Mitotic phosphorylation of Bcl-2 during normal cell cycle progression and Taxol-induced growth arrest." J Biol Chem **273**(46): 30777-30784.

Schiff, P. B. and S. B. Horwitz (1980). "Taxol stabilizes microtubules in mouse fibroblast cells." Proc Natl Acad Sci U S A **77**(3): 1561-1565.

Schulze, E., D. J. Asai, et al. (1987). "Posttranslational modification and microtubule stability." J Cell Biol **105**(5): 2167-2177.

Shepherd, P. R., B. T. Nave, et al. (1995). "Insulin stimulation of glycogen synthesis and glycogen synthase activity is blocked by wortmannin and rapamycin in 3T3-L1 adipocytes: evidence for the involvement of phosphoinositide 3-kinase and p70 ribosomal protein-S6 kinase." Biochem J **305**(Pt 1): 25-28.

Sirotnak, F. M., D. M. Moccio, et al. (1981). "Relative frequency and kinetic properties of transport-defective phenotypes among methotrexate-resistant L1210 clonal cell lines derived in vivo." Cancer Res **41**(11 Pt 1): 4447-4452.

Skipper, H. E. and D. P. Griswold (1984). "Frank M. Schabel 1918-1983." Cancer Res **44**(2): 871-872.

Skipper, H. E., F. M. Schabel, Jr., et al. (1964). "Experimental Evaluation Of Potential Anticancer Agents. Xiii. On The Criteria And Kinetics Associated With "Curability" Of Experimental Leukemia." Cancer Chemother Rep **35**: 1-111.

Strobel, T., L. Swanson, et al. (1996). "BAX enhances paclitaxel-induced apoptosis through a p53-independent pathway." Proc Natl Acad Sci U S A **93**(24): 14094-14099.

Tee, A. R. and J. Blenis (2005). "mTOR, translational control and human disease." Semin Cell Dev Biol **16**(1): 29-37.

Thirone, A. C., J. B. Carvalheira, et al. (2004). "Regulation of Cbl-associated protein/Cbl pathway in muscle and adipose tissues of two animal models of insulin resistance." Endocrinology **145**(1): 281-293.

Tishler, R. B., C. R. Geard, et al. (1992). "Taxol sensitizes human astrocytoma cells to radiation." Cancer Res **52**(12): 3495-3497.

Torres, K. and S. B. Horwitz (1998). "Mechanisms of Taxol-induced cell death are concentration dependent." Cancer Res **58**(16): 3620-3626.

Towler, M. C. and D. G. Hardie (2007). "AMP-activated protein kinase in metabolic control and insulin signaling." Circ Res **100**(3): 328-341.

Traina, F., J. B. Carvalheira, et al. (2003). "BCR-ABL binds to IRS-1 and IRS-1 phosphorylation is inhibited by imatinib in K562 cells." FEBS Lett **535**(1-3): 17-22.

Vassetzky, Y. S., G. C. Alghisi, et al. (1995). "DNA topoisomerase II mutations and resistance to anti-tumor drugs." Bioessays **17**(9): 767-774.

Venter, J. C. (2000). "Genomic impact on pharmaceutical development." Novartis Found Symp **229**: 14-15; discussion 15-18.

Venter, J. C., M. D. Adams, et al. (2001). "The sequence of the human genome." Science **291**(5507): 1304-1351.

Vivanco, I. and C. L. Sawyers (2002). "The phosphatidylinositol 3-Kinase AKT pathway in human cancer." Nat Rev Cancer **2**(7): 489-501.

Vousden, K. H. and D. P. Lane (2007). "p53 in health and disease." Nat Rev Mol Cell Biol **8**(4): 275-283.

Wang, L. G., X. M. Liu, et al. (1999). "The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review." Cancer Chemother Pharmacol **44**(5): 355-361.

Wani, M. C., H. L. Taylor, et al. (1971). "Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia." J Am Chem Soc **93**(9): 2325-2327.

Wendel, H. G., E. De Stanchina, et al. (2004). "Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy." Nature **428**(6980): 332-337.

Wills, L., P. W. Clutterbuck, et al. (1937). "A new factor in the production and cure of macrocytic anaemias and its relation to other haemopoietic principles curative in pernicious anaemia." Biochem J **31**(11): 2136-2147.

Winder, W. W. and D. G. Hardie (1996). "Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise." Am J Physiol **270**(2 Pt 1): E299-304.

Wullschleger, S., R. Loewith, et al. (2006). "TOR signaling in growth and metabolism." Cell **124**(3): 471-484.

Yamaizumi, M. and T. Sugano (1994). "U.v.-induced nuclear accumulation of p53 is evoked through DNA damage of actively transcribed genes independent of the cell cycle." Oncogene **9**(10): 2775-2784.

Zhang, C. C., J. M. Yang, et al. (1999). "DNA damage increases sensitivity to vinca alkaloids and decreases sensitivity to taxanes through p53-dependent repression of microtubule-associated protein 4." Cancer Res **59**(15): 3663-3670.

Zunino, F., P. Perego, et al. (1997). "Role of apoptotic response in cellular resistance to cytotoxic agents." Pharmacol Ther **76**(1-3): 177-185.

Zwick, E., J. Bange, et al. (2001). "Receptor tyrosine kinase signalling as a target for cancer intervention strategies." Endocr Relat Cancer **8**(3): 161-173.

APÊNDICE I

Epidemiological and molecular mechanisms aspects linking obesity and cancer

Mecanismos epidemiológicos e moleculares que associam obesidade e câncer

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ABSTRACT

About 25% of cancer cases globally are due to excess weight and a sedentary lifestyle. These results are alarming, as the world knows a pandemy of obesity and, in consequence, insulin resistance. Obesity may increase risk for various cancers by several mechanisms, including increasing sex and metabolic hormones, and inflammation. Here, we present a review of epidemiological and molecular evidences linking obesity and cancer – particularly colorectal, postmenopausal breast, endometrial, pancreatic, high grade prostate, hepatocellular, gallbladder, kidney and esophageal adenocarcinoma. The expected striking increase in the incidence of cancer in the near future related to obesity turns the knowledge of this field of great impact as it is needed to the development of strategies to prevent and treat this disease. Arq Bras Endocrinol Metab. 2009;53(2):213-226.

Keywords

Neoplasms; obesity; overweight; insulin resistance

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RESUMO

Aproximadamente 25% dos casos de câncer são decorrentes do excesso de peso e do modo de vida sedentário. Esses resultados são alarmantes, pois o mundo vive uma pandemia de obesidade e, consequentemente, de resistência à insulina. A obesidade pode aumentar o risco de vários tipos de câncer por diversos mecanismos, incluindo aumento dos hormônios sexuais e metabólicos, e de inflamação. Neste trabalho, apresentamos uma revisão das evidências epidemiológicas e moleculares que relacionam a obesidade ao câncer – em particular aos cânceres colorretal, mamário na pós-menopausa, endometrial, pancreático, prostático avançado, hepatocelular, de bexiga, renal e esofágico. O aumento esperado da incidência de câncer relacionado à obesidade em um futuro próximo torna o conhecimento dessa área de grande importância, uma vez que este é fundamental para o desenvolvimento de estratégias preventivas e terapêuticas para a doença. Arq Bras Endocrinol Metab. 2009;53(2):213-226.

Descritores

Neoplasias; obesidade; sobrepeso; resistência à insulina

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Received in Feb/13/2009
Accepted in Feb/15/2009

INTRODUCTION

Since the 1980's, the world has been living a striking increase in the prevalence of overweight and obesity. This phenomenon has had it's beginning in developed countries, but, nowadays, it is also common to many other populous regions over the world, as Asia and Latin America, and it is becoming a Public Health concern (1,2). By the end of the last millennium, nearly two-thirds of adults in the United States were overweight or obese, and their prevalence continues to increase in this decade (3). The incidence of type 2 diabetes during this same period of time has mirrored the obesity epidemic, and that is presumed to be a direct result of it.

There are epidemiologic studies linking adiposity and the risk of several adult cancers. Investigation of pathophysiological mechanisms and clinical interven-

tions applied to the process of excess body weight and carcinogenesis has, thus, become an interesting research field. This review outlines the epidemiological and clinical evidence implicating excess body weight both in increased cancer risk and its impact on mortality in certain neoplasias. Some hypothesis explaining these epidemiological observations are explored, specially the metabolic and endocrine effects of obesity, and the alterations that they induce in the production of peptide, steroid hormones and inflammation pathways.

EPIDEMIOLOGY OF ADIPOSITY AND CANCER RISK

Evidences from epidemiological studies indicate that adiposity contributes to an elevated risk of developing some cancers and may also influence disease outcomes. Both the International Agency for Research on Cancer (IARC) (4) and, more recently, the World Cancer Research Fund (WCRF) (5) have concluded that, among the different cancer sites, this association is positive for esophageal adenocarcinoma, and cancers of the pancreas, colorectum, post-menopausal breast, endometrium and kidney, beyond the evidence of a probable association with risk of gallbladder cancer.

These results were further confirmed to the high prevalent cancers listed above and extended to reveal associations with less common malignancies in a large British cohort, the Million Women Study, and in a multiethnic systematic review and meta-analysis (table 1). It is important to observe that this multiethnic analysis found a constant association between body mass index (BMI) and cancer across populations, except for pre-menopausal breast cancer, in which there has been observed a positive association with increased BMI in Asia-Pacific populations, but an inverse association in the other regions (6,7). There are also some other constant similarities in most of the epidemiologic evidences. The effect of BMI on risk differed significantly according to menopausal status (and, by extrapolation, hormonal influences) as follows: increased risk in pre-menopausal women for colorectal cancer and malignant melanoma, while increased risk in post-menopausal women for breast and endometrial cancers (6). As a concern to intergender comparisons, there are differences in associations at some sites, notably colon, rectum and kidney (8).

Table 1. Summary of estimated trend in the relative risk of cancer incidence by type, adapted from two recent large studies (6,7). Data in italic have not reached significance

	British cohort	Multiethnic meta-analysis	
Incident cases	45,037	282,137	
	Trend in relative risk per 10 kg/m²	Trend in relative risk per 5 kg/m²	
Cancer site	Female	Female	Male
Endometrium	2.89	1.59	NA
Oesophageal adenocarcinoma	2.38	1.51	1.52
Renal	1.53	1.34	1.24
Leukemia	1.50	1.17	1.08
Multiple myeloma	1.31	1.11	1.11
Non-Hodgkin lymphoma	1.17	*1.07*	1.06
Breast (post-menopausal)	1.4	1.12	NA
Colorectum (pre-menopausal)	1.61	NA	NA
Colon	NA	1.09	1.24
Rectum	NA	*1.02*	1.09
Gallbladder	NA	1.59	*1.09*
Prostate	NA	NA	*1.03*
Pancreas	1,24	1.12	*1.07*
Ovary	1,14	*1.03*	NA
Liver	NA	*1.24*	*1.07*
Stomach	*0.90*	*1.04*	*0.97*

EXCESS BODY WEIGHT AND CANCER OUTCOME

Given that BMI is consistently associated with cancer risk at several sites, it is not surprising that increased adiposity may have a negative effect on treatment outcome and ultimate survival. Data from a large American cohort published by Calle and cols. (9) was the germinal evidence that obesity may be an unfavorable prognostic factor in patients diagnosed with cancer. The heaviest members of this cohort (those with a BMI index of, at least, 40) had death rates from all cancers combined, and they were 52% higher for men, and 62% higher for women, than the rates in men and women of normal weight. These results were recently confirmed by Reeves and cols. (6) in another large British cohort. These observations in human studies are supported by pre-clinical data that observed different outcome in models of cancer. In these studies, they were worse in diet-induced obesity animal models (10), and better in energy restriction ones (11).

OBESITY RELATED CANCERS

Colorectal cancer

Obesity has been associated with higher risk of colorectal cancer. However, there are important differences related to gender and sites. The association between BMI and risk for colon cancer is positive in men, RR = 1.24, but the evidences are weaker in women, RR = 1.09 (8). Further, the association with rectal cancer is also weaker and present only in men, RR = 1.09.

There are some hypotheses for this gender difference. One of them is that central adiposity, quite more frequent in men, may have an important role in the pathophysiology underlying the association between abdominal obesity and increased colon cancer risk. This could be expected, once abdominal obesity has been shown to be more strongly associated with metabolic abnormalities than gluteofemoral obesity (12). This hypothesis has been supported by epidemiological evidences that associates increased waist circumference or increased waist-hip ratio with colon cancer risk in men and women, whereas body weight and BMI are associated with colon cancer risk in men but not in women (13,14). By contrast to colon cancer risk, these anthropometric results were not reproduced as risk factor for rectal cancer. These epidemiological data are supported by consistent results that associate insulin resistance and subsequent hyperinsulinemia as risk factors for colon cancer (15).

Increased levels of bioavailable insulin-like growth factor (IGF)-1, which is known to have cancer promoting effects, are related to hyperinsulinemia. In addition, insulin interacts with the axis, by reducing the synthesis of IGF binding proteins (IGFBP) (16). The IGF-1 system has been linked to colorectal malignancy by a convergence of data from epidemiological, clinical and laboratory-based sources (16-18). Recent results have also implicated higher levels of prediagnosis plasma C-peptide and lower levels of prediagnosis plasma IGFBP-1 with increased mortality among patients with surgically resected colorectal cancer (19). These results lead to the hypothesis that circulating insulin and IGFBP-1 are potential mediators of the association between lifestyle factors and mortality after colorectal cancer resection.

In regard to adipose tissue-derived cytokines and hormones, collectively named adipokines, recent results may involve them in tumourigenesis. First, leptin, whose circulating levels are closely related to the amount of adipose tissue and is also related to insulin resistance, has been related to progression of colon cancer in experimental studies (16,20-22). This hormone conveys information to the brain about the size of energy storage and its levels are increased by overfeeding (23). In contrast, energy restriction, a well-established protective factor against cancer (24), decreases this hormone levels (24,25). In light of this evidence, leptin was suggested as a link between obesity and colon cancer. This hypothesis is supported by two case-control studies (22,26) that demonstrated significant associations of leptin with colon cancer risk, but not rectal cancer. By contrast, adiponectin, an adipokine decreased in obesity, is inversely associated with the development of insulin resistance and has a strong anti-inflammatory function (3). There are controversial data relating low plasma adiponectin levels with higher risk of colorectal cancer in men, and there is need to further prospective studies to investigate this association (27,28).

Breast cancer

The evidence linking obesity to breast cancer risk is dependent on the menopausal status. There is consistent evidence for this association in post-menopausal women, but not in pre-menopausal ones. In fact, there are some registries of a modest reduction in risk among women with elevated BMI, which could be because of a tendency for these women to have anovulatory menstrual cycles and lower levels of circulating steroid hormones – notably of progesterone and estradiol.

In addition, studies that examined both factors have found that adult weight gain is generally more associated with increase in risk of post-menopausal breast cancer than BMI (29); this could also be an effect of anovulatory menstrual cycles in the young obese women. As regards to breast cancer outcome, BMI ≥ 40 kg/m² is associated with higher mortality, RR = 2.12 (9). This worse prognosis may be because of true biological effect of adiposity on survival or secondary to delayed diagnosis in heavier women.

As concerns to sex steroids, the higher expression of aromatase, which produces estrogens from androgenic precursors, may have an important role in the pathophysiology underlying the association between post-menopausal breast cancer and cancer risk in obese women (8). There is convincing evidence relating the association of BMI with post-menopausal breast cancer risk to be almost entirely mediated by elevated blood levels of estradiol (30). These analyses also demonstrated that elevated blood concentrations of androgens are associated with increased risk of breast cancer in both pre and post-menopausal women, and, thus, androgens may be potential candidates linking obesity and breast cancer. In contrast to men, testosterone concentrations are positively related with obesity in women (30).

Besides steroids influences, several studies associated hyperinsulinemia, measured as high circulating levels of serum C-peptide, with elevated risk of post-menopausal breast cancer (31-33), although these associations were inconsistent with pre-menopausal breast cancer (32-34). In addition, cohort studies and a meta-analysis support increased IGF-1 levels, an indirect effect of hyperinsulinemia, as a risk factor for pre-menopausal breast cancer (17,35). Furthermore, insulin resistance is an adverse prognostic factor for breast cancer (36).

In regard to adipokines, a recent review from Vona-Davis and Rose (37) has found inconsistent results related to leptin as breast cancer risk factor. In contrast, two case-control studies concurrently reported inverse association between adiponectin and breast cancer; significant for both pre and post-menopausal in one (38), and for post-menopausal women only in the other (39). Clearly, prospective studies are needed to examine the role of obesity biomarkers in the development of breast cancer.

Endometrial carcinoma

The strongest association, 2.5-3.0-fold increase in risk, between obesity and cancer risk is reported to endometrial carcinoma (6,8). The pathophysiology underlying this association, like for breast cancer, is associated to estrogens exposition. This hypothesis is supported by epidemiological studies that have related high levels of plasma estrone and estradiol as risk factors for endometrial cancer risk in post-menopausal women. In addition, hyperandrogenism may also play a central role linking obesity to endometrial cancer risk in pre-menopausal women, as it leads to diminished levels of progesterone and continuous anovulation. The hypothesis is that lack of progesterone promotes unopposed estrogen exposition and continuous proliferation stimulus to endometrial cell lines, which may increase the risk of endometrial cancer, whereas, in post-menopausal, excess weight may continue to increase the risk primarily through elevated plasma levels of bioavailable estrogens, and secondary to higher expression of aromatase, in the absence of ovarian progesterone synthesis. Furthermore, besides a rise in estrogens and androgens, excess weight leads to a decrease in plasma sex hormone-binding globulin (40). In a multicenter prospective study in post-menopausal women (41), circulating estrogens and androgens were found to be positively associated with endometrial cancer risk, and an inverse association was reported for sex hormone-binding globulin.

With regard to insulin resistance, it was speculated that elevated estrogens and low progesterone promote increase in IGF-1 bioactivity within endometrial tissue, resulting from estrogen-induced IGF-1 synthesis and reductions in IGFBP-1, leading to the development and growth of endometrial tumours (40). In addition, biomarkers of hyperinsulinemia, as C-peptide, have been shown to be associated with increased endometrial cancer risk (42).

Renal cancer

There are several evidences that link obesity as a risk factor for renal cell cancer (12,43). Most of them found a dose-response relationship with increasing weight or BMI (44). However, few studies have observed that fat distribution does not predict renal cell cancer risk beyond adiposity in general (45). As regards to gender, there is an unexplained difference with stronger association in women than in men (44).

Hypertension and diabetes, both of which are risk factors for renal cell cancer, are also associated with obesity (12,46). However, some evidence suggested that obesity

increases the risk of renal cell cancer even independently of blood pressure levels, indicating that hypertension and obesity might influence renal cell cancer through different mechanisms (12).

The pathophysiology underlying the association of obesity and renal cell cancer remains unclear. Very recently, lower adiponectin levels were observed in individuals with renal cell cancer when compared with healthy controls (47). There is clearly a need for additional studies of this issue with a larger number of patients.

Esophageal and gastric carcinoma

There is a marked change in esophageal and gastric carcinomas epidemiology. Whereas the incidence rates of esophageal adenocarcinoma and gastric cardia have risen in recent decades, they remained stable for esophageal squamous cell carcinoma and have declined steadily for noncardia gastric adenocarcinoma (48). This rise in incidence has partly been attributed to the rise in the prevalence of obesity. Some evidence from cohorts and meta-analysis have recently confirmed the association between obesity and risk of esophageal adenocarcinoma (6,7,49). Regarding the risk for gastric cardia adenocarcinoma in this analysis, a high BMI was weakly associated with the risk of cardia adenocarcinoma (RR = 1.5). Recently, these results were reinforced by a large American cohort, which has found an increased risk for gastric cardia adenocarcinoma for people with BMI ≥ 35 kg/m². In contrast, there has not been found any association with gastric non-cardia adenocarcinoma (50).

Thus, there is impressive evidence to link obesity as a risk factor for esophageal and gastric cardia cancer. However, BMI does not explain the intergender differences found in the incidence rates of these cancers, which are more frequent in white men than in women. One hypothesis is that abdominal obesity could explain this discordance. A recent case-control study examined the distribution of obesity as a risk factor for these types of cancer and has found strongly association between elevated abdominal diameter and increased risk of esophageal adenocarcinoma. However, this association was not confirmed with the risk of gastric cardia adenocarcinomas (51). Further prospective studies are necessary to confirm this hypothesis.

At present, Barrett's esophagus, characterized by replacement of squamous epithelium with columnar epithelium, is the most common cause of esophageal adenocarcinoma. The pathophysiology of Barrett's esophagus involves gastroesophageal reflux and esophagitis (52). Further, obesity is related to esophagitis, and this association is not present only in symptomatic gastroesophageal reflux obese individuals. The exact role that obesity plays within these processes remains to be defined.

Pancreatic carcinoma

For some time, there was controversy between the link of obesity and pancreatic carcinoma. Early case-control studies have found weak association, but this is believed to have been biased because of high mortality or reliance on proxy interviews (53). A considerable number of prospective studies and meta-analysis have examined this subject during the last years (9,54), and have shown positive results (13). Recently, this results were reinforced by The Women's Health Initiative in The United States, which has found a 70% excess risk of pancreatic cancer for women in the highest quintile of waist-to-hip ratio compared with women in the lowest quintile (55).

Several evidence implicates hyperinsulinemia and hyperglycemia in the pathophysiology of pancreatic cancer (56). However, as it matters to IGF axis, a nested case-control study within four large cohort studies has not found associations between pre-diagnostic plasma levels of IGF-1, IGF-2 and IGFBP-3 and pancreatic cancer risk (57). The exact role that obesity and insulin resistance play within these processes remains to be defined.

Prostate cancer

Epidemiological evidence is conflicting and has failed to show overall significant associations between obesity and the risk of prostate cancer (4), although a meta-analysis has suggested a weak significant positive association, with an estimated increase in prostate cancer risk of 1.05 per 5 kg/m² (58). However, concerning to prostate cancer outcomes, there is convincing evidence which indicates that obese men with prostate cancer are more likely to have aggressive disease that recurs after radical prostatectomy than non-obese men (59).

Some studies have positively associated high IGF-1 circulating levels with prostate cancer risk, whereas total IGFBP-3 levels were negatively associated (60). There is clearly a need for additional studies of this issue with a larger number of patients to examine the possible effects of obesity and insulin resistance in the pathophysiology of pancreatic cancer.

Hepatocellular and gallbladder cancer

The relationship between obesity and risk of gallbladder cancer has recently been investigated in some meta-analyses and cohort studies (6,7,61). Most of them have found a dose-response relationship with increasing weight or BMI (RR = 1.15 in those who were overweight, and RR = 1.66 in those who were obese). The risk was stronger for women (RR = 1.88) than for men (RR = 1.35). The mechanisms by which obesity may affect gallbladder cancer risk are unclear until now. However, gallstone formation is a major risk factor for this disease and obesity is one of the factors that increase gallstone formation.

Obesity as well as type 2 diabetes are also likely to be risk factors for hepatocellular cancer (62). It is suggested that the increase in the incidence of non-alcoholic fatty liver disease could be the underlying mechanism related to the recent increase in risk for hepatocellular carcinoma, once it may progress to steatohepatitis and cirrhosis.

OTHER CANCERS

Lung cancer

Several studies reported an inverse association between obesity and lung cancer, although this data may be secondary to confounding effects of smoking. However, in non-smoking populations, there has not been found any association to link obesity as a risk factor for lung cancer (9,63).

Cervical carcinoma

The literature is exiguous about the association between obesity and cervical cancer. Overall there is no association reported (28), however an American case-control study found an increased risk of adenocarcinomas of the cervix, not extensive to squamous cell carcinomas of the cervix (RR = 1.6) (64). Clearly, additional studies of this issue with a larger number of patients are needed to investigate the association between obesity and cervical cancer.

Ovarian cancer

A recent systematic review has found a weak (1.3-fold) increase association between obesity and risk for ovarian cancer (65). It was speculated that the association between obesity and weight gain could be restricted to some subtypes of ovarian cancer, but this hypothesis was not confirmed in an Australian case-control study, which has found no association with BMI or weight gain for any of the histological subtypes (66). These results add to the current evidence that obesity increases a woman's risk of developing distinct histological subtypes of ovarian cancer.

Hematopoietic neoplasias

Several evidence from meta-analysis or large cohort studies have found weak association between obesity and lymphohaematopoietic cancers, including lymphomas, acute and chronic leukaemia (67) and multiple myeloma (6,7,68). Further prospective studies of this issue with a larger number of patients are needed to examine the possible role of obesity as a risk factor for hematopoietic neoplasias.

SUMMARY OF EPIDEMIOLOGICAL EVIDENCE

After reviewing a large number of literatures, there is currently sufficient evidence that obesity increases the risk for esophageal adenocarcinoma, and cancers of the pancreas, colorectum, post-menopausal breast, endometrium and kidney, beyond evidence of a probable association with risk of gallbladder cancer and hepatocellular cancer. The more recently published studies suggest that obesity may also increase the risk of advanced-stage prostate cancer. It is also suggested that obesity is a risk factor for hematopoietic neoplasias. However, this association may be weak, and there is need for further studies to explore the molecular mechanisms of obesity that could explain these results.

PROPOSED MECHANISMS

Endogenous hormones

The association between excess body weight with cancer risk may be explained by alterations in the metabolism of endogenous hormones – including insulin, insulin-like growth factors and sex steroids – which can lead to distortion of the normal balance between cell proliferation, differentiation, and apoptosis. However, the pathophysiological and biological mechanisms underpinning these associations are only starting to be understood.

Tumour genesis occurs as a result of mutations that confer a set of cancer-specific hallmarks, including self-sufficiency in growth signals and evasion of apoptosis.

Many cancer-causing genes encode protein kinases; indeed, the protein kinase domain is the most commonly found functional domain known in cancer genes. As protein kinases occupy apical positions in signal-transduction cascades, integrate with many other signaling pathways and regulate the activity or abundance of transcription factors, the cellular effects of aberrant protein kinases activity are wide-ranging.

The functional importance of this acquired capability for the manifestation of the disease has been further validated by the approval of tyrosine kinase inhibitors as cancer therapeutics – most notably the ones targeting the BCR Abl and cKIT signaling pathways. The pioneer of the clinical proof-of-concept for tyrosine kinase inhibitors is Imatinib (Gleevec, Novartis) targeting the BCR Abl and cKIT receptor (69). Imatinib has been approved for treating patients with chronic myeloid leukemia and gastrointestinal stromal tumor. Numerous ongoing clinical trials seek to expand the applications of each of tyrosine kinase pathway inhibitors, and dozens of other tyrosine kinase inhibitors are being clinically evaluated.

The same is true for endogenous hormones signaling, which integrates with others signal-transduction cascades to control a variety of processes – including gene expression. Recent years have seen a growing appreciation of the extent to which components of endogenous hormones are remodeled or deregulated in cancer. Whether these changes are drivers that are required to sustain the transformed phenotype remains to be established. In this section, we review the core components of the endogenous hormones signaling systems, focusing on the role of insulin, insulin-like growth factor and sex steroids in one crucial aspect of the cancer phenotype – control of cell proliferation.

Insulin and insulin-like growth factor

In the early 1990's, McKeown-Eyssen (70) and Giovannucci (71) noted that the risk factors for Westernized cancer were remarkably similar to those for insulin resistance, and suggested that hyperinsulinemia might contribute to cancer development through the growth-promoting effect of elevated levels of insulin. In addition to its importance in glucose homeostasis, it is well established that insulin is a crucial hormone in anabolic processes involved in early growth and development, and may be also a strong mediator of the adverse effect of obesity on cancer prognosis.

Excess body weight, increased plasma triglyceride levels, low levels of physical activity and certain dietary patterns can all favor elevated circulating insulin levels. Chronically increased insulin concentrations reduce the synthesis of IGFBP-1 and 2, resulting in increased levels of free, IGF-1 Bio-Active®, and concomitant changes in the cellular environment, that favor tumor development (72). It is also suggested that IGF-1 can synergize with other growth factors to produce enhanced mitogenic effects, and may operate via an endocrine, paracrine or autocrine manner to regulate cell growth, cell survival, cell transformation, and cell differentiation.

Growth hormone (GH) provides the main stimulus for the synthesis of IGF-1 in liver, which is the source of over 80% of circulating IGF-1, and nutritional energy balance exerts profound and complex effects on the synthesis and biological activity of IGF-1. In type 1 diabetes patients, or in chronically fasting people, the low production of insulin, which causes a reduction in hepatic GH-receptor levels, also results in GH resistance and reduction of IGF-1 blood levels and synthesis. The IGF-1 bioavailability to tissue receptor is also reduced by the increased levels of IGFBP-1 and IGFBP-2. On the other hand, patients with type 2 diabetes, or in over nourished states, have high levels of endogenous insulin and hepatic GH-receptor, producing large amounts of IGF-1 (73). Paradoxically, however, obese people have lower blood levels of IGF-1 than normal-weight, well-nourished individuals (21). Recent studies have shown a non-linear relationship between IGF-1 and anthropometric indices of adiposity, with the highest levels of IGF-1 at a BMI of around 24-27 kg/m^2, and lower levels for men and women in either the lower or higher BMI categories (74,75). An explanation for the low IGF-1 blood levels in obese individuals, despite increased GH sensitivity of liver and other tissues, is that reductions in IGFBP-1 and IGFBP-2 levels increase the negative feedback by free IGF-1 on pituitary GH secretion, resulting in reduced synthesis of IGF-1 and reduced plasma IGF-1 concentrations (21).

Many *in vitro* and *in vivo* models provide convincing evidence for a role of IGF-1 receptor (IGF-1R) in different forms of cancer. Initial *in vitro* experiments demonstrated that increasing IGF-1 concentrations induced a dose-dependent increase in human breast cancer cell proliferation (76). *In vivo* models using mutations associated with low IGF-1 levels or reduced ligand levels showed that *in vivo* tumour growth is influenced by the IGF-1 physiology of the host (77,78). The work

by Creighton and cols. (79) showed that a pattern of gene expression induced by IGF-1 represents pathways of increased aggressiveness and possibly hormone independence in clinical breast cancers.

As shown in Figure 1, both insulin and IGF-1 activate the important tyrosine kinase growth receptor pathway, insulin receptor (IR) and IGF-1R, respectively, as well as the hybrid IGF-1/IR – all of which are expressed at higher levels in malignant cells and resistant to down-regulation typical of receptors in nonmalignant cells exposed to insulin (80). Activation of these receptors results in up-regulation of insulin response substrate-1 (IRS-1), leading to downstream activation of the mitogenic-activated protein (MAP) kinase pathway and the phosphoinositide-3 kinase/Akt (PI3K-Akt) pathway – two of the most important signaling cascades frequently deregulated in cancer. In addition, there is accumulating evidence that these pathways may cooperate to promote the survival of transformed cells (81).

PI3K is recruited to the membrane after stimulation by various different growth factors and cytokines. At this site, the enzyme is activated and, in proximity to its lipid substrate, phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2], generates PtdIns(3,4,5)P_3 (82). The tumor suppressor PTEN acts reversing the action of PI3K, dephosphorylating PtdIns(3,4,5)P_3 and is thus an essential suppressor of PI3K signaling. This PTEN function is lost in various advanced-stage cancers. Effector proteins with pleckstrin-homology (PH) domains, such as the AKT/PKB and 3-phosphoinositide-dependent protein kinase 1 (PDK1) protein kinases, utilizes PtdIns(3,4,5)P_3 as a docking site. PKB phosphorylates various substrates involved in diverse processes, including cell survival (inactivation of the proapoptotic protein BAD), glycogen synthesis (down-regulation of glycogen synthase kinase-3) and gene transcription (FOXO transcription factors) (82); however, PKB also promotes cell and organism growth downstream of PI3K. The mechanisms indicating how the insulin-PI3K-PKB pathway induces growth has recently been clarified by the finding that PKB phosphorylates and inactivates (83) tuberin – also known as tuberous sclerosis complex 2 (TSC2) –, an inhibitor of cell growth, thereby inactivating the function of the TSC1-TSC2 tumour suppressor complex. TSC1-TSC2 is a complex of the proteins hamartin (TSC1) and tuberin (10) that acts inhibiting GTPase-activating protein activity of the small GTPase, Rheb (84). Activation of PI3K induced by insulin has been shown to relieve this inhibitory activity (84), resulting in activation of Rheb. This induction of Rheb leads to activation of the mTOR pathway and an mTOR-regulated serine/threonine kinase, S6K.

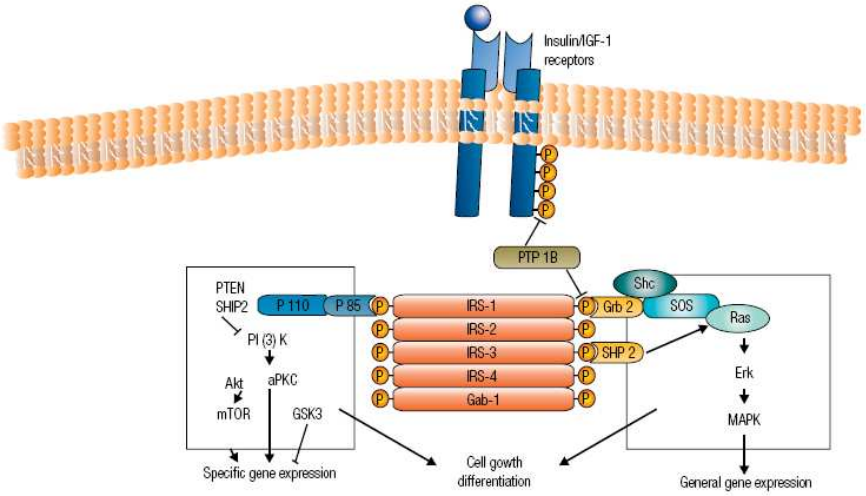

Figure 1. The insulin/IGF-1 receptor is a tyrosine kinase that undergoes autophosphorylation, and catalyses the phosphorylation of cellular proteins such as members of the IRS family and Shc. Upon tyrosine phosphorylation, these proteins interact with signaling molecules through their SH2 domains, resulting in a diverse series of signaling pathways, including activation of PI(3)K and downstream PtdIns(3,4,5)P3-dependent protein kinases, ras and the MAP kinase cascade. These pathways act in a concerted fashion to coordinate the regulation of vesicle trafficking, protein synthesis, enzyme activation and inactivation, and gene expression.

The signaling through mTOR pathway is important in ribosome biogenesis and cell growth (85), and its induction after PI3K activation and inactivation of TSC1-TSC2 by PKB might explain the sequential activation of PKB and S6K by insulin (86). As recently shown, phosphorylation of tuberin by the p90 ribosomal S6 kinase 1 (Rsk1) (87) has a similar inhibitory effect to that of PKB on TSC1-TSC2, this way promoting mTOR signaling; however, phosphorylation of tuberin by 5' AMP-activated protein kinase (AMPK) in response to reduced cellular energy levels might act in the opposite manner to regulate TSC1-TSC2 function positively (88).

Cellular growth is controlled in part by mTOR-raptor which phosphorylates the hydrophobic motif of S6K1, activating this kinase. mTOR-raptor also phosphorylates and inhibits 4E-BPs, proteins that inhibit the eIF4E-dependent translation of capped mRNAs (85). Persuasive evidence suggests the existence of a negative-feedback loop that enables the nutrient-sensitive mTOR-raptor pathway, through S6K1, to desensitize insulin signaling (89). S6K1 mediates the feedback by phosphorylating and inactivating IRS1.

The MAP kinase-Ras-Raf cascade is centrally important in driving tumor cell proliferation. The Ras/Raf/MEK/ERK cascade couples signals from cell surface receptors to transcription factors, which regulate gene expression. Furthermore, this cascade also regulates the activity of many proteins involved in apoptosis.

The *Raf* gene family consists of three proteins, termed A-Raf, B-Raf and Raf-1 (C-Raf). Raf is a serine/threonine (S/T) kinase, normally activated by a complex series of events, including: (i) interaction with Ras and subsequent recruitment to the plasma membrane; (ii) dimerization of Raf proteins; (iii) phosphorylation/dephosphorylation on different domains; (iv) disassociation from the *R*af *k*inase inhibitory protein (RKIP), and (v) association with scaffolding complexes (e.g., *k*inase *s*uppressor of *R*as, (KSR). Chaperonin proteins such as Bag1, 14-3-3 (90) and heat *s*hock *p*rotein 90 (Hsp90) modulates Raf activity (91).

When activated, Raf phosphorylates the serine residue in the activation loop of Mek (MAPKK) (92). The activated Mek1/2 phosphorylates the MAPK protein, ERK, on adjacent threonine and tyrosine residues. Active ERK phosphorylates multiple cytoplasmic and cytoskeletal proteins (93), including MAPK-activated protein kinases and the family of approximately 90-kDa ribosomal S6 kinases (Rsk). Additionally, active ERK and Rsk1/2 translocate to the nucleus, where ERK phosphorylates and activates various transcription factors, such as Sp1, E2F, Elk-1 and AP-1 (94). AP-1 is comprised of two short-lived proteins, Jun and Fos, which are the product of immediate early genes (IEGs). This pathway can control various cellular processes such as proliferation, migration and differentiation.

Part of insulin and IGF-1 action is also mediated by the crosstalk of these pathways with that of the estrogen receptor pathway in breast cells.

Sex steroids

With regard to sex steroids, adiposity influences the synthesis and bioavailability of the hormones through, at least, three mechanisms. First, adipose tissue expresses a variety of sex-steroid-metabolizing enzymes, like aromatase, that promote the formation of estrogens from androgenic precursors, which are secreted by the gonads or adrenal glands. Adipose tissue is the major site of estrogen synthesis in men and postmenopausal women, with levels of aromatase and circulating levels of estrone and estradiol strongly related to BMI (95).

A second hypothesis is that obesity results in an increase in circulating levels of insulin and IGF-1 bioactivity. Insulin and IGF-1 both inhibit the synthesis of sex hormone-binding globulin (SHBG) – the major carrier protein for testosterone and estradiol in the plasma – and may lead to an increase in the amount of unbound sex-steroid available for bioactivity (96). In one study, obese women (BMI > 30 kg/m²) had average SHBG concentration that was half of that of women with a BMI of < 22 kg/m² (97).

SHBG can act directly on breast cancer cells inhibiting the cell proliferation induced by estradiol. Binding of this protein to cell membrane induces the second messenger cAMP on MCF-7 breast cancer cell line (98), and leads to a complete inhibition of estradiol-induced cell proliferation (99). In addition, the pre-incubation of MCF-7 cells with SHBG before estradiol treatment counteracts the antiapoptotic effect of the hormone (100). Thus, SHBG prevents estradiol action in breast cancer cells, acting as an anti-proliferative factor, loss of which could contribute to tumorigenesis in obese women.

Estradiol (E2) and IGFs also act as mitogens in breast cancer cells trough the activation of ERalpha transcriptional activity. They act together and reciprocally. In addition, insulin at supraphysiologic doses may reproduce the IGF-1 effects on breast cells. It was shown in breast can-

cer cell lines that E2 interacts with the whole transduction pathways of IGF (101). IGFs act through two membrane receptors: IGF-1R and type 2 receptors (IGF-2R). However, most of the effects of IGFs are mediated by the IGF-1R. Activation by the ligand leads to an autophosphorylation and binding to signaling adaptor proteins, like insulin receptor substrate (IRS)-1 and Shc, which activate the ERK and PI3Kinase pathways.

Finally, high insulin levels can increase ovarian, and possibly also adrenal, androgen synthesis, and can cause the development of the polycystic ovary syndrome in some genetically susceptible pre-menopausal women (102). PCOS is characterized by ovarian hyperandrogenism and chronic anovulation, which results in continuous estrogen stimulation of the endometrium unopposed by progesterone (103). In PCOS pre-menopausal women, ovarian hyperandrogenism likely increases risk of endometrial cancer by decreasing local uterine IGFBP-1 synthesis, which in turn increases bio-availability of IGF-1, favoring tumor formation (104). Polycystic ovary syndrome and obesity are both associated with increased risk of endometrial cancer in pre and post-menopausal women, respectively (73,105), and share mechanistic pathways that overlap between the estrogen, progesterone, androgen, and IGF systems.

Breast cancer risk and most established risk factors for endometrial cancer, such as early menarche, late menopause and obesity, probably act through pathways reflecting greater life-time exposure to estrogens. At a molecular level, estrogen actions are mediated by the estrogen receptors (ERalpha or ERbeta). The ER genomic actions involve its role as a transcription factor by binding directly to DNA through estrogen response elements, or by tethering to DNA through interaction with other proteins. However, estrogen can also exhibit pleiotropic effects trough nongenomic interactions with growth factor signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)/Akt and the mitogenic-activated protein kinase pathways (106). Thus, besides ER is predominantly localized in the nucleus in steroid-deprived MCF-7 breast cancer cells, a substantial proportion is translocated to the plasma membrane upon E2 stimulation, contributing to growth factor receptor signaling.

Obesity inflammation and cancer

A causal relationship between cancer and inflammation is suspected for thousands of years. Galen was the first to note the relationship and Virchow, in the 19th century, demonstrated that leukocytes were present in malignant tissues, claiming that tumors arise from chronic inflammation sites (107). Chronic infection and the consecutive inflammation that occurs may affect normal tissue cells, transforming these cells and might even affect tumors cells trough interaction with surrounding cells. Cancer deaths (15%-20%) can be linked to inflammation and infection. For example, the major risk factors for hepatocellular carcinoma (HCC) are hepatitis B virus (HBV) and hepatitis C virus (HCV) chronic infections, and most gastric cancers are associated with *Helicobacter pylori* infections (108). Ulcerative colitis and other inflammatory bowel diseases are thought to increase the risk of colorectal cancer (109), and irritation and inflammation of airways by airborne particles and tobacco smoke might be important promoters of lung carcinogenesis (110).

In parallel, there is a large amount of evidence that links obesity, inflammation and the development of insulin resistance (111,112). In obese adipose tissue there is macrophage recruitment, which, in turn, results in a pro-inflammatory state. Macrophages that are infiltrated in other tissues are known to secrete large amounts of tumor-necrosis factor (TNF), leading to a chronic inflammatory state with impaired triglyceride deposition and increased lipolysis, the excess of circulating triglyceride and free fatty acids results in disruption of normal metabolic functions such as mitochondrial oxidative phosphorylation and insulin-stimulated glucose transport, thus triggering insulin resistance.

Serine phosphorylation of IRS are major mechanisms that suppresses the insulin pathway leading to insulin resistance (113). In this regard, JNK, a member of the MAP kinase family that can be activated by TNFalpha, might serve as a feedback inhibitor during insulin stimulation. JNK activation induces inhibitory serine 307 (Ser307) phosphorylation of IRS-1. Ser307 is located next to the phosphotyrosine-binding (PTB) domain in IRS-1 and its phosphorylation inhibits the interaction of the PTB domain with the phosphorylated NPEY motif in the activated IR, causing insulin resistance (114). Previous studies suggest that, in addition to JNK, IKKbeta phoshorylation also increases serine phosphorylation of IRS-1. Thus, the IKK complex appears to be another candidate that plays a key role in the phosphorylation of IRS-1 and in the regulation of insulin sensitivity.

The nuclear factor kappaB (NF-kappaB) pathway plays a central role in inflammation and immunity. Transcription factors, which integrate stress signals and orchestrate immune responses known as NF-

kappaB proteins, have recently been linked to the hallmarks of carcinogenesis, and recent experimental studies have demonstrated the mechanistic pathways by which NF-kappaB signaling (Figure 2) and JNK contributes to these aspects (115,116). Greten and cols. (115) utilized the IKKbeta conditional knockout to test the role of the NF-kappaB activation pathway in controlling tumorigenesis in a colitis-associated model for cancer. In another related study, Maeda and cols. (116) showed that loss of IKKbeta in hepatocytes actually promoted chemical-induced hepatocarcinogenesis through a mechanism involving enhanced ROS production and JNK activation with associated cell death, leading to a compensatory response in surviving hepatocytes.

The consistent results from epidemiologic studies linking adiposity and the risk of several adult cancers turns plausible the hypothesis that the molecular mechanisms of carcinogenesis may be mediated by inflammatory pathways. In face of the striking prevalence of obesity, understanding the exact molecular mechanisms connecting the two may be crucial to the treatment of this pathology.

CONCLUSION

In several studies, obesity has been associated with risk and prognosis for various cancers, and several mechanisms have been proposed to explain the links between obesity and cancer. Confirmation of the role of obesity on cancer risk and prognosis has emerged from clinical trials and meta-analyses in the last decades. Improvement of the knowledge of the pathophysiological mechanisms linking obesity and cancer would be necessary to establish Public Health interventions for reducing the impact of cancer.

Disclosure: No potential conflict of interest relevant to this article was reported.

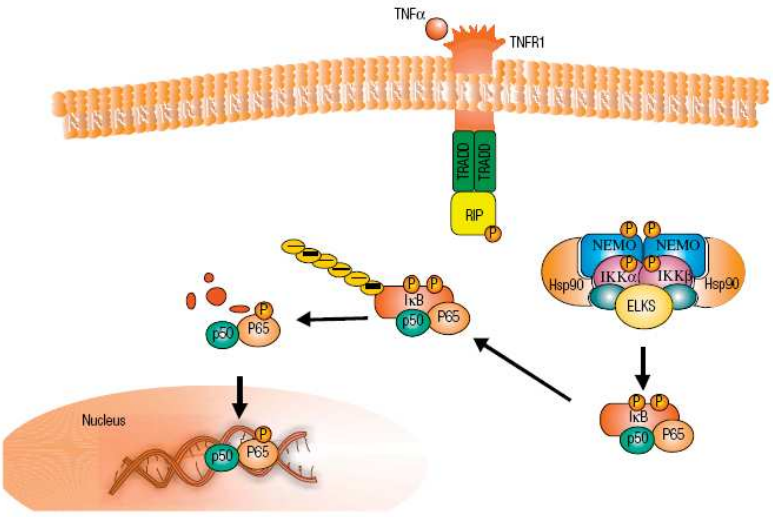

Figure 2. Activation of tumour-necrosis factor receptor 1 (TNFR) by binding of TNFα results in rapid assembly of complex I, which is composed of TNF receptor 1-associated protein (TRADD), receptor-interacting protein 1 (RIP1). This complex leads to activation of inhibitor of NFκB kinase (IKK). Activation of IKK leads to IκB phosphorylation and degradation that culminate in nuclear translocation of NFκB.

REFERENCES

1. Filozof C, Gonzalez C, Sereday M, Mazza C, Braguinsky J. Obesity prevalence and trends in Latin-American countries. Obes Rev. 2001;2(2):99-106.
2. Carvalheira JB, Saad MJ. [Insulin resistance/hyperinsulinemia associated diseases not included in the metabolic syndrome]. Arq Bras Endocrinol Metabol. 2006;50(2):360-7. Epub 2006 May 23. Review. Portuguese.
3. den CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM. Prevalence of overweight and obesity in the United States, 1999-2004. JAMA. 2006;295(13):1549-55.
4. IARC – International Agency for Research on Cancer Working Group on the Evaluation of Cancer-Preventive Strategies. Weight control and physical activity. Lyon, France: IARC Press; 2002.
5. WCRF – World Cancer Reserach Fund. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. Washington: American Institute for Cancer Research; 2007.
6. Reeves GK, Pirie K, Beral V, Green J, Spencer E, Bull D. Cancer incidence and mortality in relation to body mass index in the Million Women Study: cohort study. BMJ. 2007;335(7634):1134. Epub 2007 Nov 6.
7. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet. 2008;371(9612):569-78. Review.
8. Renehan AG, Roberts DL, Dive C. Obesity and cancer: pathophysiological and biological mechanisms. Arch Physiol Biochem. 2008;114(1):71-83. Review.
9. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N Engl J Med. 2003;348(17):1625-38.
10. Yakar S, Nunez NP, Pennisi P, Brodt P, Sun H, Fallavollita L, et al. Increased tumor growth in mice with diet-induced obesity: impact of ovarian hormones. Endocrinology. 2006;147 (12):5826-34. Epub 2006 Sep 7.
11. Dirx MJ, Zeegers MP, Dagnelie PC, van den Bogaard T, van den Brandt PA. Energy restriction and the risk of spontaneous mammary tumors in mice: a meta-analysis. Int J Cancer. 2003;106(5):766-70.
12. Chow WH, Gridley G, Fraumeni JF Jr, Järvholm B. Obesity, hypertension, and the risk of kidney cancer in men. N Engl J Med. 2000;343(18):1305-11.
13. Larsson SC, Orsini N, Wolk A. Body mass index and pancreatic cancer risk: a meta-analysis of prospective studies. Int J Cancer. 2007;120(9):1993-8.
14. Pischon T, Lahmann PH, Boeing H, Friedenreich C, Norat T, Tjønneland A, et al. Body size and risk of colon and rectal cancer in the European Prospective Investigation Into Cancer and Nutrition (EPIC). J Natl Cancer Inst. 2006;98(13):920-31.
15. Jenab M, Riboli E, Cleveland RJ, Norat T, Rinaldi S, Nieters A, et al. Serum C-peptide, IGFBP-1 and IGFBP-2 and risk of colon and rectal cancers in the European Prospective Investigation into Cancer and Nutrition. Int J Cancer. 2007;121(2):368-76.
16. Sandhu MS, Dunger DB, Giovannucci EL. Insulin, insulin-like growth factor-I (IGF-I), IGF binding proteins, their biologic interactions, and colorectal cancer. J Natl Cancer Inst. 2002;94(136):972-80.
17. Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM, Egger M. Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet. 2004;363(9418):1346-53.
18. Ma J, Pollak MN, Giovannucci E, Chan JM, Tao Y, Hennekens CH, et al. Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. J Natl Cancer Inst. 1999;91(7):620-5.
19. Wolpin BM, Meyerhardt JA, Chan AT, Ng K, Chan JA, Wu K, et al. Insulin, the insulin-like growth factor axis, and mortality in patients with nonmetastatic colorectal cancer. J Clin Oncol. 2009;27(2):176-85. Epub 2008 Dec 8.
20. Aaronson SA. Growth factors and cancer. Science. 1991;254(5035):1146-53. Review.
21. Kaaks R, Lukanova A. Energy balance and cancer: the role of insulin and insulin-like growth factor-I. Proc Nutr Soc. 2001;60(1):91-106. Review.
22. Stattin P, Lukanova A, Biessy C, Söderberg S, Palmqvist R, Kaaks R, et al. Obesity and colon cancer: does leptin provide a link? Int J Cancer. 2004;109(1):149-52.
23. Carvalheira JB, Siloto RM, Ignacchitti I, Brenelli SL, Carvalho CR, Leite A, et al. Insulin modulates leptin-induced STAT3 activation in rat hypothalamus. FEBS Lett. 2001;500(3):119-24.
24. Björntorp P. Obesity. Lancet. 1997;350(9075):423-6.
25. Giovannucci E, Colditz GA, Stampfer MJ, Willett WC. Physical activity, obesity, and risk of colorectal adenoma in women (United States). Cancer Causes Control. 1996;7(2):253-63.
26. Stattin P, Palmqvist R, Söderberg S, Biessy C, Ardnor B, Hallmans G, et al. Plasma leptin and colorectal cancer risk: a prospective study in Northern Sweden. Oncol Rep. 2003;10(6):2015-21.
27. Wei EK, Giovannucci E, Fuchs CS, Willett WC, Mantzoros CS. Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. J Natl Cancer Inst. 2005;97(22):1688-94.
28. Lukanova A, Söderberg S, Kaaks R, Jellum E, Stattin P. Serum adiponectin is not associated with risk of colorectal cancer. Cancer Epidemiol Biomarkers Prev. 2006;15:401-2.
29. Huang Z, Hankinson SE, Colditz GA, Stampfer MJ, Hunter DJ, Manson JE, et al. Dual effects of weight and weight gain on breast cancer risk. JAMA. 1997;278(17):1407-11.
30. Travis RC, Key TJ. Oestrogen exposure and breast cancer risk. Breast Cancer Res. 2003;5(5):239-47.
31. Schairer C, Hill D, Sturgeon SR, Fears T, Pollak M, Mies C, et al. Serum concentrations of IGF-I, IGFBP-3 and c-peptide and risk of hyperplasia and cancer of the breast in postmenopausal women. Int J Cancer. 2004;108(5):773-9.
32. Toniolo P, Bruning PF, Akhmedkhanov A, Bonfrer JM, Koenig KL, Lukanova A, et al. Serum insulin-like growth factor-I and breast cancer. Int J Cancer. 2000;88(5):828-32.
33. Yang G, Lu G, Jin F, Dai Q, Best R, Shu XO, et al. Population-based, case-control study of blood C-peptide level and breast cancer risk. Cancer Epidemiol Biomarkers Prev. 2001;10(11):1207-11.
34. Hirose K, Toyama T, Iwata H, Takezaki T, Hamajima N, Tajima K. Insulin, insulin-like growth factor-I and breast cancer risk in Japanese women. Asian Pac J Cancer Prev. 2003;4(3):239-46.
35. Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, et al. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. Lancet. 1998;351(9113):1393-6.
36. Goodwin PJ, Ennis M, Pritchard KI, Trudeau ME, Koo J, Hartwick W, et al. Insulin-like growth factor binding proteins 1 and 3 and breast cancer outcomes. Breast Cancer Res Treat. 2002;74(1):65-76.
37. Vona-Davis L, Rose DP. Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression. Endocr Relat Cancer. 2007;14(2):189-206.
38. Miyoshi Y, Funahashi T, Kihara S, Taguchi T, Tamaki Y, Matsuzawa Y, et al. Association of serum adiponectin levels with breast cancer risk. Clin Cancer Res. 2003;9(15):5699-704.
39. Mantzoros C, Petridou E, Dessypris N, Chavelas C, Dalamaga M, Alexe DM, et al. Adiponectin and breast cancer risk. J Clin Endocrinol Metab. 2004;89(3):1102-7.
40. Kaaks R, Lukanova A, Kurzer MS. Obesity, endogenous hormones, and endometrial cancer risk: a synthetic review. Cancer Epidemiol Biomarkers Prev. 2002;11(12):1531-43. Review.

41. Lukanova A, Lundin E, Micheli A, Arslan A, Ferrari P, Rinaldi S, et al. Circulating levels of sex steroid hormones and risk of endometrial cancer in postmenopausal women. Int J Cancer. 2004;108(3):425-32.
42. Lukanova A, Zeleniuch-Jacquotte A, Lundin E, Micheli A, Arslan AA, Rinaldi S, et al. Prediagnostic levels of C-peptide, IGF-I, IGFBP -1, -2 and -3 and risk of endometrial cancer. Int J Cancer. 2004;108(2):262-8.
43. Nicodemus KK, Sweeney C, Folsom AR. Evaluation of dietary, medical and lifestyle risk factors for incident kidney cancer in postmenopausal women. Int J Cancer. 2004;108(1):115-21.
44. Bergstrom A, Hsieh CC, Lindblad P, Lu CM, Cook NR, Wolk A. Obesity and renal cell cancer–a quantitative review. Br J Cancer. 2001;85(7):984-90.
45. Pischon T, Lahmann PH, Boeing H, Tjonneland A, Halkjaer J, Overvad K, et al. Body size and risk of renal cell carcinoma in the European Prospective Investigation into Cancer and Nutrition (EPIC). Int J Cancer. 2006;118(3):728-38.
46. Lindblad P, Chow WH, Chan J, Bergström A, Wolk A, Gridley G, et al. The role of diabetes mellitus in the aetiology of renal cell cancer. Diabetologia. 1999;42(1):107-12.
47. Spyridopoulos TN, Petridou ET, Skalkidou A, Dessypris N, Chrousos GP, Mantzoros CS. Low adiponectin levels are associated with renal cell carcinoma: a case-control study. Int J Cancer. 2007;120(7):1573-8.
48. Pera M, Manterola C, Vidal O, Grande L. Epidemiology of esophageal adenocarcinoma. J Surg Oncol. 2005;92(3):151-9.
49. Kubo A, Corley DA. Body mass index and adenocarcinomas of the esophagus or gastric cardia: a systematic review and meta-analysis. Cancer Epidemiol Biomarkers Prev. 2006;15(5):872-8.
50. Abnet CC, Freedman ND, Hollenbeck AR, Fraumeni JF Jr, Leitzmann M, Schatzkin A. A prospective study of BMI and risk of oesophageal and gastric adenocarcinoma. Eur J Cancer. 2008;44(3):465-71.
51. Corley DA, Kubo A, Zhao W. Abdominal obesity and the risk of esophageal and gastric cardia carcinomas. Cancer Epidemiol Biomarkers Prev. 2008;17(2):352-8.
52. Hampel H, Abraham NS, El-Serag HB. Meta-analysis: obesity and the risk for gastroesophageal reflux disease and its complications. Ann Intern Med. 2005;143(3):199-211.
53. Giovannucci E, Michaud D. The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. Gastroenterology. 2007;132(6):2208-25.
54. Samanic C, Gridley G, Chow WH, Lubin J, Hoover RN, Fraumeni JF Jr. Obesity and cancer risk among white and black United States veterans. Cancer Causes Control. 2004;15(1):35-43.
55. Luo J, Margolis KL, Adami HO, LaCroix A, Ye W. Obesity and risk of pancreatic cancer among postmenopausal women: the Women's Health Initiative (United States). Br J Cancer. 2008;99(3):527-31. Epub 2008 Jul 15.
56. Gapstur SM, Gann PH, Lowe W, Liu K, Colangelo L, Dyer A. Abnormal glucose metabolism and pancreatic cancer mortality. JAMA. 2000;283(19):2552-8.
57. Wolpin BM, Michaud DS, Giovannucci EL, Schernhammer ES, Stampfer MJ, Manson JE, et al. Circulating insulin-like growth factor axis and the risk of pancreatic cancer in four prospective cohort. Br J Cancer. 2007;97(1):98-104. Epub 2007 May 29.
58. MacInnis RJ, English DR. Body size and composition and prostate cancer risk: systematic review and meta-regression analysis. Cancer Causes Control. 2006;17(8):989-1003.
59. Potischman N, Swanson CA, Siiteri P, Hoover RN. Reversal of relation between body mass and endogenous estrogen concentrations with menopausal status. J Natl Cancer Inst. 1996;88(11):756-8.
60. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, et al. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science. 1998;279(5350):563-6.
61. Larsson SC, Wolk A. Obesity and the risk of gallbladder cancer: a meta-analysis. Br J Cancer. 2007;96(9):1457-61.
62. Caldwell SH, Crespo DM, Kang HS, Al-Osaimi AM. Obesity and hepatocellular carcinoma. Gastroenterology. 2004;127(5 Suppl 1):S97-103. Review.
63. Pan SY, Johnson KC, Ugnat AM, Wen SW, Mao Y. Association of obesity and cancer risk in Canada. Am J Epidemiol. 2004;159(3):259-68.
64. Lahmann PH, Hoffmann K, Allen N, van Gils CH, Khaw KT, Tehard B, et al. Body size and breast cancer risk: findings from the European Prospective Investigation into Cancer and Nutrition (EPIC). Int J Cancer. 2004;111(5):762-71.
65. Olsen CM, Green AC, Whiteman DC, Sadeghi S, Kolahdooz F, Webb PM. Obesity and the risk of epithelial ovarian cancer: a systematic review and meta-analysis. Eur J Cancer. 2007;43(4):690-709. Epub 2007 Jan 12.
66. Olsen CM, Nagle CM, Whiteman DC, Purdie DM, Green AC, Webb PM. Body size and risk of epithelial ovarian and related cancers: a population-based case-control study. Int J Cancer. 2008;123(2):450-6.
67. Engeland A, Tretli S, Hansen S, Bjørge T. Height and body mass index and risk of lymphohematopoietic malignancies in two million Norwegian men and women. Am J Epidemiol. 2007;165(1):44-52. Epub 2006 Oct 13.
68. Larsson SC, Wolk A. Body mass index and risk of multiple myeloma: a meta-analysis. Int J Cancer. 2007;121(11):2512-6.
69. Druker BJ. Imatinib as a paradigm of targeted therapies. Adv Cancer Res. 2004;91:1-30.
70. McKeown-Eyssen G. Epidemiology of colorectal cancer revisited: are serum triglycerides and/or plasma glucose associated with risk? Cancer Epidemiol Biomarkers Prev. 1994;3(8):687-95.
71. Giovannucci E. Insulin and colon cancer. Cancer Causes Control. 1995;6(2):164-79. Review.
72. Giovannucci E. Nutrition, insulin, insulin-like growth factors and cancer. Horm Metab Res. 2003;35(11-12):694-704.
73. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer. 2004;4(8):579-91.
74. Allen NE, Appleby PN, Kaaks R, Rinaldi S, Davey GK, Key TJ. Lifestyle determinants of serum insulin-like growth-factor-I (IGF-I), C-peptide and hormone binding protein levels in British women. Cancer Causes Control. 2003;14(1):65-74.
75. Holmes MD, Pollak MN, Hankinson SE. Lifestyle correlates of plasma insulin-like growth factor I and insulin-like growth factor binding protein 3 concentrations. Cancer Epidemiol Biomarkers Prev. 2002;11(9):862-7.
76. Myal Y, Shiu RP, Bhaumick B, Bala M. Receptor binding and growth-promoting activity of insulin-like growth factors in human breast cancer cells (T-47D) in culture. Cancer Res. 1984;44(12 Pt 1):5486-90.
77. Wu Y, Cui K, Miyoshi K, Hennighausen L, Green JE, Setser J, et al. Reduced circulating insulin-like growth factor I levels delay the onset of chemically and genetically induced mammary tumors. Cancer Res. 2003;63(15):4384-8.
78. Pollak M, Blouin MJ, Zhang JC, Kopchick JJ. Reduced mammary gland carcinogenesis in transgenic mice expressing a growth hormone antagonist. Br J Cancer. 2001;85(3):428-30.
79. Creighton CJ, Casa A, Lazard Z, Huang S, Tsimelzon A, Hilsenbeck SG, et al. Insulin-like growth factor-I activates gene transcription programs strongly associated with poor breast cancer prognosis. J Clin Oncol. 2008;26:4078-85.

80. Carvalheira JB, Zecchin HG, Saad MJ. [Vias de sinalização da insulina]. Arq Bras Endocrinol Metabol. 2002;46(4):419-25.
81. Moore T, Beltran L, Carbajal S, Strom S, Traag J, Hursting SD, et al. Dietary energy balance modulates signaling through the akt/mammalian target of rapamycin pathways in multiple epithelial tissues. Cancer Prev Res. 2008;1(1):65-76. Epub 2008 Mar 31.
82. Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002;296(5573):1655-7.
83. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. 2002;4(9):648-57.
84. Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, et al. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell. 2003;11(6):1457-66.
85. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev. 2004;18(16):1926-45.
86. McManus EJ, Alessi DR. TSC1-TSC2: a complex tale of PKB-mediated S6K regulation. Nat Cell Biol. 2002;4(9):E214-6.
87. Roux PP, Ballif BA, Anjum R, Gygi SP, Blenis J. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc Natl Acad Sci U S A. 2004;101(37):13489-94. Epub 2004 Sep 1.
88. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003;115(5):577-90.
89. Harrington LS, Findlay GM, Lamb RF. Restraining PI3K: mTOR signalling goes back to the membrane. Trends Biochem Sci. 2005;30(1):35-42.
90. Fantl WJ, Muslin AJ, Kikuchi A, Martin JA, MacNicol AM, Gross RW, et al. Activation of Raf-1 by 14-3-3 proteins. Nature. 1994;371(6498):612-4.
91. Blagosklonny MV. Hsp-90-associated oncoproteins: multiple targets of geldanamycin and its analogs. Leukemia. 2002;16(4):455-62.
92. Kyriakis JM, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR, et al. Raf-1 activated MAP kinase kinase. Nature. 1992;358(6385):417-21.
93. Yoon S, Seger R. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors. 2006;24(1):21-44.
94. Murphy LO, Blenis J. MAPK signal specificity: the right place at the right time. Trends Biochem Sci. 2006;31(5):268-75. Epub 2006 Apr 17.
95. Key TJ, Appleby PN, Reeves GK, Roddam A, Dorgan JF, Longcope C, et al. Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. J Natl Cancer Inst. 2003;95(16):1218-26.
96. Pugeat M, Crave JC, Elmidani M, Nicolas MH, Garoscio-Cholet M, Lejeune H, et al. Pathophysiology of sex hormone binding globulin (SHBG): relation to insulin. J Steroid Biochem Mol Biol. 1991;40(4-6):841-9.
97. McTiernan A, Rajan KB, Tworoger SS, Irwin M, Bernstein L, Baumgartner R, et al. Adiposity and sex hormones in postmenopausal breast cancer survivors. J Clin Oncol. 2003;21(10):1961-6.
98. Fortunati N, Fissore F, Fazzari A, Piovano F, Catalano MG, Becchis M, et al. Estradiol induction of cAMP in breast cancer cells is mediated by foetal calf serum (FCS) and sex hormone-binding globulin (SHBG). J Steroid Biochem Mol Biol. 1999;70(1-3):73-80.
99. Fortunati N, Fissore F, Fazzari A, Becchis M, Comba A, Catalano MG, et al. Sex steroid binding protein exerts a negative control on estradiol action in MCF-7 cells (human breast cancer) through cyclic adenosine 3',5'-monophosphate and protein kinase A. Endocrinology. 1996;137(2):686-92.
100. Catalano MG, Frairia R, Boccuzzi G, Fortunati N. Sex hormone-binding globulin antagonizes the anti-apoptotic effect of estradiol in breast cancer cells. Mol Cell Endocrinol. 2005;230(1-2):31-7.
101. Lee AV, Jackson JG, Gooch JL, Hilsenbeck SG, Coronado-Heinsohn E, Osborne CK, et al. Enhancement of insulin-like growth factor signaling in human breast cancer: estrogen regulation of insulin receptor substrate-1 expression in vitro and in vivo. Mol Endocrinol. 1999;13(5):787-96.
102. Dunaif A. Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. Endocr Rev. 1997;18(6):774-800.
103. Ehrmann DA, Barnes RB, Rosenfield RL. Polycystic ovary syndrome as a form of functional ovarian hyperandrogenism due to dysregulation of androgen secretion. Endocr Rev. 1995;16(3):322-53. Review.
104. Druckmann R, Rohr UD. IGF-1 in gynaecology and obstetrics: update 2002. Maturitas. 2002;41 Suppl 1:S65-83.
105. Colangelo LA, Gapstur SM, Gann PH, Dyer AR, Liu K. Colorectal cancer mortality and factors related to the insulin resistance syndrome. Cancer Epidemiol Biomarkers Prev. 2002;11(4):385-91.
106. Schiff R, Massarweh SA, Shou J, Bharwani L, Mohsin SK, Osborne CK. Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. Clin Cancer Res. 2004;10(1 Pt2):S331-6.
107. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet. 2001;357(9255):539-45.
108. Roder DM. The epidemiology of gastric cancer. Gastric Cancer. 2002;5 Suppl 1:5-11.
109. Ekbom A. Risk of cancer in ulcerative colitis. J Gastrointest Surg. 1998;2(4):312-3. Review.
110. Dhala A, Pinsker K, Prezant DJ. Respiratory health consequences of environmental tobacco smoke. Med Clin North Am. 2004;88(6):1535-52, xi.
111. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993;259(5091):87-91.
112. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, et al. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes. 2007;56(8):1986-98. Epub 2007 May 22.
113. Greene MW, Sakaue H, Wang L, Alessi DR, Roth RA. Modulation of insulin-stimulated degradation of human insulin receptor substrate-1 by Serine 312 phosphorylation. J Biol Chem. 2003;278(10):8199-211. Epub 2003 Jan 1.
114. Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF. Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J Biol Chem. 2002;277(2):1531-7. Epub 2001 Oct 17.
115. Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, et al. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell. 2004;118(3):285-96.
116. Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. Cell. 2005;121(7):977-90.

APÊNDICE II

J Physiol 587.10 (2009) pp 2341–2351

The Journal of Physiology

Inhibition of hypothalamic Foxo1 expression reduced food intake in diet-induced obesity rats

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Insulin signalling in the hypothalamus plays a role in maintaining body weight. The forkhead transcription factor Foxo1 is an important mediator of insulin signalling in the hypothalamus. Foxo1 stimulates the transcription of the orexigenic neuropeptide Y and Agouti-related protein through the phosphatidylinositol-3-kinase/Akt signalling pathway, but the role of hypothalamic Foxo1 in insulin resistance and obesity remains unclear. Here, we identify that a high-fat diet impaired insulin-induced hypothalamic Foxo1 phosphorylation and degradation, increasing the nuclear Foxo1 activity and hyperphagic response in rats. Thus, we investigated the effects of the intracerebroventricular (I.C.V.) microinfusion of Foxo1-antisense oligonucleotide (Foxo1-ASO) and evaluated the food consumption and weight gain in normal and diet-induced obese (DIO) rats. Three days of Foxo1-ASO microinfusion reduced the hypothalamic Foxo1 expression by about 85%. I.C.V. infusion of Foxo1-ASO reduced the cumulative food intake (21%), body weight change (28%), epididymal fat pad weight (22%) and fasting serum insulin levels (19%) and increased the insulin sensitivity (34%) in DIO but not in control animals. Collectively, these data showed that the Foxo1-ASO treatment blocked the orexigenic effects of Foxo1 and prevented the hyperphagic response in obese rats. Thus, pharmacological manipulation of Foxo1 may be used to prevent or treat obesity.

(Received 29 January 2009; accepted after revision 25 March 2009; first published online 30 March 2009)
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Abbreviations AgRP, Agouti-related peptide; Akt, protein kinase B; ASO, antisense oligonucleotide; CBP, citrate binding protein; DIO, diet-induced obesity; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; Foxo1, forkhead box protein; I.C.V., intracerebroventricular; IR, insulin receptor; IRS-1, insulin receptor substrate 1; IRS-2, insulin receptor substrate 2; Kitt, glucose disappearance rate; NPY, neuropeptide Y; PI3K, phosphatidylinositol-3-kinase; POMC, proopiomelanocortin; RIA, radioimmunossay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; STAT3, signal transducer-activated transcript-3; PMSF, phenylmethanesulphonylfluoride; RNA, ribonucleic acid.

Obesity is a major public health problem, associated with morbidity and mortality, and continues to increase worldwide (Zimmet *et al.* 2001). Food intake and energy expenditure are tightly regulated by complex physiological mechanisms, and a disturbance in these processes may lead to obesity (Spiegel *et al.* 2005). The hypothalamus is critical in the regulation of food intake-controlling neural circuits, which produce a number of peptides that influence food intake. The hypothalamus receives and integrates neural, metabolic and humoral signals from the periphery, such as insulin and leptin from pancreatic and adipose tissue, respectively (Schwartz *et al.* 2000). However, the underlying mechanisms by which these hormones regulate the food intake are unclear.

Insulin acts at the same hypothalamic areas as leptin to suppress feeding (Carvalheira *et al.* 2003). The insulin receptor (IR) is a protein with endogenous tyrosine kinase activity that, following activation by insulin, undergoes rapid autophosphorylation and, subsequently, phosphorylates intracellular protein substrates, including IRS-1 and IRS-2 (Cheatham & Kahn, 1995). After stimulation by insulin, IRS-1 and IRS-2 associate with

DOI: 10.1113/jphysiol.2009.170050

Table 1. Components of rat chow and high fat diet

Ingredients	Standard chow (g kg^{-1})	kcal kg^{-1}	High fat diet (g kg^{-1})	kcal kg^{-1}
Cornstarch (Q.S.P.)	397.5	1590	115.5	462
Casein	200	800	200	800
Sucrose	100	400	100	400
Dextrinated starch	132	528	132	528
Lard	—	—	312	2808
Soybean oil	70	630	40	360
Cellulose	50	—	50	—
Mineral mix	35	—	35	—
Vitamin mix	10	—	10	—
L-Cysteine	3	—	3	—
Choline	2.5	—	2.5	—
Total	1000	3948	1000	5358

several proteins, including phosphatidylinositol 3-kinase (PI3K) (Folli *et al.* 1992; Saad *et al.* 1993; Williamson *et al.* 2003). Downstream from PI3K, the serine threonine kinase, Akt, is activated and plays a pivotal role in the regulation of various biological processes, including apoptosis, proliferation, differentiation, and intermediary metabolism (Downward, 1998; Chen *et al.* 2001). Among the targets of activated Akt is forkhead transcriptional factor subfamily forkhead box O1 (Foxo1 or FKHR), which is inhibited by Akt-mediated phosphorylation (Tang *et al.* 1999).

It has been suggested that hypothalamic Foxo1 is an important regulator of food intake and energy balance (Kim *et al.* 2006; Kitamura *et al.* 2006). Nuclear Foxo1 expression stimulates the transcription of the orexigenic neuropeptide Y and Agouti-related protein and suppresses the transcription of anorexigenic proopiomelanocortin (POMC) by antagonizing the activity of signal transducer-activated transcript-3 (STAT3) (Kim *et al.* 2006; Kitamura *et al.* 2006). In addition, the PI3K/Akt signalling pathway can exclude the Foxo1 from the nucleus and lead Foxo1 to proteosomal degradation (Matsuzaki *et al.* 2003; Aoki *et al.* 2004). These molecular events can repress Foxo1-induced orexigenic signals in the hypothalamus. In the present study, we sought to determine the contribution of the hypothalamic Foxo1 on food intake and body weight in diet-induced obesity (DIO) rats. Moreover, we examined the effects of intracerebroventricular (I.C.V.) microinfusion of Foxo1 antisense oligonucleotide (Foxo1-ASO) in the hypothalamus of control and DIO rats and evaluated the food intake and body weight.

Methods

Experimental animals

Male 4-week-old Wistar rats from the University of Campinas Breeding Center were randomly divided into two groups: control, fed standard rodent chow *ad libitum*, and DIO, fed a fat-rich diet *ad libitum* (Table 1). This diet composition has been previously used (Ropelle *et al.* 2006; Pauli *et al.* 2008). For Western blot analysis, hypothalami and other tissues were obtained from six to eight rats of each group after 8 weeks of dieting. The University's Ethical Committee approved the protocols. The animals were maintained on a 12-h artificial light, 12-h dark cycle and kept in individual cages.

Physiological and metabolic parameters

After 6 h of fasting, control and DIO rats were submitted to an insulin tolerance test (1 U (kg body weight)$^{-1}$ of insulin). Rats were injected with insulin and blood samples were collected at 0, 4, 8, 12 and 16 min from the tail for serum glucose determination. The rate constant for plasma glucose disappearance was calculated using the formula 0.693/biological half life ($t_{1/2}$). The plasma glucose $t_{1/2}$ was calculated from the slope of last square analysis of the plasma glucose concentration during the linear phase of decline (Bonora *et al.* 1989). Plasma glucose was determined using a glucose meter (Roche Diagnostic, Rotkreuze, Switzerland), and RIA was used to measure serum insulin, according to a previous description (Scott *et al.* 1981). Following the experimental procedures, the rats were killed under anaesthesia (200 mg kg^{-1} thiopental) following the recommendations of the NIH.

Intracerebroventricular cannulation

The Wistar rats were stereotaxically instrumented under sodium amobarbital (15 mg (kg body weight)$^{-1}$) anaesthesia with chronic unilateral 26-gauge stainless steel indwelling guide cannulae, aseptically placed into the lateral ventricle (0.2 mm posterior, 1.5 mm lateral, and 4.2 mm ventral to bregma), as previously described

(Pereira-da-Silva *et al.* 2003). After a 1 week recovery period, all rats were submitted to experimental protocols.

Foxo1 antisense oligonucleotide (Foxo1-ASO)

Sense and antisense Foxo1 oligonucleotides were diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA) and injected (Hamilton syringe) twice a day at 08.00 h and 16.00 h, with a total volume of 2.0 μl per dose (final concentrations of 0.1, 0.2 and 0.4 nmol), 24, 48 and 72 h after the onset of the experimental period. Rats were randomly assigned to treatment conditions: rats without oligonucleotide treatment (control); rats with sense (5′GAT GCT GGA CAT GGG AGA T 3′) oligonucleotide treatment (Sense) and rats with antisense (5′ATC TCC CAT GTC CAG CAT C 3′) oligonucleotide treatment (ASO).

Treatments and measurement of food intake

Rats deprived of food for 6 h with free access to water were I.C.V. injected (2 μl) with saline or insulin (10^{-6} M). Thereafter standard chow was given and food intake was determined by measuring the difference between the weight of chow given and the weight of chow at the end of a 12 h period. Similar studies were carried out in rats that were initially injected I.C.V. with sense or Foxo1-ASO (4 nmol).

Protein analysis by immunoblotting

As soon as anaesthesia was assured by the loss of pedal and corneal reflexes, 2.0 μl of normal saline or insulin (10^{-6} M) was injected I.C.V. using a Hamilton syringe. Ten minutes after insulin injection, the cranium was opened and the basal diencephalon, including the preoptic area and hypothalamus, was excised. The hypothalami were pooled and 200 μg of protein was used as whole tissue extract. For evaluated insulin-induced Foxo1 expression, hypothalamus was excised 30 min after insulin injection, as shown for Kim and colleagues (Kim *et al.* 2006). Tissues were pooled, minced coarsely and homogenized immediately in extraction buffer (1% Triton X-100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethanesulphonylfluoride (PMSF) and 0.1 mg ml^{-1} aprotinin) at 4°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 15 s. The extracts were centrifuged at 9 000 *g* and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA, USA) for 45 min to remove insoluble material, and the supernatants of these tissues were used for protein quantification, using the Bradford method (Bradford, 1976). Proteins were denatured by boiling in Laemmli sample buffer (Laemmli, 1970) containing 100 mM DTT, run on SDS-PAGE, transferred to nitrocellulose membranes. Antibodies used for immunoprecipitation and immunoblotting were anti-phosphotyrosine, anti-IR, anti-IRS-2 anti-Akt, anti-phospho-Akt, anti-α-tubulin and anti-histone (Santa Cruz Biotechnology, Inc., CA, USA), anti-phospho-Foxo1 and anti-Foxo1 (Cell Signaling Technology, MA, USA). Blots were exposed to preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at −80°C for 12–48 h. Band intensities were analysed by optical densitometry (Scion Image software, ScionCorp, Frederick, MD, USA) of the developed autoradiographs.

Nuclear extraction

To characterize the expression and subcellular localization of Foxo1, a subcellular fractionation protocol was employed as described previously (Prada *et al.* 2006). The hypothalami from untreated rats or rats treated with Foxo1-ASO (0.4 nmol), according to the protocols described above, were minced and homogenized in 2 vol. of STE buffer (0.32 M sucrose, 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 1 mM DTT, 100 mM sodium fluoride, 100 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 1 mM PMSF, and 0.1 mg ml^{-1} aprotinin) at 4°C with a Polytron homogenizer. The homogenates were centrifuged (1000 *g*, 25 min, 4°C) to obtain pellets. The pellets were washed once with STE buffer (1000 *g*, 10 min, 4°C) and suspended in Triton buffer (1% Triton X-100, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 200 mM EDTA, 10 mM sodium orthovanadate, 1 mM PMSF, 100 mM NaF, 100 mM sodium pyrophosphate, and 0.1 mg ml^{-1} aprotinin), kept on ice for 30 min, and centrifuged (15 000 *g*, 30 min, 4°C) to obtain the nuclear fraction. The samples were used for immunoprecipitation with Foxo1 antibody and Protein A-Sepharose 6MB (Amersham Pharmacia Biotech UK Ltd). Thereafter they were treated with Laemmli buffer with 100 mM DTT and heated in a boiling water bath for 5 min, and aliquots (100 μg of protein) were subjected to SDS-PAGE and Western blotting with anti-CBP/p300 or Foxo1 antibodies, as described elsewhere (Gasparetti *et al.* 2003).

Statistical analysis

All numerical results are expressed as the means ± S.E.M. of the indicated number of experiments. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the Scion Image software. Data were analysed by ANOVA followed by *post hoc* analysis of significance (Bonferroni test) when appropriate, comparing experimental and control groups. The level of significance was set at $P < 0.05$.

Results

Metabolic parameters and energy intake of control and DIO rats

Figure 1 shows comparative data regarding control and DIO rats. Rats fed on the high-fat diet for 8 weeks had a greater body weight, epididymal fat pad weight and fasting serum insulin than age-matched controls. The glucose disappearance rate (Kitt) was decreased in DIO animals when compared to control groups (Fig. 1*A–D*). The fasting glucose concentration was similar between the groups (data not shown).

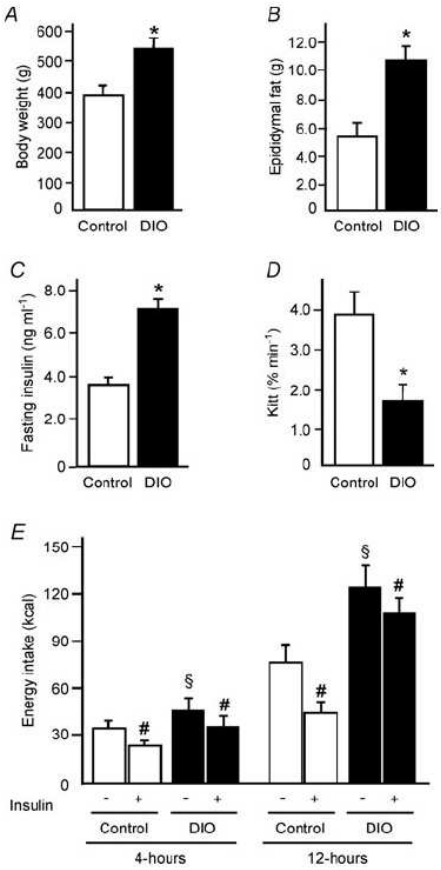

Figure 1. Metabolic parameters and energy intake of control and diet-induced obese (DOI) rats
A, total body weight. *B*, epididymal fat pad weight. *C*, fasting serum insulin. *D*, insulin tolerance test. *E*, energy intake 4 and 12 h after saline or insulin infusion in the hypothalamus of control and DOI rats. Bars represent means ± S.E.M. of eight rats. **P* < 0.004 *versus* control, #*P* < 0.04 *versus* respective saline groups, §*P* < 0.01 obese rats plus saline *versus* control plus saline.

Next, we evaluated the effect of insulin (2 μl, 10^{-6} M) or its saline vehicle on the control of food intake. In the control and DIO rats treated with saline, we observed that the energy intake was higher in DIO rats, 27% and 63% during 4 and 12 h, respectively, when compared to control animals. Insulin induced reductions in the 4 and 12 h of food intake of both control and DIO rats. In the control group, insulin reduced food intake by about 31% and 41% during 4 and 12 h, respectively, while in the obese rats the same dose induced reductions of about 21% and 14% respectively, indicating that insulin was much less effective in DIO rats (Fig. 1*E*).

Intracerebroventricular insulin activates the hypothalamic IR/IRS-2/Akt/Foxo1 pathway in control rats to a greater extent than in DIO rats

Insulin (2 μl, 10^{-6} M) I.C.V. induced increases in IR, IRS-2, Akt and Foxo1 phosphorylation in hypothalamus from both control and DIO rats. However, insulin was much less effective in inducing IR, IRS-2, Akt and Foxo1 phosphorylation in DIO rats when compared to control rats (Fig. 2*A–D*, upper panels). We did not observe differences in IR, IRS-2, Akt and Foxo1 phosphorylation between control and DIO groups treated with saline.

In addition, we evaluated the Foxo1 nuclear expression and the association between Foxo1 and CBP/p300 in control and DIO rats after insulin and saline injection. In the hypothalamus of control rats, insulin reduced Foxo1 expression in the nuclear fraction by 89%, compared with a 22% reduction in the hypothalamus from DIO rats (Fig. 2*E*, upper panel). Insulin also reduced Foxo1/CBP interaction by 89% in control rats and 29% in the hypothalamus of DIO rats (Fig. 2*F*, upper panel). We did not observe differences in Foxo1 nuclear expression and Foxo1/CBP binding between control and DIO groups treated with saline.

Insulin reduced Foxo1 expression in the hypothalamus of control but not in DIO rats

Previous studies suggested that insulin controlled the Foxo1 expression in the hypothalamus of lean mice (Kim *et al.* 2006). We sought to determine the effects of I.C.V. infusion of insulin on the expression of Foxo1 in the hypothalamus of control and DIO rats. First, we observed that insulin did not change the IR, IRS-2 and Akt protein expression in the hypothalamus of control and DIO rats (Fig. 3*A*). However, 30 min of insulin I.C.V. infusion reduced Foxo1 expression in the

hypothalamus of control rats by about 27%. Interestingly, in the hypothalamus of DIO rats, insulin did not change the Foxo1 expression (Fig. 3*B*). We did not observe differences in Foxo1 expression between control and DIO groups treated with saline.

Determining the dose–response and time course effects of I.C.V.-injected Foxo1 antisense upon hypothalamic Foxo1 expression

To evaluate the effect of Foxo1-ASO on Foxo1 expression in the hypothalamus, I.C.V.-cannulated control rats were treated for 1 day with antisense Foxo1 at low doses (0.1 and 0.2 nmol Foxo1-ASO) or at a high dose (0.4 nmol Foxo1-ASO). As shown in Fig. 4, a single infusion of Foxo1-ASO (0.4 nmol) induced a reduction of Foxo1 expression by about 50% in the hypothalamus of control animals. In addition, injection of a high dose (0.4 nmol) of Foxo1-ASO for 1, 2 and 3 days was able to reduce Foxo1 expression in a time-dependent manner. Three days after Foxo1-ASO treatment we observed a great reduction (85%) in the expression of Foxo1 in the hypothalamus of control rats (Fig. 4*B*). This treatment with Foxo1-ASO did not change the IR, IRS-2 and Akt protein expression in the hypothalamus of control rats (Fig. 4*D*, upper panels) and did not change the Foxo-1 protein expression in the peripheral tissues such as gastrocnemius muscle, liver and white adipose tissue (Fig. 4*C*).

Effects of Foxo1-ASO treatment on food intake and body weight in control and DIO rats

To determine the impact of Foxo1-ASO treatment on food intake and body weight, we injected Foxo1-ASO

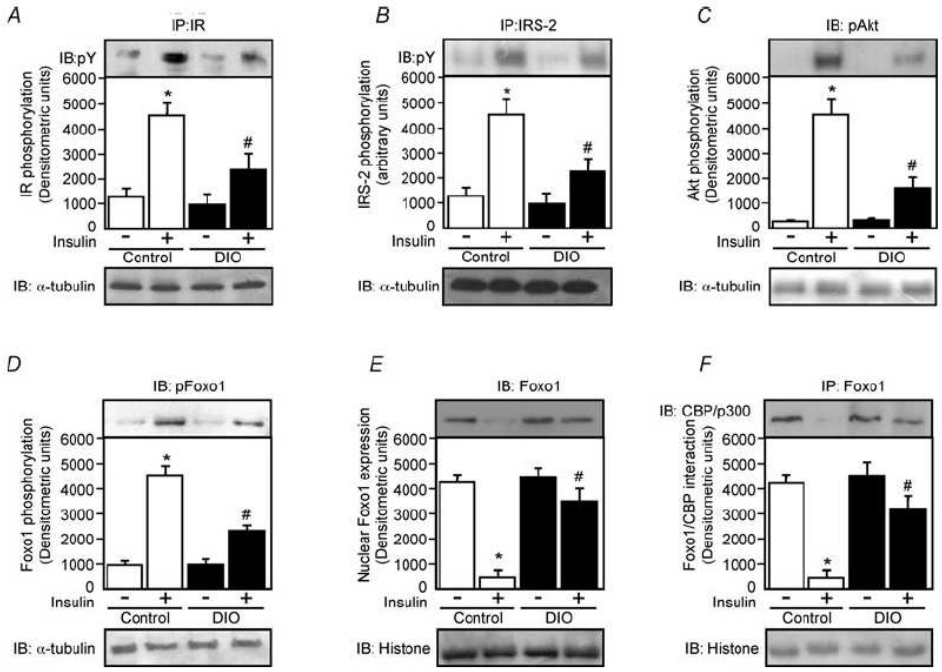

Figure 2. Effects of I.C.V. infusion of insulin on insulin signalling in the hypothalamus of control and DIO rats

A–D, upper panels, representative blots show the insulin-stimulated tyrosine phosphorylation of insulin receptor (IR; *A*), insulin receptor substrate-2 (IRS-2; *B*), Akt serine phosphorylation (*C*) and Foxo1 phosphorylation of control and DIO rats (*D*). *A–D*, lower panels, total protein expression of α-tubulin. The evaluation of nuclear Foxo1 expression (*E*) and the association between Foxo1 and CBP/p300 (*F*), were performed using nuclear extract of control and DIO rats as described in Methods. The results of scanning densitometry were expressed as arbitrary units. Bars represent means ± S.E.M. of six rats. **P* < 0.001 *versus* control plus saline, #*P* < 0.01 *versus* DIO plus saline.

(0.4 nmol) in the hypothalamus of control and DIO rats during 3 days. Foxo1-ASO reduced Foxo1 expression in control and DIO rats in a time-dependent manner (Fig. 5*A*). The nuclear Foxo1 expression was reduced by about 71 and 64% in control and DIO rats, respectively, when compared to the respective control group treated with sense after 3 days (Fig. 5*B*). We also observed that Foxo1-ASO reduced Foxo1/CBP interaction by about 70% and 73% in control and DIO rats, respectively, when compared to the respective control group treated with sense after 3 days (Fig. 5*C*).

Three days after Foxo1-ASO treatment the cumulative food intake, body weight, epididymal fat pad weight, fasting insulin levels and insulin sensitivity were similar in control animals when compared to sense-injected control rats (Fig. 5*D*–*H*). However, 3 days after Foxo1-ASO treatment in DIO rats, there was a reduction in cumulative food intake (21%), body weight change (28%), epididymal fat pad weight (22%) and fasting serum insulin levels (19%) and an increase in insulin sensitivity (34%) when compared to sense-injected DIO rats (Fig. 5*D*–*H*). The fasting glucose concentration was similar between the groups (data not shown).

Discussion

During the last decade, advances have been made in the characterization of the role played by the hypothalamus in the coordination of food intake and energy expenditure (Friedman, 2000; Flier, 2004). Multiple hypothalamic neuronal signalling pathways and the cross talk between these pathways are involved in the control of energy intake (Schwartz *et al.* 2000; Minokoshi *et al.* 2004; Cota *et al.* 2006; Ropelle *et al.* 2007, 2008*b*). In addition to leptin, insulin is also able to reduce food intake (Schwartz *et al.* 2000; Carvalheira *et al.* 2001; Niswender *et al.* 2003). It has been demonstrated that the increased responsiveness of leptin and insulin action in the hypothalamus could be pathophysiologically important in the prevention of obesity (Picardi *et al.* 2008; Ropelle *et al.* 2008*a*) and Foxo1 is a distal protein of the insulin signalling that contributed to anorexigenic effects of insulin (Romanatto *et al.* 2007; Belgardt *et al.* 2008). In the present study, we demonstrate that intracerebroventricular (I.C.V.) microinfusion of insulin reduced Foxo1 expression in the hypothalamus of control but not in rats with diet-induced obesity (DIO). We showed that the injection of Foxo1 antisense oligonucleotide (Foxo1-ASO) in the hypothalamus leads to reduced Foxo1 expression in both control and DIO rats, and reduced food consumption and body weight gain in DIO but not in control rats.

As in peripheral tissues, neuronal insulin action involves the IR/IRS/phosphatidylinositol 3-kinase (PI3K) signal transduction pathway (Schwartz *et al.* 2000). The hypothalamic insulin signalling increases after either I.C.V. or systemic insulin administration (Niswender *et al.* 2003) and the inhibitory effect of I.C.V. insulin on both food intake (Carvalheira *et al.* 2001; Niswender *et al.* 2003) and the impairment in this pathway contribute to the hyperphagic response (Carvalheira *et al.* 2003; De Souza *et al.* 2005). On the other hand, the improvement in the hypothalamic IR/IRS-2/Akt pathway increases insulin-induced anorexigenic signals (Flores *et al.* 2006). Foxo1 is a downstream target of Akt. Activation of Akt phosphorylates Foxo1, leading to its nuclear exclusion and proteosomal degradation (Matsuzaki *et al.* 2003; Aoki

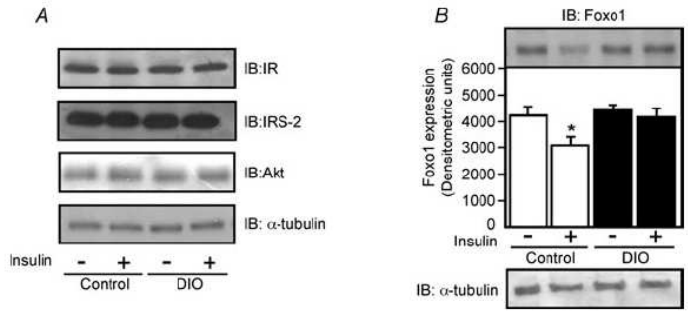

Figure 3. Effects of I.C.V. insulin infusion on Foxo1 expression in the hypothalamus of control and DIO rats

A, representative blots show the protein expression of insulin receptor (IR), insulin receptor substrate-2 (IRS-2) and Akt in the hypothalamus of control and DIO rats injected with saline (2.0 μl) or insulin (2.0 μl, 10^{-6} M). *B*, representative blots show the protein expression of Foxo1 in the hypothalamus of control and DIO rats injected with saline (2.0 μl) or insulin (2.0 μl, 10^{-6} M). The results of scanning densitometry were expressed as arbitrary units. Bars represent means ± S.E.M. of six rats. *$P < 0.03$ *versus* control plus saline.

et al. 2004), and thereby inhibiting its anorexigenic actions. Here, we showed that I.C.V. insulin infusion increased the IR/IRS-2/Akt/Foxo1 phosphorylation in control rats to a greater extent than in DIO animals. This aberrant molecular signalling also reduced insulin-induced Foxo1 degradation in the hypothalamus of DIO rats compared with control rats. These data suggest that hypothalamic Foxo1 phosphorylation and degradation are required for the induction of anorexia by insulin, establishing a signalling pathway through which insulin acts in hypothalamus neurons to control food intake.

Foxo1 is expressed in cells in the hypothalamus, in POMC and Agouti-related protein (AgRP) neurons (Kitamura *et al.* 2006). In these neurons, Foxo1 stimulates the transcription of the orexigenic neuropeptide Y (NPY) and AgRP through the PI3K/Akt signalling pathway, and suppresses the transcription of anorexigenic POMC by antagonizing the activity of STAT3 in lean mice (Kim *et al.* 2006; Kitamura *et al.* 2006). Different groups have demonstrated that the hypothalamic Foxo1 expression controls food intake in rodents (Kim *et al.* 2006; Kitamura *et al.* 2006). It has been proposed that insulin and leptin decrease hypothalamic Foxo1 expression. In agreement with this, activation of insulin signalling by expression of PI3K and Akt or treatment with insulin and leptin inhibits Foxo1-stimulated NPY transcription (Kim *et al.* 2006).

In the present study, we sought to determine the effects of Foxo1-ASO in the hypothalamus of DIO rats. The I.C.V. treatment with Foxo1-ASO promoted reduction of Foxo1 protein expression in a time- and dose-dependent manner. Foxo1-ASO also reduced

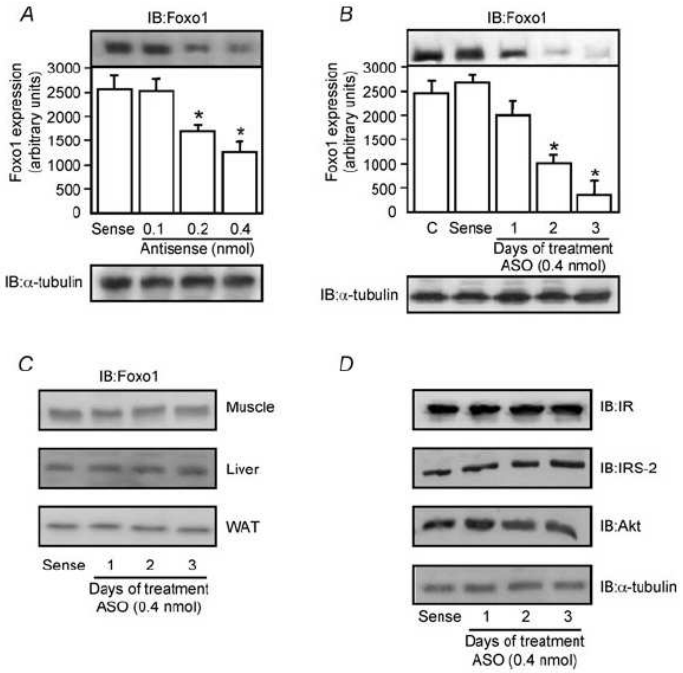

Figure 4. Effects of I.C.V. Foxo1-ASO injection in hypothalamic Foxo1 expression in lean rats
Representative Western blots demonstrating the dose-dependent effect of 24 h of treatment with saline, sense or Foxo1-ASO in lean rats (*A*) and hypothalamic Foxo1 expression in a time-dependent manner after central infusions of Foxo1-ASO in lean rats (*B*). *C*, representative Western blots show the protein expression of Foxo1 in the gastrocnemius muscle, hepatic tissue and white adipose tissue after I.C.V. infusion of Foxo1-ASO in lean rats during 3 days (0.4 nmol). *D*, representative Western blots show the protein expression of IR, IRS-2, Akt and α-tubulin in the hypothalamus of lean rats after I.C.V. infusion of Foxo1-ASO in lean rats during 3 days (0.4 nmol). The results of scanning densitometry were expressed as arbitrary units. Bars represent means ± S.E.M. of eight rats. *$P < 0.01$ *versus* sense treatment.

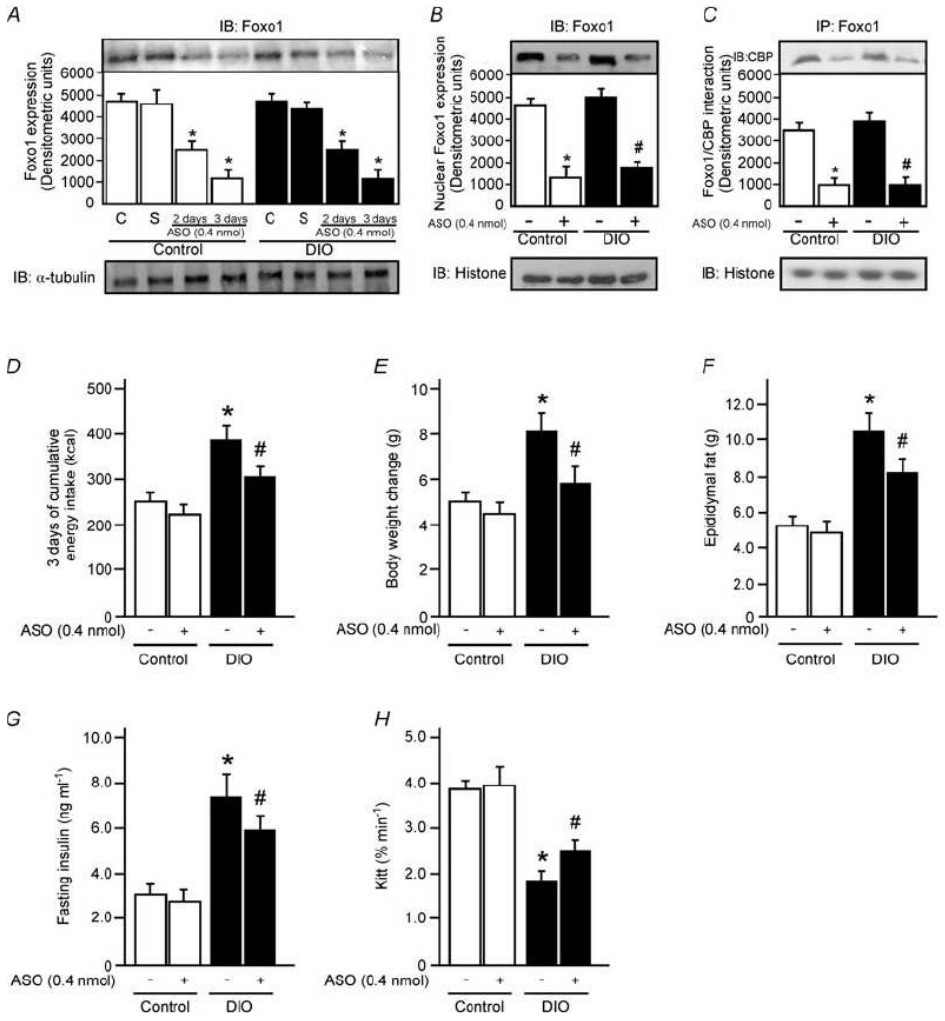

Figure 5. The effect of central infusion of Foxo1-ASO on total Foxo1 expression, food intake and body weight in control and DIO rats

Hypothalamic Foxo1 expression was evaluated after 72 h of central infusions of Foxo1-ASO in lean and DIO rats. Rats received I.C.V. saline (C), sense (S) or Foxo1-ASO (0.4 nmol) during 2 or 3 days (*A*). The evaluation of nuclear Foxo1 expression (*B*) and the association between Foxo1 and CBP/p300 (*C*) were performed using nuclear extract of control and DIO rats as described in Methods. Determination of 3 days of cumulative energy intake (*D*), body weight change (*E*), epididymal fat pad weight (*F*), fasting serum insulin (*G*) and insulin tolerance test (*H*) of control and DIO rats 3 days after Foxo1-ASO or sense I.C.V. infusion. Bars represent means ± S.E.M. of 6–8 rats; $^{*}P < 0.001$, ASO *versus* control plus sense; #$P < 0.03$, *versus* DIO plus sense.

nuclear interaction between Foxo1 and CBP/p300 in the hypothalamus of control and DIO rats. Three days of treatment with Foxo-ASO (4 nmol) reduced the cumulative energy intake, body weight gain and epididymal fat pad weight, fasting insulin levels and increased insulin sensitivity in DIO but not in control animals. Although Kim and colleagues showed that bilateral injection of the Foxo1 siRNA into the arcuate nucleus of hypothalamus decreased daily food intake and body weight in lean mice (Kim *et al.* 2006), our results showed that a daily infusion of Foxo1-ASO during 3 days reduced the cumulative food intake in DIO but not in control animals. Our data are in accordance with Kitamura and coworkers who explored the contribution of loss-of-function of Foxo1 on orexigenic signalling and food intake using two different approaches in lean mice. The injection of a dominant-negative of Foxo1 prevented induction of AgRP expression caused by fasting but the AgRP expression was similar in mice under normal conditions. Furthermore, in Foxo1 haploinsufficient mice ($Foxo1^{+/-}$), the food intake was similar under basal conditions when compared to wild-type littermate controls and the difference in food intake occurred only after I.C.V. leptin infusion in $Foxo1^{+/-}$ mice when compared to control mice (Kitamura *et al.* 2006). These apparent contradictory results in lean animals may be related to the different approaches and model of rodent and deserves further investigation.

The *in vivo* physiological importance of Foxo transcription factors in the brain has been reported. Several studies showed that Foxo1 is expressed in different areas of murine brain, including hippocampus, neocortex, hypothalamus and other areas (Fukunaga *et al.* 2005; Hoekman *et al.* 2006; Kitamura *et al.* 2006). In the hypothalamus of rodents, Foxo1 is expressed in a majority of cells in the arcuate nucleus, ventromedial hypothalamus and dorsomedial hypothalamus (Kitamura *et al.* 2006). Although the Foxo1-ASO injection into the third ventricle of rats reduced Foxo1 expression in a tissue-specific manner as demonstrated in Fig. 4, we cannot exclude the possibility that Foxo1-ASO reduced the Foxo1 expression in other areas of the brain.

Collectively, these data showed that central insulin resistance diminished insulin-induced Foxo1 phosphorylation and insulin-induced Foxo1 degradation. Thus, we identify that a high-fat diet impaired the hypothalamic Foxo1 phosphorylation and increased the nuclear activity, increasing the hyperphagic response in rats. Foxo1-ASO treatment blocked the orexigenic effects of hypothalamic Foxo1 and prevented the hyperphagic response in these animals. Thus, pharmacological manipulation of Foxo1 may be used to prevent or treat obesity.

References

Aoki M, Jiang H & Vogt PK (2004). Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. *Proc Natl Acad Sci U S A* **101**, 13613–13617.

Belgardt BF, Husch A, Rother E, Ernst MB, Wunderlich FT, Hampel B, Klockener T, Alessi D, Kloppenburg P & Bruning JC (2008). PDK1 deficiency in POMC-expressing cells reveals FOXO1-dependent and -independent pathways in control of energy homeostasis and stress response. *Cell Metab* **7**, 291–301.

Bonora E, Moghetti P, Zancanaro C, Cigolini M, Querena M, Cacciatori V, Corgnati A & Muggeo M (1989). Estimates of in vivo insulin action in man: comparison of insulin tolerance tests with euglycemic and hyperglycemic glucose clamp studies. *J Clin Endocrinol Metab* **68**, 374–378.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Carvalheira JB, Ribeiro EB, Araujo EP, Guimaraes RB, Telles MM, Torsoni M, Gontijo JA, Velloso LA & Saad MJ (2003). Selective impairment of insulin signalling in the hypothalamus of obese Zucker rats. *Diabetologia* **46**, 1629–1640.

Carvalheira JB, Siloto RM, Ignacchitti I, Brenelli SL, Carvalho CR, Leite A, Velloso LA, Gontijo JA & Saad MJ (2001). Insulin modulates leptin-induced STAT3 activation in rat hypothalamus. *FEBS Lett* **500**, 119–124.

Cheatham B & Kahn CR (1995). Insulin action and the insulin signaling network. *Endocr Rev* **16**, 117–142.

Chen R, Kim O, Yang J, Sato K, Eisenmann KM, McCarthy J, Chen H & Qiu Y (2001). Regulation of Akt/PKB activation by tyrosine phosphorylation. *J Biol Chem* **276**, 31858–31862.

Cota D, Proulx K, Smith KA, Kozma SC, Thomas G, Woods SC & Seeley RJ (2006). Hypothalamic mTOR signaling regulates food intake. *Science* **312**, 927–930.

De Souza CT, Araujo EP, Bordin S, Ashimine R, Zollner RL, Boschero AC, Saad MJ & Velloso LA (2005). Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. *Endocrinology* **146**, 4192–4199.

Downward J (1998). Mechanisms and consequences of activation of protein kinase B/Akt. *Curr Opin Cell Biol* **10**, 262–267.

Flier JS (2004). Obesity wars: molecular progress confronts an expanding epidemic. *Cell* **116**, 337–350.

Flores MB, Fernandes MF, Ropelle ER, Faria MC, Ueno M, Velloso LA, Saad MJ & Carvalheira JB (2006). Exercise improves insulin and leptin sensitivity in hypothalamus of Wistar rats. *Diabetes* **55**, 2554–2561.

Folli F, Saad MJ, Backer JM & Kahn CR (1992). Insulin stimulation of phosphatidylinositol 3-kinase activity and association with insulin receptor substrate 1 in liver and muscle of the intact rat. *J Biol Chem* **267**, 22171–22177.

Friedman JM (2000). Obesity in the new millennium. *Nature* **404**, 632–634.

Fukunaga K, Ishigami T & Kawano T (2005). Transcriptional regulation of neuronal genes and its effect on neural functions: expression and function of forkhead transcription factors in neurons. *J Pharmacol Sci* **98**, 205–211.

Gasparetti AL, de Souza CT, Pereira-da-Silva M, Oliveira RL, Saad MJ, Carneiro EM & Velloso LA (2003). Cold exposure induces tissue-specific modulation of the insulin-signalling pathway in *Rattus norvegicus*. *J Physiol* **552**, 149–162.

Hoekman MF, Jacobs FM, Smidt MP & Burbach JP (2006). Spatial and temporal expression of FoxO transcription factors in the developing and adult murine brain. *Gene Expr Patterns* **6**, 134–140.

Kim MS, Pak YK, Jang PG, Namkoong C, Choi YS, Won JC, Kim KS, Kim SW, Kim HS, Park JY, Kim YB & Lee KU (2006). Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. *Nat Neurosci* **9**, 901–906.

Kitamura T, Feng Y, Kitamura YI, Chua SC Jr, Xu AW, Barsh GS, Rossetti L & Accili D (2006). Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. *Nat Med* **12**, 534–540.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Matsuzaki H, Daitoku H, Hatta M, Tanaka K & Fukamizu A (2003). Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *Proc Natl Acad Sci U S A* **100**, 11285–11290.

Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B, Mu J, Foufelle F, Ferre P, Birnbaum MJ, Stuck BJ & Kahn BB (2004). AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* **428**, 569–574.

Niswender KD, Morrison CD, Clegg DJ, Olson R, Baskin DG, Myers MG Jr, Seeley RJ & Schwartz MW (2003). Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. *Diabetes* **52**, 227–231.

Pauli JR, Ropelle ER, Cintra DE, Carvalho-Filho MA, Moraes JC, De Souza CT, Velloso LA, Carvalheira JB & Saad MJ (2008). Acute physical exercise reverses *S*-nitrosation of the insulin receptor, insulin receptor substrate 1 and protein kinase B/Akt in diet-induced obese Wistar rats. *J Physiol* **586**, 659–671.

Pereira-da-Silva M, Torsoni MA, Nourani HV, Augusto VD, Souza CT, Gasparetti AL, Carvalheira JB, Ventrucci G, Marcondes MC, Cruz-Neto AP, Saad MJ, Boschero AC, Carneiro EM & Velloso LA (2003). Hypothalamic melanin-concentrating hormone is induced by cold exposure and participates in the control of energy expenditure in rats. *Endocrinology* **144**, 4831–4840.

Picardi PK, Calegari VC, Prada PO, Moraes JC, Araujo E, Marcondes MC, Ueno M, Carvalheira JB, Velloso LA & Saad MJ (2008). Reduction of hypothalamic protein tyrosine phosphatase improves insulin and leptin resistance in diet-induced obese rats. *Endocrinology* **149**, 3870–3880.

Prada PO, Pauli JR, Ropelle ER, Zecchin HG, Carvalheira JB, Velloso LA & Saad MJ (2006). Selective modulation of the CAP/Cbl pathway in the adipose tissue of high fat diet treated rats. *FEBS Lett* **580**, 4889–4894.

Romanatto T, Cesquini M, Amaral ME, Roman EA, Moraes JC, Torsoni MA, Cruz-Neto AP & Velloso LA (2007). TNF-α acts in the hypothalamus inhibiting food intake and increasing the respiratory quotient – effects on leptin and insulin signaling pathways. *Peptides* **28**, 1050–1058.

Ropelle ER, Fernandes MF, Flores MB, Ueno M, Rocco S, Marin R, Cintra DE, Velloso LA, Franchini KG, Saad MJ & Carvalheira JB (2008*a*). Central exercise action increases the AMPK and mTOR response to leptin. *PLoS ONE* **3**, e3856.

Ropelle ER, Pauli JR, Fernandes MF, Rocco SA, Marin RM, Morari J, Souza KK, Dias MM, Gomes-Marcondes MC, Gontijo JA, Franchini KG, Velloso LA, Saad MJ & Carvalheira JB (2008*b*). A central role for neuronal AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) in high-protein diet-induced weight loss. *Diabetes* **57**, 594–605.

Ropelle ER, Pauli JR, Prada PO, de Souza CT, Picardi PK, Faria MC, Cintra DE, Fernandes MF, Flores MB, Velloso LA, Saad MJ & Carvalheira JB (2006). Reversal of diet-induced insulin resistance with a single bout of exercise in the rat: the role of PTP1B and IRS-1 serine phosphorylation. *J Physiol* **577**, 997–1007.

Ropelle ER, Pauli JR, Zecchin KG, Ueno M, de Souza CT, Morari J, Faria MC, Velloso LA, Saad MJ & Carvalheira JB (2007). A central role for neuronal adenosine 5′-monophosphate-activated protein kinase in cancer-induced anorexia. *Endocrinology* **148**, 5220–5229.

Saad MJ, Folli F, Kahn JA & Kahn CR (1993). Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. *J Clin Invest* **92**, 2065–2072.

Schwartz MW, Woods SC, Porte D Jr, Seeley RJ & Baskin DG (2000). Central nervous system control of food intake. *Nature* **404**, 661–671.

Scott AM, Atwater I & Rojas E (1981). A method for the simultaneous measurement of insulin release and B cell membrane potential in single mouse islets of Langerhans. *Diabetologia* **21**, 470–475.

Spiegel A, Nabel E, Volkow N, Landis S & Li TK (2005). Obesity on the brain. *Nat Neurosci* **8**, 552–553.

Tang ED, Nunez G, Barr FG & Guan KL (1999). Negative regulation of the forkhead transcription factor FKHR by Akt. *J Biol Chem* **274**, 16741–16746.

Williamson D, Gallagher P, Harber M, Hollon C & Trappe S (2003). Mitogen-activated protein kinase (MAPK) pathway activation: effects of age and acute exercise on human skeletal muscle. *J Physiol* **547**, 977–987.

Zimmet P, Alberti KG & Shaw J (2001). Global and societal implications of the diabetes epidemic. *Nature* **414**, 782–787.

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E.R.R.: conception and design, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, final approval of the version to be published; J.R.P.: conception and design; P.P.: conception and design; D.E.C.: conception and design; G.Z.R.: conception and design; J.C.M.: conception and design; M.J.S.F.: conception and design;

G.d.L.: conception and design; R.A.P.: final approval of the version to be published; J.B.C.C.: drafting the article or revising it critically for important intellectual content; L.A.V.: drafting the article or revising it critically for important intellectual content; M.J.S.: drafting the article or revising it critically for important intellectual content; C.T.D.S: conception and design, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, final approval of the version to be published.

Acknowledgements

These studies were supported by grants from Fundacão de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de desenvolvimento científico e tecnológico (CNPq).

APÊNDICE III

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IL-6 and IL-10 Anti-Inflammatory Activity Links Exercise to Hypothalamic Insulin and Leptin Sensitivity through IKKβ and ER Stress Inhibition

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Abstract

Overnutrition caused by overeating is associated with insulin and leptin resistance through IKKβ activation and endoplasmic reticulum (ER) stress in the hypothalamus. Here we show that physical exercise suppresses hyperphagia and associated hypothalamic IKKβ/NF-κB activation by a mechanism dependent upon the pro-inflammatory cytokine interleukin (IL)-6. The disruption of hypothalamic-specific IL-6 action blocked the beneficial effects of exercise on the re-balance of food intake and insulin and leptin resistance. This molecular mechanism, mediated by physical activity, involves the anti-inflammatory protein IL-10, a core inhibitor of IKKβ/NF-κB signaling and ER stress. We report that exercise and recombinant IL-6 requires IL-10 expression to suppress hyperphagia-related obesity. Moreover, in contrast to control mice, exercise failed to reverse the pharmacological activation of IKKβ and ER stress in C3H/HeJ mice deficient in hypothalamic IL-6 and IL-10 signaling. Hence, inflammatory signaling in the hypothalamus links beneficial physiological effects of exercise to the central action of insulin and leptin.

Citation: Ropelle ER, Flores MB, Cintra DE, Rocha GZ, Pauli JR, et al. (2010) IL-6 and IL-10 Anti-Inflammatory Activity Links Exercise to Hypothalamic Insulin and Leptin Sensitivity through IKKβ and ER Stress Inhibition. PLoS Biol 8(8): e1000465. doi:10.1371/journal.pbio.1000465

Academic Editor: Antonio J. Vidal-Puig, University of Cambridge, United Kingdom

Received October 23, 2009; **Accepted** July 15, 2010; **Published** August 24, 2010

Funding: This study was supported by grants from Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP) and Conselho Nacional de desenvolvimento cientifico e tecnológico (CNPq). The funders had no role in study design, data lection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: ASO IL-10, IL-10 antisense oligonucleotide; CNS, central nervous system; DIO, diet-induced obese; ER, endoplasmatic reticulum; IL, interleukin; IL-1ra, IL-1 receptor antagonist; IL-6R, IL-6 Receptor; IL-10R, IL-10 Receptor; IRS-1, insulin receptor substrate-1; Jak-2, Janus Kinase-2; NPY, Neuropeptide-Y; POMC, Proopiomelanocortin; sTNF-R, soluble TNF-receptors; SW Exe, swimming exercise; T2D, type 2 diabetes; TG, thapsigargin; TR Exe, treadmill running exercise; WT, wild type.

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Introduction

Overnutrition and sedentary lifestyle are among the most important factors that lead to an unprecedented increase in the prevalence of obesity. In mammals, food intake and energy expenditure are tightly regulated by specific neurons localized in the hypothalamus. The hypothalamus can gather information on the body's nutritional status by integrating multiple signals, including potent hormonal signals such as insulin and leptin [1,2]. The impairment of hypothalamic insulin and leptin signaling pathways is sufficient to promote hyperphagia, obesity, and type 2 diabetes (T2D) in different genetic rodent models with neuronal ablation of insulin and leptin signaling [1,3,4]. We and others have proposed that overnutrition induces the central insulin and leptin resistance through the aberrant hypothalamic activation of proinflammatory molecules, including TLR4 and IKK [5–7].

IKKβ is a key player in controlling both innate and adaptive immunity. Activation of IKKβ by phosphorylation at S177 and S181 induces phosphorylation, ubiquitination, and subsequent proteosomal degradation of its substrate IκBα. The degradation of IκBα allows NF-κB proteins to translocate to the nucleus and bind their cognate DNA binding sites to regulate the transcription of a large number of genes, including stress-response proteins and cytokines [8]. Growing evidence provides an intriguing link between metabolic inflammation and dysfunction of insulin and leptin signaling via activation of IKKβ and endoplasmatic reticulum (ER) stress [9–14]. Examination of ER stress markers in different tissues of dietary (high-fat diet-induced) and genetic (*ob/ob*) mouse models of obesity demonstrated increased levels of PERK phosphorylation and JNK and IKKβ activity [7,12]. In addition, a recent study showed the activation of hypothalamic IKKβ/NF-κB, at least in part, through elevated endoplasmic reticulum stress in the hypothalamus and that these phenomena are associated with central insulin and leptin resistance, hyperphagia, and body weight gain in mice [7]. Thus, strategies to reduce the aberrant activation of inflammatory signaling and/or ER stress in hypothalamic neurons are of great interest to improve the central insulin and leptin action and prevent or treat obesity and related diseases.

Author Summary

The hypothalamus is a brain region that gathers information on the body's nutritional status and governs the release of multiple metabolic signaling molecules such as insulin and leptin to maintain homeostasis. Overeating and obesity are associated with insulin and leptin resistance in the hypothalamus, and recent studies provide an intriguing link between inflammation and dysfunction of hypothalamic insulin and leptin signaling through activation of IKKβ, a key player in immune response, and endoplasmic reticulum (ER) stress. This means that strategies to reduce the aberrant activation of inflammatory signaling in the hypothalamus are of great interest to improve the central insulin and leptin action and prevent or treat related metabolic diseases. Using a combination of pharmacological, genetic, and physiological approaches, our study indicates that physical activity reorganizes the set point of nutritional balance through anti-inflammatory signaling mediated by interleukin (IL)-6 and IL-10 in the hypothalamus of rodents. Hence, IL-6 and IL-10 are important physiological contributors to the central insulin and leptin action mediated by exercise, linking it to hypothalamic ER stress and inflammation.

Physical activity is considered a cornerstone of the treatment for obesity. Exercise has long been reported to reduce body weight and visceral adiposity, increasing the energy expenditure and improving glycaemic control in overweight or T2D patients [15,16]. Since the discovery of interleukin (IL)-6 releases from contracting skeletal muscle, accumulating evidence indicates that exercise induces metabolic changes in other organs, such as the liver, the adipose tissue, and hypothalamus, in an IL-6 dependent manner. IL-6 is most often classified as a pro-inflammatory cytokine, although consistent data also demonstrate that IL-6 has an anti-inflammatory effect and may negatively regulate the inflammation of acute phase response by increasing IL-10, IL-1 receptor antagonist (IL-1ra), and soluble TNF-receptors (sTNF-R) [17]. Moreover, IL-6 appears to play a central role in the regulation of appetite, energy expenditure, and body composition [18,19]. However, the effects of physical activity in the metabolic regulatory pathways in the central nervous system (CNS) remain unexplored. Thus, we hypothesized that exercise could exert its effects in the CNS by modulating the specific hypothalamic neurons responsible for the control of food consumption. In the present study, we investigated the effect of the anti-inflammatory response, mediated by IL-6, on hypothalamic IKKβ activation and ER stress, central insulin and leptin sensitivity, and food intake in diet-induced rats after physical activity.

Results

Exercise Suppresses Hyperphagia Mediated by Overnutrition

It has been demonstrated that physical activity may contribute to the energy balance by increasing energy expenditure. Although the energy expenditure aspects of such exercise may contribute to the effects of weight loss, the effect of exercise on the control of energy intake remains unclear. To evaluate the impact of physical activity on food consumption, we measured the 12-h total energy intake in lean and diet-induced obese (DIO) rats after one bout of swimming (SW Exe) and treadmill running (TR Exe) exercise. Neither of the exercise protocols changed the energy intake in lean animals; however, exercise suppressed the hyperphagic response, mediated by chronic overnutrition, restoring the energy intake to the levels of lean animals (Figure 1A). To assess whether the effects of exercise on food intake are dependent on the neuropeptides modulation, we performed a real time PCR assay to determine the mRNA levels of Neuropeptide-Y (NPY) and Proopiomelanocortin (POMC). After 9 h of fasting, we found that chronic overnutrition increased NPY mRNA and reduced POMC mRNA levels, while physical activity restored the NPY (Figure 1B) and POMC mRNA levels (Figure 1C) in obese animals; on the other hand, exercise did not change the NPY and POMC mRNA levels in lean rats (Figure 1B and C).

Chronic overnutrition increased body weight, epididymal fat (Figure 1D and E), serum insulin, leptin, triglycerides, and free fatty acid levels (Table 1), compared to age-matched controls. No significant variations were found in body weight, epididymal fat serum leptin, triglycerides, and urinary corticosterone levels between exercised and obese animals under resting conditions (Figure 1D, E and Table 1). The insulin levels were lower in both lean and obese rats after the exercise protocols (Table 1) and exercise increased the free fatty acid in obese animals (Table 1). To determine whether lean and obese rodents were swimming or running in the same fashion, we evaluated lactate production every 15 min during the SW Exe and TR Exe. We did not find any difference in the lactate production between lean and obese rats. Table 1 depicts the final values obtained in this test. These results reinforce the negative relationship between body weight change and stress related with the appetite-suppressive actions mediated by exercise.

To extend our hypothesis, we investigated food intake in leptin-deficient mice (*ob/ob*) after physical activity. Acute SW Exe and TR Exe did not change the food intake in wild type (WT) mice, however the food consumption was reduced in *ob/ob* mice (Figure 1F). After 9 h of fasting, we found that NPY mRNA was increased and POMC mRNA levels were reduced in *ob/ob* mice, while physical activity restored the NPY (Figure 1G) and POMC mRNA levels (Figure 1H) in obese animals; on the other hand, exercise did not change the NPY and POMC mRNA levels in control mice (Figure 1G and H). Exercise did not change the total body weight and epididymal fat pad weight in WT and *ob/ob* mice (Figure 1I and J). In addition, we observed that the exercise protocols did not change the triglycerides and free fatty acid levels but reduced the insulin levels in WT and *ob/ob* mice (Table 2). The lactate production was similar between lean and obese mice during the respective exercise protocols (Table 2). These exercise protocols did not evoke any significant stressful effect in these animals, as demonstrated by urinary corticosterone levels (Table 2). Thus, our data demonstrate that exercise modulates hypothalamic neuropeptides (NPY and POMC) and suppresses food intake in obese, but not in lean, rodents without changing the adipose tissue content and corticosterone levels.

Exercise Restores Insulin and Leptin Sensitivity in the Hypothalamus

Next, we evaluated whether exercise modulates insulin signaling in the hypothalamus. Western blot analysis revealed that IRβ, IRS-1, IRS-2, Akt, and FOXO1 phosphorylation were similar between the groups (Figure 2A and B). Although exercise did not change the basal levels of insulin signaling, we next performed intrahypothalamic insulin (200 mU) or its vehicle injection to evaluated food intake and insulin sensitivity after the SW Exe protocol. Overnutrition markedly reduced the ability of intrahypothalamic insulin infusion to reduce food intake, when compared to chow-fed animals; however, exercise restored the central effects of insulin on reduced food intake (Figure 2C). Using Western

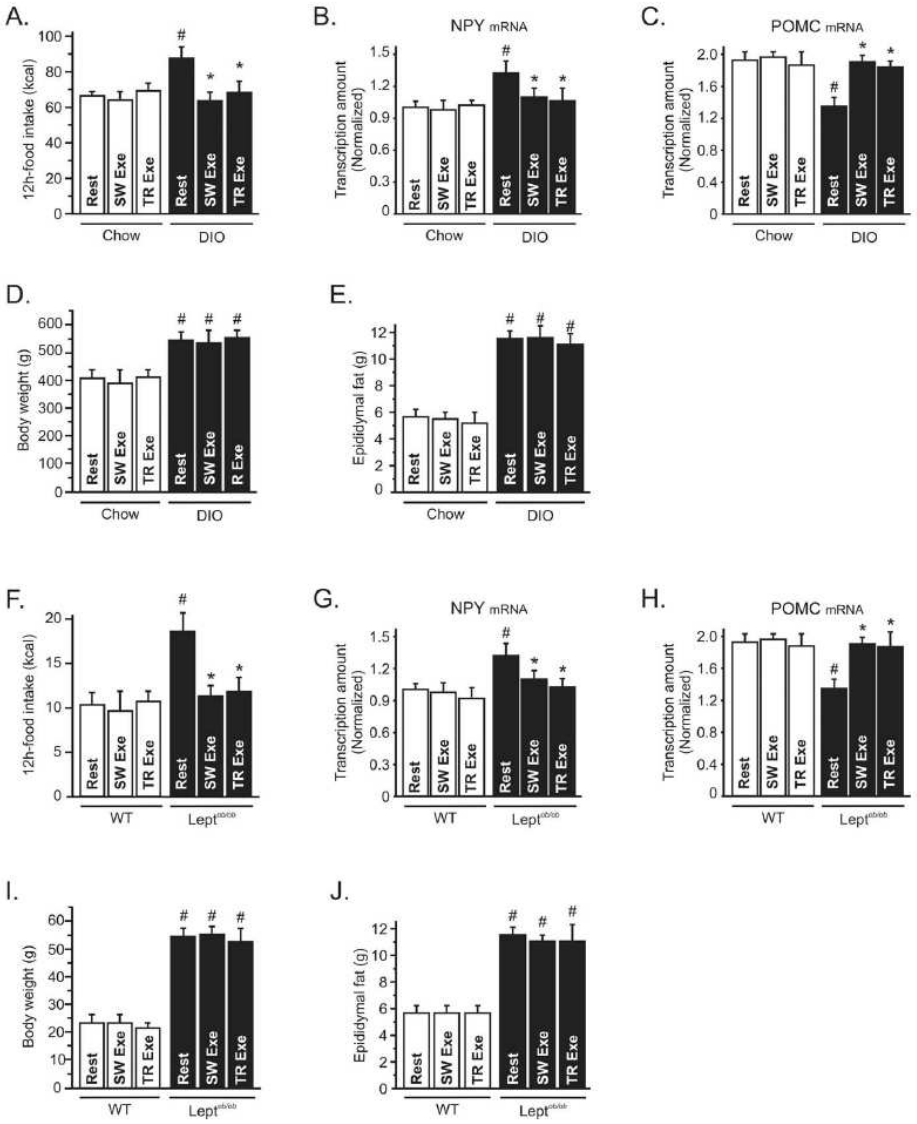


Figure 1. Exercise induces appetite-suppressive actions in different models of obesity. (A) 12 h of food intake (kcal) in lean and diet-induced obesity (DIO) Wistar rats under resting conditions or after swimming exercise (SW Exe) or treadmill running (TR Exe) ($n = 20–35$ animals per group). Rats were fasted during 9 h and the hypothalamic levels (B) NPY and (C) POMC mRNA were examined using real time PCR assay. (D) Body weight, (E) epididymal fat pad weight, (F) 12-h food intake of leptin-deficient mice ($Lept^{ob/ob}$) and respective wild type group. (G) NPY and (H) POMC mRNA were examined using real time PCR assay. (I) Body weight and (J) epididymal fat pad weight of wild type and leptin-deficient mice under resting conditions or immediately after the exercise protocols ($n = 10$ animals per group). Data are the means ± SEM. # $p<0.05$ versus respective lean group at rest; * $p<0.05$ versus respective obese group at rest. Lean animals (white bars) and obese animals (black bars).
doi:10.1371/journal.pbio.1000465.g001

Table 1. Metabolic parameters of lean and DIO rats after acute exercise protocols.

Groups	Glucose (mg/dL)	Insulin (ng/mL)	Leptin (ng/mL)	Cholesterol (mg/dL)	TG (mg/dL)	FFA (mmol/L)	Corticost. (ng/mL)	Lactate (mmol/L)
Chow rest	97±5	4.0±0.2	2.0±0.2	129.3±8.5	94.0±1,4	0.64±0.2	11.1±0.6	ND
Chow SW exe	108±9	2.8±0.3#	2.1±0.1	123.7±6.4	93,7±7,2	0.81±0.1	11.0±0.4	3.6±0.6
Chow TR exe	118±12	2.9±0.2#	2.1±0.2	121.8±6.3	97.3±7.5	0.79±0.2	11.4±0.5	4.20±0.4
DIO rest	115±5	7.8±0.4#	3.6±0.3#	141.0±10.1	152.5±7.8#	1.75±0.5#	11.2±0.7	ND
DIO SW exe	117±9	6.1±0.3#*	3.7±0.2#	141.6±9.5	141.7±9.5#	2.89±0.3#*	10.4±0.8	4.0±0.5
DIO TR exe	112±15	6.2±0.2#*	3.6±0.3#	145.2±12.5	150.3±8.0#	2.65±0.4#*	10.5±0.7	3.9±0.3

# $p<0.05$ versus chow rest and * $p<0.05$ versus DIO rest ($n=8$–10).
doi:10.1371/journal.pbio.1000465.t001

blotting analysis, we determined the effects of exercise on the insulin sensitivity in hypothalamic tissue. The high-fat diet impaired insulin-induced tyrosine phosphorylation of insulin receptor β (IRβ), insulin receptor substrate-1 (IRS-1), and IRS-2 in the hypothalamus (Figure 2D). Similar results were observed for the serine phosphorylation of Akt and FOXO1 (Figure 2D). Physical activity was able to restore insulin-induced hypothalamic IRβ, IRS-1, and IRS-2 tyrosine phosphorylation and insulin-induced hypothalamic Akt and FOXO1 serine phosphorylation in DIO rats (Figure 2D). Subcellular fraction of hypothalamic extract was then performed to evaluate the nuclear FOXO1 expression. Intrahypothalamic infusion of insulin reduced the nuclear FOXO1 expression in control rats, but insulin failed to reduce the nuclear FOXO1 expression in rats after overnutrition (Figure 2E). After exercise, insulin reduced the nuclear FOXO1 expression in neuronal cells of obese animals (52%), when compared to DIO at rest (Figure 2E).

We then explored the effects of exercise on hypothalamic leptin action, monitoring Janus Kinase-2 (Jak-2) and STAT-3 tyrosine phosphorylation. Exercise did not change the Jak-2 and STAT-3 phosphorylation in lean animals; however, overnutrition reduced Jak-2 and STAT-3 phosphorylation when compared to lean animals. Interestingly, physical activity was able to increase the neuronal Jak-2 and STAT-3 tyrosine phosphorylation in obese animals (Figure 2F and G). In addition we investigated the effects of exercise on leptin sensitivity. Intrahypothalamic infusion of leptin markedly reduced the 12-h total energy intake in control rats; however, the anorexigenic effects of leptin were attenuated in obese rats. In contrast, exercise restored the central effects of leptin on reduced food intake (Figure 2H). We noted that leptin modestly promoted the hypothalamic tyrosine phosphorylation of Jak-2, IRS-1, IRS-2, and STAT-3 after high-fat diet treatment. Conversely, exercise restored leptin-induced hypothalamic Jak-2, IRS-1, IRS-2, and STAT-3 tyrosine phosphorylation in obese animals (Figure 2I).

We also evaluated nuclear STAT3 expression after intrahypothalamic leptin infusion. After overnutrition, leptin failed to increase the expression of nuclear STAT3 in the hypothalamus. On the other hand, exercise increased the ability of leptin to increase the nuclear expression of STAT3 (48%) in the hypothalamus of obese animals (Figure 2J).

Increasing Hypothalamic Levels of IL-6 Reverses IKKβ and ER Stress Caused by Obesity

Recently, IL-6 was reported as the first myokine that is produced and released by contracting skeletal muscle fibers, exerting its effects on other organs of the body [20], including the hypothalamus [18,21]. Thus, we evaluated the central role of IL-6 in the control of food intake. Firstly, the serum level of IL-6 was observed to be slightly up-regulated after high-fat diet treatment and was dramatically increased immediately after SW Exe and TR Exe, but we observed that, in exercised obese animals, the serum levels of IL-6 were higher when compared to exercised lean ones (Figure S1A). Similar results were found when IL-6 protein expression in the hypothalamic tissue was evaluated (Figure S1B). To investigate whether neuronal cells were producing IL-6 in response to exercise, we performed real time PCR to evaluate IL-6 mRNA levels in the hypothalamic tissue. IL-6 mRNA levels were slightly up-regulated after the high-fat diet treatment and were increased by about 53% and 64% immediately after physical activity in lean and obese rats, respectively (Figure 3A). Thus, these data demonstrate that exercise increases the serum and hypothalamic levels of IL-6.

Table 2. Metabolic parameters of control and *ob/ob* mice after acute exercise protocols.

Groups	Glucose (mg/dL)	Insulin (ng/mL)	Leptin (ng/mL)	Cholesterol (mg/dL)	TG (mg/dL)	Corticosterone (ng/mL)	Lactate (mmol/L)
WT rest	94±3	3.9±0.4	1.9±0.2	126.7±6.1	73.3±12.6	11.0±0.7	ND
WT SW exe	93±2	2.5±0.3#	2.1±0.3	123.5±3.5	79.0±11.5	11.1±0.5	4.2±0.3
WT TR exe	94±2	2.7±0.3#	2.0±0.3	128.5±3.5	77.33±12.7	11.5±0.8	5.3±1.3
$Lept^{a\Delta a\Delta}$ rest	284±18#	8.0±0.4#	ND	156.7±3.8#	194.5±32.9#	11.2±0.4	ND
$Lept^{a\Delta a\Delta}$ SW exe	154±8#*	6.2±0.4#*	ND	154.7±2.5#	176.7±14.9#	11.4±0.5	4.9±0.7
$Lept^{a\Delta a\Delta}$ TR exe	175±17#*	6.5±0.3#*	ND	153.2±2.7#	173.7±16.5#	11.2±0.6	5.3±0.8

# $p<0.05$ versus WT rest and *$p<0.05$ versus $Lept^{a\Delta a\Delta}$ rest ($n=6$–8). ND, no detected.
doi:10.1371/journal.pbio.1000465.t002

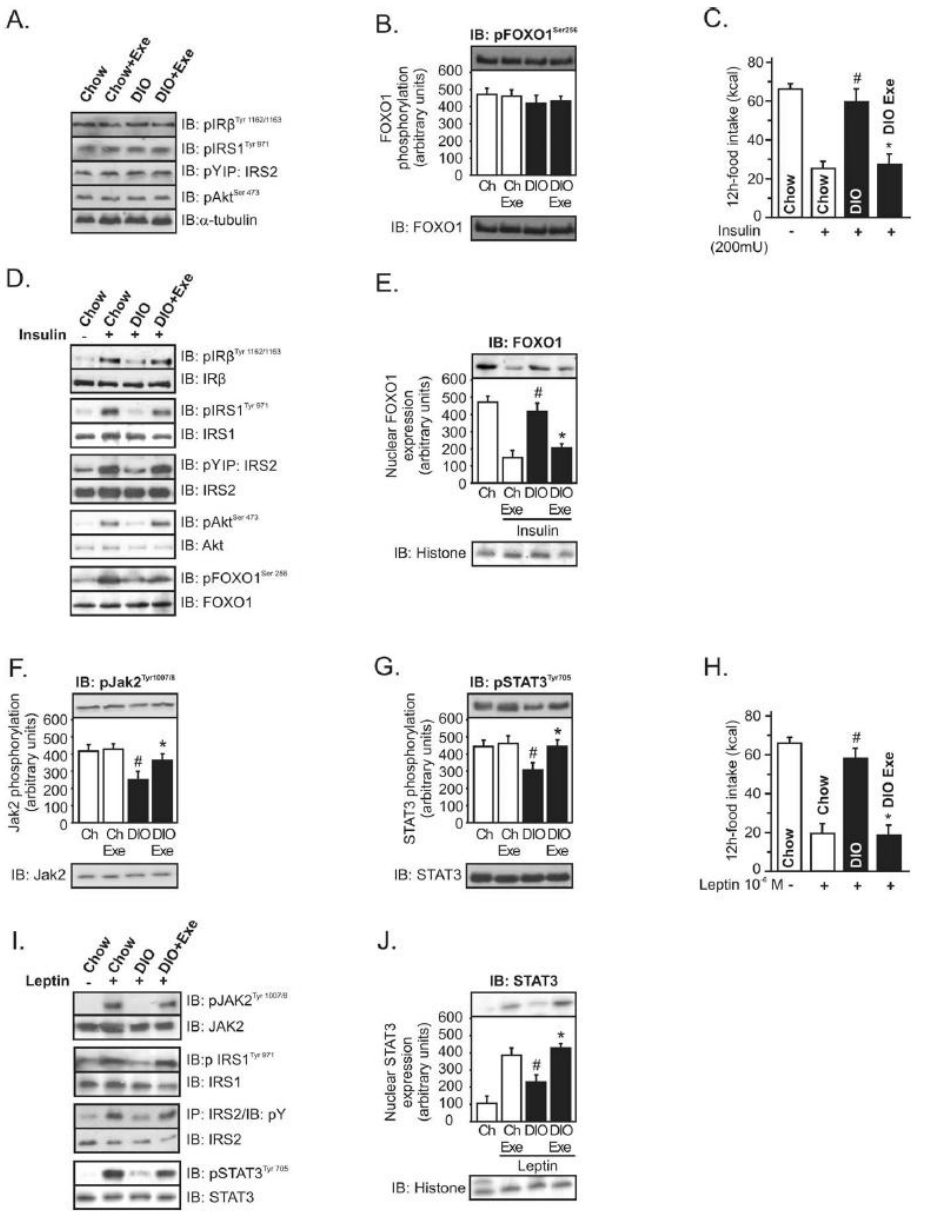

A.
Chow
Chow+Exe
DIO
DIO+Exe
IB: pIRβ Tyr 1162/1163
IB: pIRS1 Tyr 971
IB: pYIP: IRS2
IB: pAkt Ser 473
IB:α-tubulin
B.
IB: pFOXO1 Ser256
FOXO1 phosphorylation (arbitrary units)
Ch
Ch Exe
DIO
DIO Exe
IB: FOXO1
C.
12h-food intake (kcal)
Chow
Chow
DIO
DIO Exe
Insulin (200mU)
D.
Insulin
IB: pIRβ Tyr 1162/1163
IB: IRβ
IB: pIRS1 Tyr 971
IB: IRS1
IB: pYIP: IRS2
IB: IRS2
IB: pAkt Ser 473
IB: Akt
IB: pFOXO1 Ser 256
IB: FOXO1
E.
IB: FOXO1
Nuclear FOXO1 expression (arbitrary units)
Insulin
IB: Histone
F.
IB: pJak2 Tyr1007/8
Jak2 phosphorylation (arbitrary units)
IB: Jak2
G.
IB: pSTAT3 Tyr705
STAT3 phosphorylation (arbitrary units)
IB: STAT3
H.
12h-food intake (kcal)
Leptin 10-6 M
I.
Leptin
IB: pJAK2 Tyr 1007/8
IB: JAK2
IB:p IRS1 Tyr 971
IB: IRS1
IP: IRS2/IB: pY
IB: IRS2
IB: pSTAT3 Tyr 705
IB: STAT3
J.
IB: STAT3
Nuclear STAT3 expression (arbitrary units)
Leptin
IB: Histone

Figure 2. Hypothalamic insulin and leptin signaling after exercise. Western blots showing hypothalamic lysates from Wistar rats; (A) Hypothalamic IRβ, IRS-1, IRS-2, and Akt phosphorylation, (B) Hypothalamic Foxo1 phosphorylation. (C) 12-h food intake (kcal) after intrahypothalamic infusion of insulin in lean and diet-induced obesity (DIO) Wistar rats under resting conditions or after exercise ($n = 6$–8 animals per group). Western blots of five independent experiments showing hypothalamic lysates from Wistar rats; (D) Insulin-induced IRβ, IRS-1, IRS-2, Akt, and Foxo1 phosphorylation in the hypothalamus. (E) Subcellular fractionation was performed to evaluate the nuclear Foxo1 expression in the hypothalamus of lean and obese rats at 30 min after insulin infusion. (F) Hypothalamic Jak-2 and (G) STAT-3 tyrosine phosphorylation. (H) 12-h food intake (kcal) after intrahypothalamic infusion of leptin ($n = 6$–8 animals per group). Western blots showing hypothalamic lysates from Wistar rats; (I) Leptin-induced Jak2, IRS-1, IRS-2, and STAT3 tyrosine phosphorylation in the hypothalamus. (J) Subcellular fractionation was performed to evaluate the nuclear STAT3 expression in the hypothalamic cells of lean and obese rats 30 min after leptin infusion. Data are the means ± SEM. # $p < 0.05$ versus respective lean group at rest; * $p < 0.05$ versus obese group at rest. Lean animals (white bars) and obese animals (black bars).
doi:10.1371/journal.pbio.1000465.g002

Next, we sought to determine whether exercise requires IL-6 to mediate the anti-hyperphagic response. First we showed that the infusion of recombinant IL-6 into the third ventricle of obese animals under resting conditions reduced the food intake in a dose-dependent manner (Figure 3B) and restored the anorexigenic effects of insulin and leptin (Figure S2A and B). Although we used recombinant IL-6 to mimic the effects of exercise, in obese rats, the dose of recombinant IL-6 used (200 ng) is relatively high and this pharmacological approach does not reflect the same physiological conditions observed after exercise. Thus, we hypothesized that if exercise requires hypothalamic IL-6 activity to reduce food intake, inhibiting the hypothalamic effects of this cytokine, under physiological conditions, should diminish the appetite suppressive action mediated by exercise. To address this hypothesis, we developed an experimental strategy aimed at antagonizing the central action of IL-6 in the presence of a systemic elevation in plasma IL-6 concentration after physical activity. For this, we injected an anti-IL-6 antibody into the third-hypothalamic ventricle in obese animals at 15 min before the exercise protocol. Interestingly, pretreatment with anti-IL-6 antibody blocked the anorexigenic effects of insulin and leptin in exercised DIO rats (Figure 3C and D).

We then explored the mechanism by which IL-6 improves insulin and leptin signaling in the hypothalamus, evaluating the pro-inflammatory pathway. Firstly, we demonstrated that acute exercise did not change the expression or activity of the proteins involved in inflammatory signaling and in an ER stress in the hypothalamus of lean rats, when compared to control animals at rest (Figure 3E). However, high-fat diet consumption induced the aberrant activation of the NF-κB pathway components in the hypothalamic tissue, increasing the TLR4 expression, IKKβ serine phosphorylation, and the IκBα degradation (Figure 3F–H). We also monitored PERK phosphorylation and CHOP protein expression in the hypothalamus to evaluate ER stress. High-fat diet also activated ER stress, increasing PERK phosphorylation and CHOP protein expression in the hypothalamus (Figure 3I and J). In addition, high-fat diet increased IRS-1 serine 307 phosphorylation (Figure 3K). Neither acute exercise nor the single injection of recombinant IL-6 was able to reduce the TLR4 expression in the hypothalamic tissue of obese animals (Figure 3F). On the other hand, exercise and the intrahypothalamic injection of recombinant IL-6, in obese rats at rest, markedly reduced the hypothalamic IKKβ serine phosphorylation (~60%) and prevented IκBα degradation in obese animals (Figure 3G and H). The recombinant IL-6 injection and exercise reduced PERK phosphorylation by about 60% and CHOP protein expression by about 45% (Figure 3I and J) and IRS-1 serine phosphorylation by about 60% (Figure 3K) in the hypothalamic tissue of hyperphagic animals. In addition, recombinant IL-6 and exercise restored insulin-induced Akt and leptin-induced and STAT-3 phosphorylation in the hypothalamus of obese animals (Figure S3A and B). Interestingly, our results show that the intrahypothalamic injection of anti-IL-6 antibody before the exercise protocol attenuated the ability of exercise to reduce the IKKβ/IκBα pathway, ER stress, and IRS1 serine phosphorylation in the hypothalamus (Figure 3G–K). The pretreatment with anti-IL6 antibody also blocked insulin-induced Akt and leptin-induced and STAT-3 phosphorylation, mediated by exercise in the hypothalamus of obese animals (Figure S3A and B).

Immunohistochemistry with an anti-IL-6 Receptor (IL-6R)-specific antibody showed that IL-6R is expressed in a majority of neurons in the arcuate nucleus (Figure 4A). These data were confirmed when we quantified the positive cells in arcuate (Arc), dorsomedial and ventromedial (DMH/VMH), paraventricular (PVN), and lateral (LH) nuclei of hypothalamus (Figure 4B). The in situ hybridization experiment revealed that IL-6R is expressed in both anorexigenic and orexigenic neurons of rats (Figure 4C).

Since IL-6R is expressed in a majority of neurons in the arcuate nucleus, we dissected this specific hypothalamic region to evaluate the modulation of the neuropeptides in response to exercise in lean and obese rats. We found that exercise did not change the POMC, NPY, and AgRP mRNA in the arcuate nucleus of lean rats but increased the POMC and reduced the NPY mRNA levels in the arcuate nucleus of obese animals (Figure 4D).

Double-staining confocal microscopy showed that most neurons expressing IL-6R in the arcuate nucleus were shown to possess IKKβ, PERK, and IRS-1 in obese rats, showing a possible interaction between these molecules (Figure 4E).

Pharmacological Activation of IKKβ and ER Stress Is Suppressed by IL-6

To further support data indicating that IL-6 may modulate ER stress, we performed an acute intrahypothalamic injection of an ER stress inducer, thapsigargin (TG), in lean rats. Acute intrahypothalamic infusion of thapsigargin did not change food intake in lean animals by itself (Figure 5A). However, our results revealed that intrahypothalamic infusion of thapsigargin blocked the anorexigenic effects mediated by insulin and leptin in lean rats and that the injection of recombinant IL-6 and exercise restored the suppressive appetite action of insulin and leptin (Figure 5B and C). In addition, the infusion of anti-IL6 antibody blocked the improvement in insulin and leptin action mediated by exercise (Figure 5B and C).

In accordance with previous studies [7,14,22], we observed that thapsigargin markedly activated inflammatory signaling and ER stress in lean rats, as reflected by increased levels of hypothalamic IKKβ and PERK phosphorylation, respectively (Figure 5D and E), and induced central insulin and leptin resistance, increasing IRS-1 serine phosphorylation (Figure 5F) and reducing insulin-induced Akt serine phosphorylation and leptin-induced STAT-3 tyrosine phosphorylation (Figure 5G and H). Intrahypothalamic infusion of recombinant IL-6 and physical activity were sufficient to reverse all these phenomena (Figure 5D–H). Conversely, the infusion of intrahypothalamic anti-IL6 antibody before exercise protocol blocked these effects mediated by exercise (Figure 5D–H).

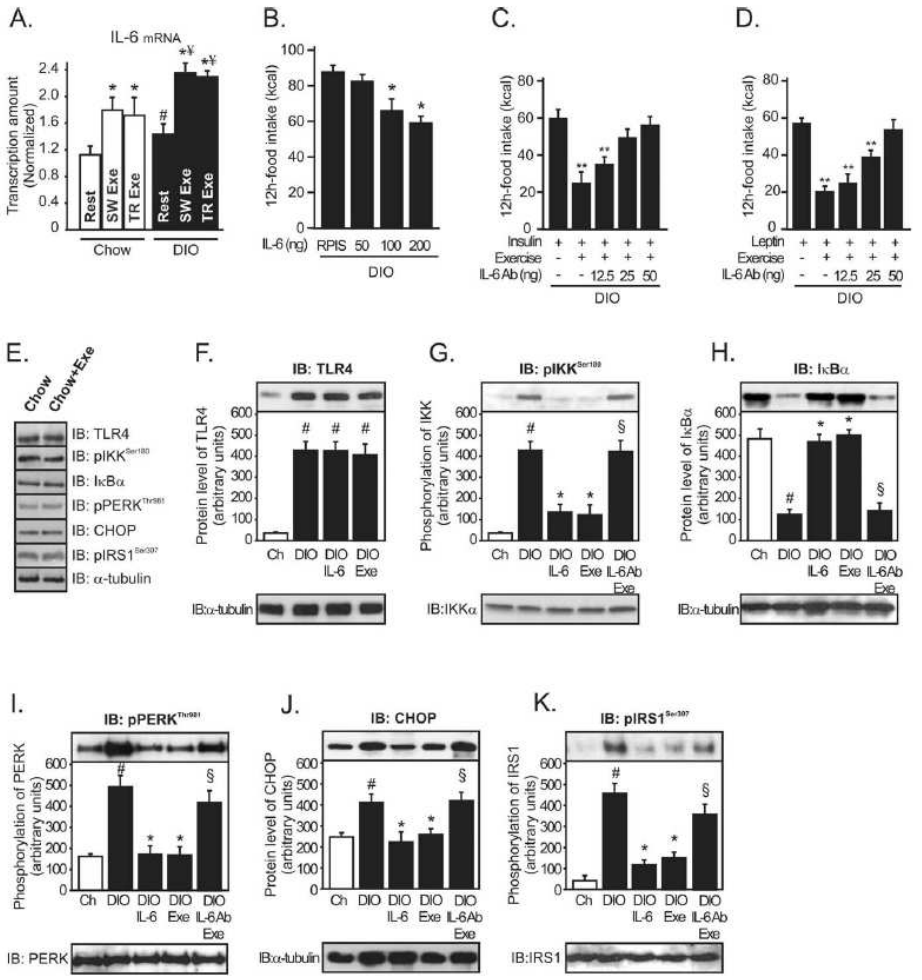


Figure 3. Anti-hyperphagic response mediated by IL-6. (A) IL-6 mRNA in the hypothalamus of lean or diet-induced obesity (DIO) rats under resting conditions and lean obese rats immediately after the swimming exercise (SW Exe) or treadmill running (TR Exe). (B) 12 h of food intake in obese rats under resting conditions following intrahypothalamic infusion of different doses of recombinant IL-6. Counter-regulatory effects of anti-IL-6 antibody on food intake in exercised obese rats after (C) insulin or (D) leptin infusion. Western blots of five independent experiments showing hypothalamic lysates from Wistar rats; (E) Expression and activity of protein involved in the inflammatory signaling or ER stress in control animals at rest condition or after acute exercise (F) TLR4 expression, (G) IKKβ phosphorylation, (H) IκBα expression, (I) PERK phosphorylation, (J) CHOP expression, and (K) IRS-1Ser307 phosphorylation from lean, obese, obese injected with recombinant IL-6, obese after exercise, and obese pretreated with anti-IL-6 antibody before the exercise protocol. Data are the means ± SEM. # $p<0.05$ versus lean group; * $p<0.05$ versus obese group at rest; ¥ $p<0.05$ versus respective exercised control rats; ** $p<0.01$ versus stimulated obese group at rest; § $p<0.05$ versus obese group injected with recombinant IL-6 and exercised obese rats ($n=8$–10 animals per group). Swimming Exercise (SW Exe) or Treadmill Running (TR Exe). Lean animals (white bars) and obese animals (black bars).
doi:10.1371/journal.pbio.1000465.g003

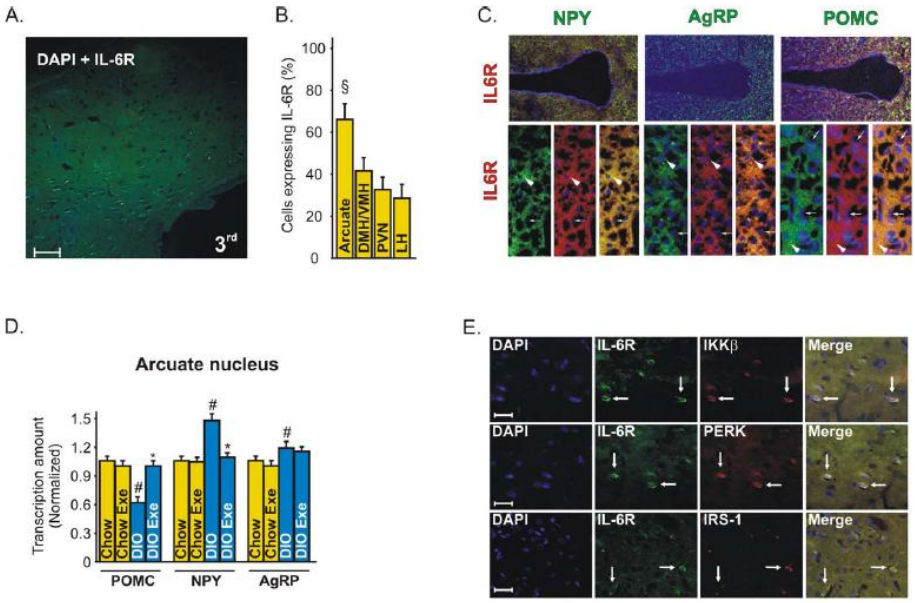


Figure 4. IL-6R localization in the hypothalamus of rats. (A) Immunohistochemistry was performed in the hypothalamic tissue of control rats, using IL-6 receptor (IL-6R)-specific antibody (green) and DAPI (blue), with 50× magnification. (B) Positive cells were quantified in different hypothalamic nuclei, § $p<0.05$ versus the other nuclei. (C) In situ hybridization showing the co-localization of IL-6R (red) with POMC, NPY, and AgRP (green) neuropeptides in the hypothalamus of control rats. Head arrows show neurons and arrows show endothelial cells using 20× and 63× magnification. (D) The dissection of hypothalamic arcuate nucleus of lean and obese rats was obtained as described in Experimental Procedures to evaluate the mRNA of POMC, NPY, and AgRP, using the real time PCR. Data are the means ± SEM. # $p<0.05$ versus respective control group at rest; * $p<0.05$ versus obese rats at rest. Lean animals (yellow bars) and obese (blue bars). (E) Confocal microscopy was performed to evaluate the co-localization of IL-6R (green) and IKKβ, PERK, and IRS-1 (red) in the arcuate nuclei of obese rats, with 200× magnification (scale bar, 20 μm).
doi:10.1371/journal.pbio.1000465.g004

There were no differences in the basal levels of Akt and STAT-3 phosphorylation between the groups (Figure 5I).

Low dose TNF-α has been reported to induce insulin and leptin resistance in the hypothalamus [23]. We injected a low dose of TNF-α into the hypothalamus of lean rats to investigate the effects of IL-6 on low-grade inflammation. First we observed that acute intrahypothalamic infusion of TNF-α did not change the food consumption in lean rats (unpublished data); however, TNF-α infusion blocked the anorexigenic actions of insulin and leptin in these animals (Figure S4A and B). The anorexigenic actions of these hormones were restored with the central infusion of recombinant IL-6 or after exercise in lean rats injected with TNF-α. In addition, the pretreatment with anti-IL6 antibody into the third ventricle blocked the improvement in insulin and leptin action mediated by exercise (Figure S4A and B).

The single injection of TNF-α also induced IKKβ serine, PERK threonine, and IRS-1 serine phosphorylation and reduced insulin-induced Akt serine phosphorylation and leptin- induced STAT-3 tyrosine phosphorylation in the hypothalamus of lean rats (Figure S4C–G). Intrahypothalamic infusion of recombinant IL-6 and physical activity were also sufficient to reverse all these phenomena. On the other hand, the central infusion of anti-IL6 antibody before the exercise protocol blocked the effects of physical activity (Figure S4C–G). There were no differences in the basal levels of Akt and STAT-3 phosphorylation between the groups (Figure S4H).

IL-6 Requires IL-10 to Reduce IKKβ and ER Stress in the Hypothalamus

Next, we sought to determine how IL-6 reduces the inflammatory response and ER stress in the hypothalamus after exercise. Several studies have reported that exercise-induced increases in plasma IL-6 levels are followed by increased circulating levels of well-known anti-inflammatory cytokines such as the IL-1ra and IL-10 [24,25]. We found that the IL-1ra protein level was not changed in the hypothalamus after chronic overnutrition or after acute exercise protocols (Figure 6A); however, IL-10 protein expression was slightly increased in the hypothalamus in obese animals; both of the exercise protocols increased IL-10 expression in a similar fashion, but the induction of IL-10 expression, mediated by exercise, was higher in the hypothalamus of obese when compared to exercised lean animals (Figure 6B). The increase in hypothalamic IL-10 levels mediated by physical activity was confirmed by real time PCR assay (Figure 6C).

We then investigated whether IL-10 reduced the energy intake in rodents. Intrahypothalamic injection of recombinant IL-10

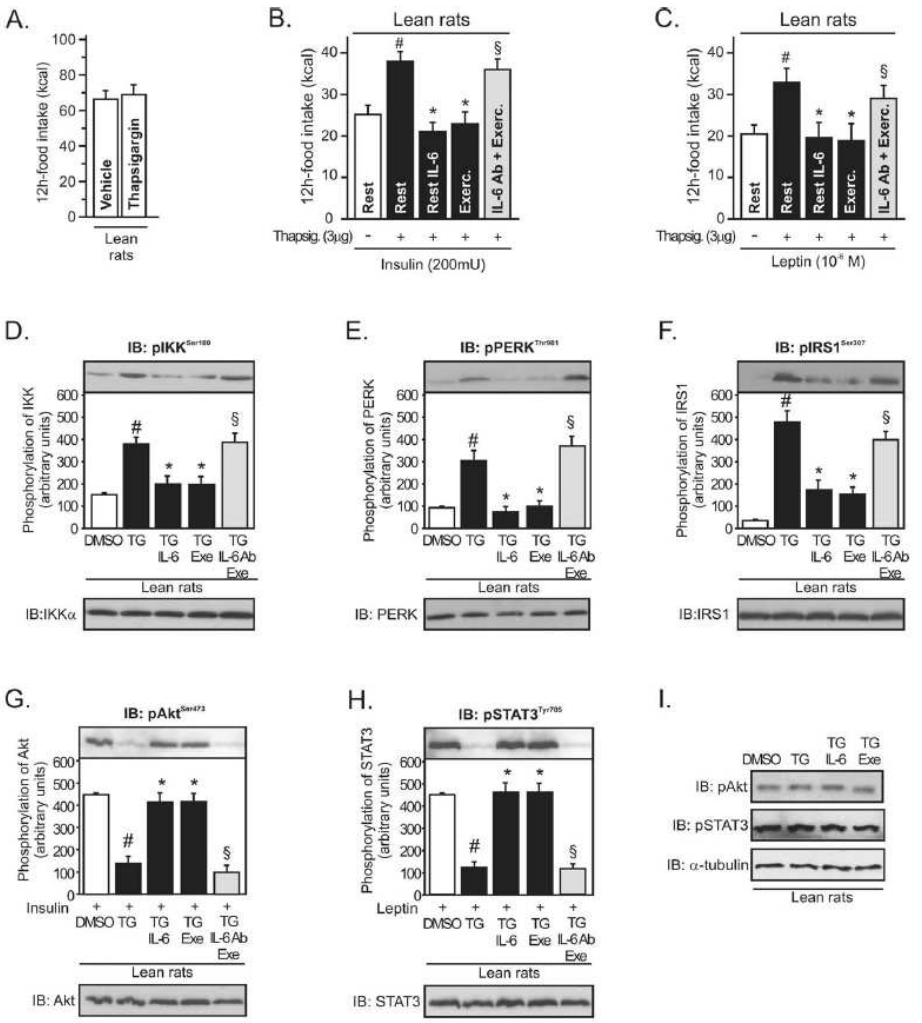
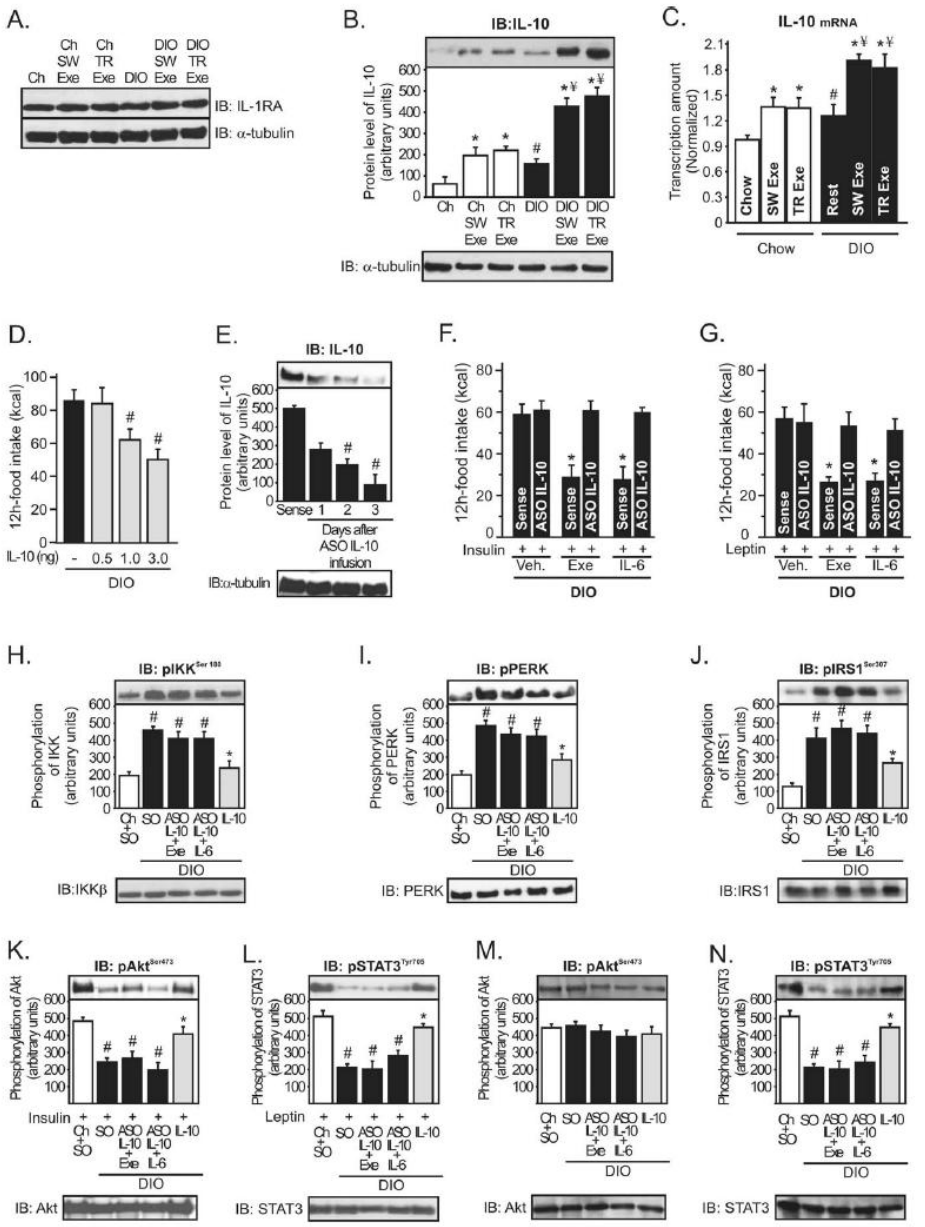


Figure 5. IL-6 reversed pharmacological endoplasmatic reticulum stress induction in the hypothalamus. (A) 12 h of food intake in lean rats after thapsigargin infusion (3 µg). (B) Anorexigenic effects of insulin in the hypothalamus of lean rats pretreated with thapsigargin. (C) Anorexigenic effects of leptin in the hypothalamus of lean rats pretreated with thapsigargin. Western blots showing hypothalamic lysates from Wistar rats; (D) IKKβ, (E) PERK, and (F) IRS-1Ser307 phosphorylation from lean rats pretreated with thapsigargin. (G) Insulin-induced Akt serine phosphorylation, (H) leptin-induced STAT3 tyrosine phosphorylation in the hypothalamus of lean animals pretreated with thapsigargin, and (I) basal levels of Akt and STAT3 phosphorylation. Data are the means ± SEM. # $p<0.05$ versus DMSO group; * $p<0.05$ versus lean plus thapsigargin; § $p<0.05$ versus thapsigargin plus recombinant IL-6 or thapsigargin plus exercised ($n=8$–10 animals per group).
doi:10.1371/journal.pbio.1000465.g005

reduced food intake in obese animals in a dose-dependent manner (Figure 6D). To explore whether IL-6 requires IL-10 expression to improve insulin and leptin action in the hypothalamus, we used an IL-10 antisense oligonucleotide (ASO IL-10) in the hypothalamus of obese rats to keep the expression levels of IL-10 low, even in the presence of high levels of IL-6 in the hypothalamus. Three days


A.
Ch
Ch SW Exe
Ch TR Exe
DIO
DIO SW Exe
DIO TR Exe
IB: IL-1RA
IB: α-tubulin
B.
IB:IL-10
Protein level of IL-10 (arbitrary units)
IB: α-tubulin
C.
IL-10 mRNA
Transcription amount (Normalized)
Chow
SW Exe
TR Exe
Rest
SW Exe
TR Exe
Chow
DIO
D.
12h-food intake (kcal)
IL-10 (ng)
DIO
E.
IB: IL-10
Protein level of IL-10 (arbitrary units)
Days after ASO IL-10 infusion
IB:α-tubulin
F.
12h-food intake (kcal)
Sense
ASO IL-10
Insulin
Veh.
Exe
IL-6
DIO
G.
12h-food intake (kcal)
Leptin
DIO
H.
IB: pIKK
Phosphorylation of IKK (arbitrary units)
IB:IKKβ
I.
IB: pPERK
Phosphorylation of PERK (arbitrary units)
IB: PERK
J.
IB: pIRS1
Phosphorylation of IRS1 (arbitrary units)
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K.
IB: pAkt
Phosphorylation of Akt (arbitrary units)
Insulin
IB: Akt
L.
IB: pSTAT3
Phosphorylation of STAT3 (arbitrary units)
Leptin
IB: STAT3
M.
IB: pAkt
Phosphorylation of Akt (arbitrary units)
IB: Akt
N.
IB: pSTAT3
Phosphorylation of STAT3 (arbitrary units)
IB: STAT3

Figure 6. Role of hypothalamic IL-10 in the control of energy intake during obesity. Western blots showing hypothalamic lysates from Wistar rats; (A) IL-1ra and (B) IL-10 expression in the hypothalamus. (C) IL-10 mRNA in the hypothalamus was examined using real time PCR assay. (D) 12 h food intake (kcal) in obese rats under resting conditions after intrahypothalamic infusion of different doses of recombinant IL-10. Western blots showing hypothalamic lysates from Wistar rats; (E) IL-10 expression after ASO IL-10 treatment in obese animals. (F) Intrahypothalamic treatment with ASO IL-10 blocked the anorexigenic response mediated by (F) insulin and (G) leptin in exercised obese animals or obese animals at rest injected with recombinant IL-6. Western blots showing hypothalamic lysates from Wistar rats; (H) IKKβ, (I) PERK, and (J) IRS-1Ser307 phosphorylation after ASO IL-10 treatment or after acute recombinant IL-10 infusion. (K) Insulin-induced Akt serine phosphorylation and (L) leptin-induced STAT3 tyrosine phosphorylation in the hypothalamus after ASO IL-10 treatment or after acute recombinant IL-10 infusion. (M) Basal levels of Akt serine phosphorylation and (N) STAT3 tyrosine phosphorylation in the hypothalamus after ASO IL-10 treatment or after acute recombinant IL-10 infusion. Data are the means ± SEM. # $p<0.05$ versus chow group; * $p<0.05$ versus DIO; * $p<0.05$ versus exercised control animals; $n=8$–10 animals per group. Lean animals (white bars), obese animals (black bars), and exercised obese plus recombinant IL-10 (grey bars). SO, sense oligonucleotide; ASO, antisense oligonucleotide.
doi:10.1371/journal.pbio.1000465.g006

after ASO IL-10 treatment, IL-10 protein expression was reduced by about 75% in the hypothalamus of obese animals (Figure 6E). Thereafter, exercise and recombinant IL-6 infusion failed to improve the anorexigenic effects of insulin and leptin in obese animals treated with ASO IL-10 (Figure 6F and G).

IL-10 is a pleiotropic cytokine that controls inflammatory processes by suppressing the production of proinflammatory cytokines and blocking IKK/NF-κB signaling and ER stress [26,27]. Thus, we investigated whether exercise and IL-6 requires IL-10 expression to reduce IKKβ activation and ER stress in the hypothalamus of obese animals. As demonstrated above, recombinant IL-6 infusion and exercise reduced IKKβ, PERK, and IRS-1^{Ser307} phosphorylation (Figure 3G, I, and K) and restored insulin and leptin signaling in the hypothalamus of obese animals (Figure S3), but the intrahypothalamic IL-10 ASO treatment abolished all these parameters mediated by recombinant IL-6 and exercise (Figure 6H–L). Conversely, the injection of recombinant IL-10 in the hypothalamus of obese animals at rest markedly reduced IKKβ, PERK, and IRS-1^{Ser307} phosphorylation and increased insulin-induced Akt and leptin-induced STAT-3 phosphorylation in the hypothalamic tissue of obese rats (Figure 6H–L). There were no differences in the basal levels of Akt (Figure 6M). However, STAT3 tyrosine phosphorylation was reduced in the hypothalamus of obese rats, but neither exercise nor IL-6 intrahypothalamic injection was able to increase the STAT-3 phosphorylation after IL-10 ASO treatment (Figure 6N).

Attenuating TLR-4-Dependent IL-6 and IL-10 Production Abolishes Exercise Sensitization of Insulin and Leptin in the Hypothalamus

Several studies showed that Toll-like receptor inactivation results in an attenuation of the secretion of several cytokines. TLR4- and MyD88-deficient mice sustain significantly lower levels of serum cytokines such as IL-1β, IL-6, TNFα, and IL-10 after different pro-inflammatory stimuli [28–30]. Since TLR4 mediates IL-6 transcriptional responses in myocytes and in the skeletal muscle of C3H/HeJ mice [31], we investigated whether exercise restores insulin and leptin signaling in the hypothalamus of TLR4-deficient mice (C3H/HeJ) injected with thapsigargin (TG, an endoplasmic reticulum stress inducer).

In contrast to WT mice, TLR4-deficient mice were found to sustain significantly lower hypothalamic levels of IL-6 (Figure 7A) and IL-10 (Figure 7B) after exercise. The food consumption was similar between C3H/HeN and C3H/HeJ under basal conditions, and acutely, thapsigargin alone did not affect the food intake in these mice (unpublished data); however, the intrahypothalamic administration of TG impaired the anorexigenic effects of insulin and leptin in WT (C3H/HeN) and in TLR4-deficient mice; while physical activity restored the appetite suppressive actions of insulin and leptin in WT but not in TLR4-deficient mice (Figure 7C and D). Furthermore, the intrahypothalamic injection of either recombinant IL-6 or IL-10 restored the anorexigenic actions of insulin and leptin in both WT and TLR4-deficient mice injected with TG (Figure 7C and D). We also observed that the intrahypothalamic infusion of recombinant IL-6 was able to increase the IL-10 protein expression in the hypothalamus of WT and TLR4-deficient mice (Figure 7E). Moreover, exercise failed to reduce inflammation and ER stress and failed to improve insulin and leptin sensitivity in the hypothalamus of TLR4-deficient mice injected with TG (Figure 7F–J). On the other hand, the intrahypothalamic injection of recombinant IL-6 or IL-10 reduced IKKβ, PERK, and IRS-1^{Ser307} phosphorylation and restored insulin and leptin signaling in the hypothalamus of TLR4-deficient mice injected with TG (Figure 7F–J). There were no differences in the basal levels of Akt and STAT-3 phosphorylation between the groups (unpublished data). The in situ hybridization experiment revealed that IL-10R is expressed in NPY, POMC, and AgRP neurons of rats (Figure 7K). Finally, immunohistochemistry with anti-IL-6R and anti-IL-10 Receptor (IL-10R)-specific antibodies revealed that IL-6R and IL-10R are expressed in the same specific neuronal subtypes in the arcuate nucleus (Figure 7L).

Effects of Chronic Exercise on Food Intake and Body Weight

We then investigated the effects of chronic SW Exe on food intake and body weight in lean and obese rats. As observed in acute exercise, the chronic exercise protocol did not change the food consumption in lean animals; however, we observed that the food intake was reduced in obese animals after onset of the chronic exercise protocol, for 3 d, but thereafter, the food intake returned to basal levels on the sixth day and was maintained similar to that of obese rats at rest (Figure 8A). Exercised obese animals showed a significant reduction of the total body weight between the third and the sixth days, but this phenomenon was not observed in control animals (Figure 8B). We also evaluated the weight gain by analyzing the variation of the body weight between the 1st and 24th days. We observed a slight weight gain in control animals at rest, but the chronic exercise protocol did not attenuate the weight gain in lean animals (Figure 8C). On the other hand, overnutrition induced a great weight gain in the group under resting conditions, while chronic exercise attenuated the weight gain in obese animals (Figure 8C). We did not observe a statistical difference in the absolute values of the epididymal fat mass between the exercised obese animals and the obese animals at rest at the end of chronic exercise protocol (Figure 8D).

Chronic overnutrition increased serum insulin, leptin, triglycerides, and free fatty acid levels, compared to age-matched controls; however, chronic exercise reduced serum insulin, triglycerides, and free fatty acid levels in obese animals (Table 3). To determine whether lean and obese rodents were swimming or running in the same fashion, we evaluated lactate production every 15 min during the SW Exe. We did not find any difference

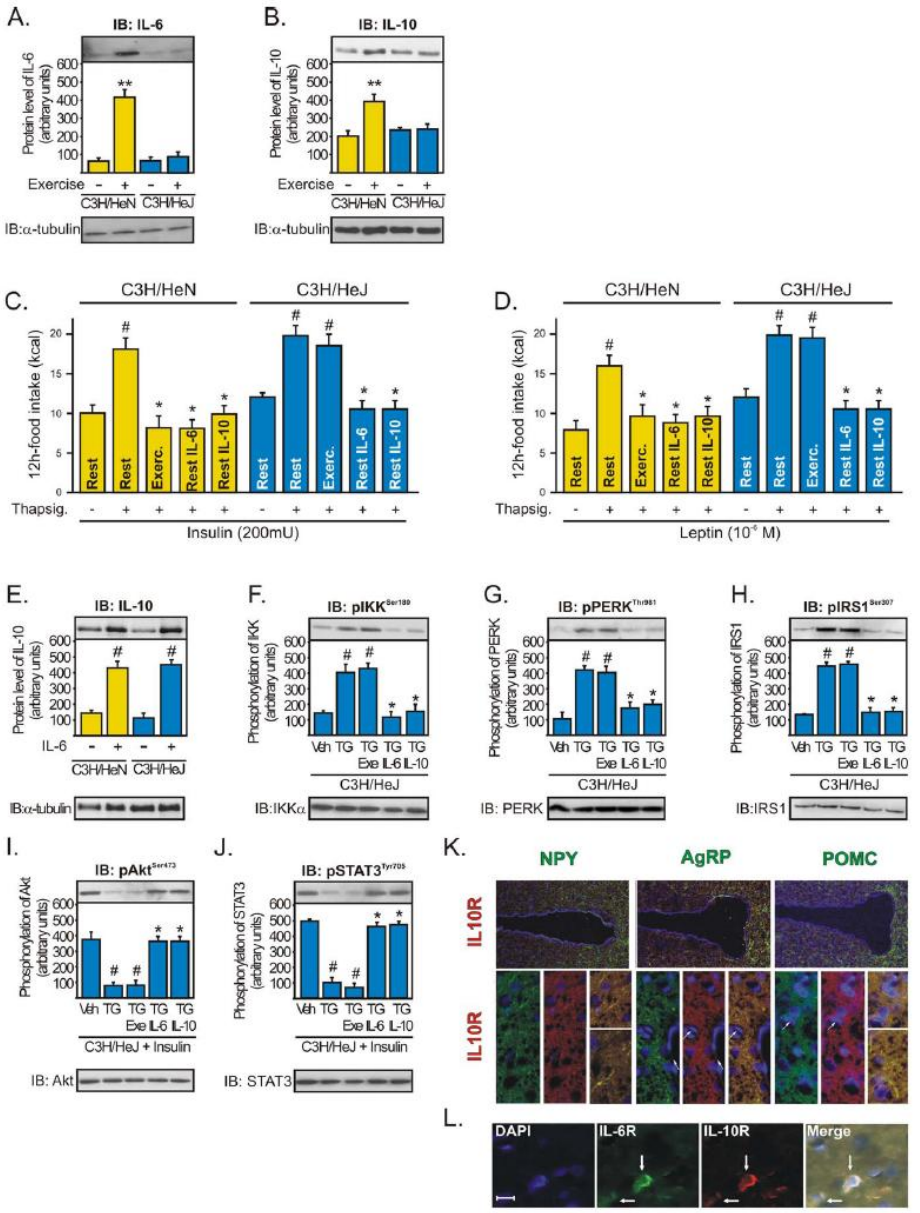
A.
IB: IL-6
Protein level of IL-6 (arbitrary units)
Exercise
C3H/HeN
C3H/HeJ
IB:α-tubulin
B.
IB: IL-10
Protein level of IL-10 (arbitrary units)
C.
C3H/HeN
C3H/HeJ
12h-food intake (kcal)
Rest
Exerc.
Rest IL-6
Rest IL-10
Thapsig.
Insulin (200mU)
D.
Leptin (10⁻⁵ M)
E.
IB: IL-10
IL-6
IBα-tubulin
F.
IB: pIKK Ser180
Phosphorylation of IKK (arbitrary units)
Veh TG TG TG TG
Exe IL-6 IL-10
IB:IKKα
G.
IB: pPERK Thr981
Phosphorylation of PERK (arbitrary units)
IB: PERK
H.
IB: pIRS1 Ser307
Phosphorylation of IRS1 (arbitrary units)
IB:IRS1
I.
IB: pAkt Ser473
Phosphorylation of Akt (arbitrary units)
C3H/HeJ + Insulin
IB: Akt
J.
IB: pSTAT3 Tyr705
Phosphorylation of STAT3 (arbitrary units)
IB: STAT3
K.
NPY
AgRP
POMC
IL10R
L.
DAPI
IL-6R
IL-10R
Merge

Figure 7. The central anti-inflammatory response mediated by exercise requires augmented hypothalamic levels of IL-6 and IL-10. Western blots showing hypothalamic lysates from C3H/NeN and C3H/HeJ mice under resting conditions or after physical activity; (A) IL-6 and (B) IL-10 expression. Anorexigenic effects of insulin (C) or leptin (D) in C3H/NeN and C3H/HeJ mice under resting conditions, after thapsigargin, thapsigargin plus exercise, and thapsigargin plus recombinant IL-6 or IL-10. Western blots showing hypothalamic lysates from mice; (E) IL-10 expression at 2 h after intrahypothalamic injection of recombinant IL-6 (200 ng) in C3H/NeN and C3H/HeJ mice under resting conditions. (F) IKKβ, (G) PERK, and (H) IRS-1Ser307 phosphorylation and (I) Insulin-induced Akt serine phosphorylation and (J) leptin-induced STAT3 tyrosine phosphorylation in the hypothalamus of C3H/HeJ mice after intrahypothalamic infusion of DMSO, thapsigargin, thapsigargin plus exercise, and thapsigargin plus recombinant IL-6 or IL-10. Data are the means ± SEM. ** $p<0.05$ versus respective control group at rest; # $p<0.05$ versus respective control group non-stimulated or stimulated with DMSO; * $p<0.05$ versus thapsigargin; $n=5$–6 animals per group. C3H/NeN (yellow bars) and C3H/HeJ (blue bars). (K) Co-localization of IL-10R (red) with NPY, AgRP, and POMC (green) was evaluated using in situ hybridization technique in the hypothalamus of lean rats, with 20× and 63× magnification. (L) Co-localization of IL-6R (green) and IL-10R (red) in the arcuate nuclei of lean rats, with 200× magnification (scale bar, 10 μm).
doi:10.1371/journal.pbio.1000465.g007

in the lactate production between lean and obese rats. Table 3 depicts the final values obtained in this test. We also determined that this exercise protocol did not change the corticosterone levels in lean and obese animals 3 d after the onset of this exercise protocol (Table 3).

We also evaluated IL-6 and IL-10 mRNA levels in the hypothalamic tissue during the chronic exercise protocol. Interestingly, we observed that the levels of IL-6 mRNA in the hypothalamus were higher on the first day of exercise, when compared to the 15th and 24th days of exercise; this phenomenon was observed in lean and obese exercised rats (Figure 8E). Similar results were found when we analyzed the levels of IL-10 mRNA during chronic exercise (Figure 8F). Finally, the chronic exercise protocol reduced IKKβ phosphorylation and increased IκBα expression in the hypothalamus of obese rats; however, this anti-inflammatory response was more evident on the first day of exercise (Figure 8G). Similar results were found when we analyzed the ER stress markers, such as PERK phosphorylation and CHOP expression (Figure 8H).

Discussion

Exercise as a Potential Target for Countering Hyperphagia and Obesity

Physical activity is a cornerstone in the prevention of obesity and related diseases. Although the energy expenditure aspects of such exercise may contribute to the effects of weight loss, it has been suggested that physical exercise may also contribute to negative energy balance by altering appetite and reducing food intake in rodents [21,32] and humans [33,34]. Our study shows that acute exercise per se did not evoke any meaningful effect, in terms of food intake in lean animals, but interestingly, it was crucial for suppressing hyperphagia mediated by overnutrition, reducing hypothalamic IKKβ/NF-κB activation and ER stress, thus improving insulin and leptin action in an IL-6- and IL-10-dependent manner (Figure 9).

In the absence of obesity, exercise does not affect food behavior, as the anorexigenic or orexigenic pathways remain unchanged in rats. Several experimental studies have demonstrated that physical activity does not activate anorexigenic pathways, such as PI3-K or mTOR/p70S6K [18,21], and does not inhibit the orexigenic pathways, such as AMPK signaling in the hypothalamus of control rodents [35]. On the other hand, the present study provides substantial evidence that physical activity could help to reorganize the set point of nutritional balance and, therefore, aid in counteracting the energy imbalance induced by overnutrition-related obesity. These data are in accordance with Park and colleagues [36], who showed that exercise improved insulin and leptin signaling, increased STAT3, and reduced AMPK phosphorylation in the cerebral cortex and hypothalamus of diabetic rats, contributing to the regulation of body weight and glucose homeostasis. These data demonstrate that exercise increases the anorexigenic pathways and attenuates the orexigenic signals, only in obese and diabetic animals, changing the anorexigenic and orexigenic signaling pathways in the hypothalamus. We also reported that physical activity reduced the hyperphagic response by reducing NPY mRNA and increasing POMC mRNA predominantly in the arcuate nucleus of obese animals. It is important to emphasize that acute exercise did not change the total body weight or epididymal fat pad weight, showing that physical activity can induce the anorexigenic response in the hypothalamus, independently of the body weight change. Our data showed that the reduction on food intake observed in obese animals after both exercise protocols was not related to stress as demonstrated by costicosterone levels. In opposite fashion, it has been demonstrated that NPY mediates stress-induced exacerbation of diet-induced obesity and metabolic syndrome after different stressor agents such as exposure to cold water or aggression in mice [37]. Thus, we hypothesized that some factors, produced during the exercise session, could be involved in this anorexigenic response.

IL-6 Is a Crucial Cytokine for Exercise to Restore Hypothalamic Insulin and Leptin Signaling

Skeletal muscle is an endocrine organ that, upon contraction, stimulates the production and release of cytokines, also called myokines, which can influence metabolism and modify cytokine production in tissue and organs. IL-6 is the first cytokine present in the circulation during exercise [17]. IL-6 can elicit proinflammatory or anti-inflammatory effects, depending on the in vivo environmental circumstances. Although IL-6 has been associated with low-grade inflammation and insulin resistance, it has been demonstrated that acute IL-6 treatment enhances insulin-stimulated glucose disposal in humans [38].

Centrally acting IL-6 appears to play a role in the regulation of appetite, energy expenditure, and body composition. Wallenius and colleagues elegantly showed that long-term peripheral IL-6 treatment to IL6−/− mice caused a decrease in body weight. In addition to increasing energy expenditure, IL-6 may prevent obesity by inhibiting feeding as obese IL-6−/− mice had increased absolute food intake [39]. In accordance with these data, mice fed on a high-fat diet with sustained circulating human IL-6 secreted predominantly from brain and lung ($hIL6^{tg}$) had low leptin concentrations, consumed less food, and expended more energy than wild-type mice [40]. In addition, the intercrossing of $hIL6^{tg}$ and *ob/ob* mice increased the leptin sensitivity in these mice, when compared to *ob/ob* mice [40]. Recently, we demonstrated that exercise requires IL-6 to increase hypothalamic insulin and leptin sensitivity [18] and increase the effects of leptin on the AMPK/mTOR pathway in the hypothalamus of rodents [21]. Furthermore, IL-6 is also released from the brain during prolonged exercise in humans [41]. In the present study, we

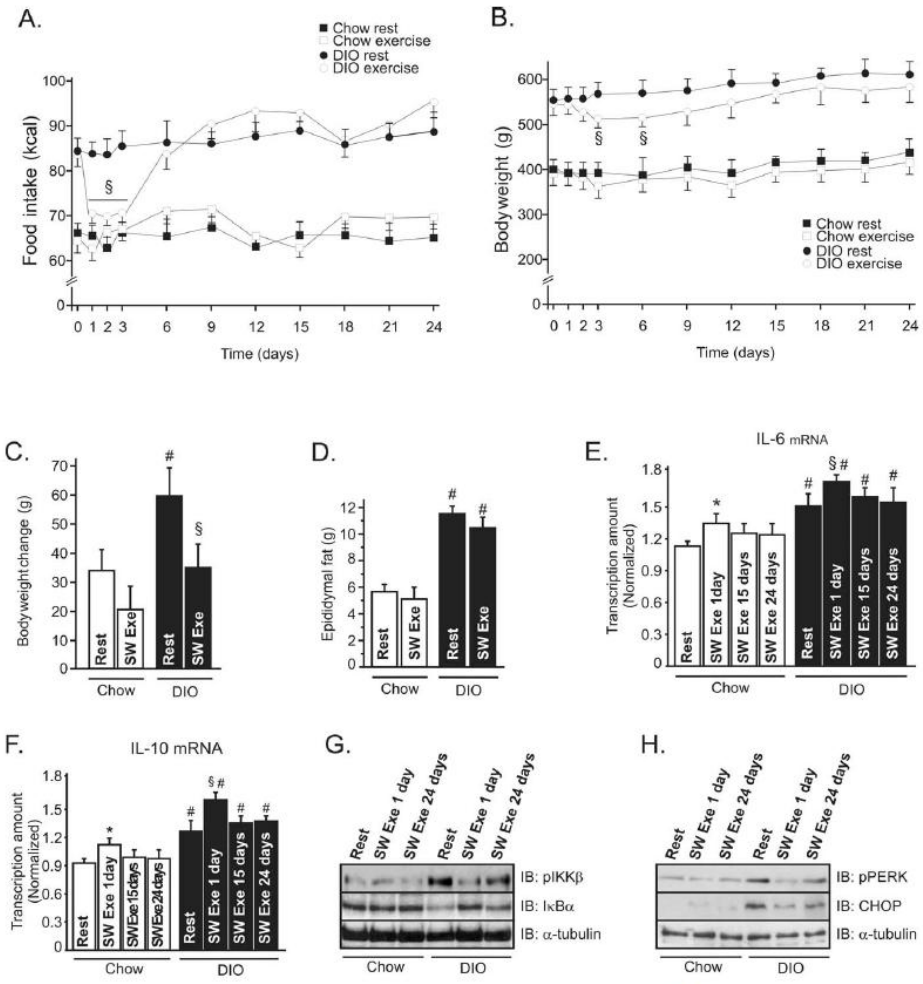

Figure 8. Effects of chronic exercise on food consumption, body weight, and IL-6 and IL-10 production. Evaluation of (A) food intake (kcal) and (B) body weight in control and obese animals during chronic exercise protocol. Chow rest (black square), chow exercise (white square), DIO rest (black ball), and DIO exercise (white ball). (C) Body weight change between the 1st and 24th day. (D) Epididymal fat pad weight after chronic exercise, (E) IL-6 and (F) IL-10 mRNA levels in the hypothalamus of lean and obese rats at rest or after chronic exercise. Western blots showing hypothalamic lysates from lean and obese Wistar rats; (G) IKKβ phosphorylation and IκBα expression and (H) PERK phosphorylation and CHOP expression 1 and 24 d after the chronic exercise protocol. Data are the means ± SEM. * $p<0.05$ versus chow at rest; § $p<0.05$ versus DIO at rest; # $p<0.05$ versus chow group (rest); $n=8–10$ animals per group. Lean animals (white bars) and obese animals (black bars).
doi:10.1371/journal.pbio.1000465.g008

showed that the increment of IL-6 expression in the hypothalamus was crucial to exercise for reducing the inflammation and ER stress activation induced by overnutrition. However, these effects, promoted by exercise, were not observed when we used an intrahypothalamic infusion of anti-IL-6 antibody before the exercise protocol. In addition, the infusion of recombinant IL-6 into the third hypothalamic ventricle reduced the energy intake in obese animals under resting conditions, in a dose-dependent manner, and reduced hypothalamic IKKβ and ER stress activation.

Table 3. Metabolic parameters of lean and DIO rats after chronic exercise.

Groups	Glucose (mg/dL)	Insulin (ng/mL)	Leptin (ng/mL)	Cholesterol (mg/dL)	TG (mg/dL)	FFA (mmol/L)	Corticost. (ng/mL)	Lactate (mmol/L)
Chow rest	98±4	4.0±0.2	2.0±0.2	132.9±9.3	94.0±1,4	0.64±0.2	11.1±0.6	ND
Chow SW exe	99±8	3.1±0.4#	2.2±0.2	134.5±6.2	92.3±6,3	0.64±0.2	11.6±0.7	5.2±0.5
DIO rest	115±5	7.8±0.4#	3.6±0.3#	149.6±10.8	152.5±7.8#	1.75±0.5#	11.2±0.7	ND
DIO SW exe	114±7	5.1±0.5#*	3.1±0.3#	144.6±10.1	102.3±10.7#*	0.89±0.3#*	11.5±0.9	5.3±0.7

# $p<0.05$ versus chow rest and *$p<0.05$ versus DIO rest ($n=8$–10).
doi:10.1371/journal.pbio.1000465.t003

In another approach, we used an ER stress inducer in lean rats to evaluate the effects of exercise/IL-6 on hypothalamic ER stress. We demonstrated that acute thapsigargin injection increased IKKβ and PERK phosphorylation and reduced insulin and leptin action in the hypothalamus and that exercise and the infusion of recombinant IL-6 were able to reduce thapsigargin-induced inflammation, ER stress, and insulin and leptin resistance, whereas the IL-6 antibody pretreatment reversed the effects of exercise. Although thapsigargin increased the hypothalamic IKKβ and PERK phosphorylation, we did not observe any difference in the basal levels of Akt serine 473 and STAT3 tyrosine 705 phosphorylation and in food intake in rats injected with thapsigargin alone. These data are in accordance with a previous study that reported that the ER-stress inhibitor, tauroursodeoxycholic acid (TUDCA), acutely reduced the hypothalamic PERK phosphorylation and NF-kB activation but did not change the food intake in mice fed on a high-fat diet [7]. Thus, our data demonstrate that IL-6 plays an important role in the control of the ER stress effects in the hypothalamus of rats.

All these results are significant, since IKKβ and ER stress activation were strongly associated with insulin and leptin resistance in the hypothalamic tissue. Although we showed a consistent anti-inflammatory effect, mediated by IL-6, in the hypothalamus, we cannot exclude the possibility that IL-6 acts directly as an anorexigenic factor.

Hypothalamic IL-10: A Core Anti-Inflammatory Cytokine Induced by IL-6

Although our findings clearly show that IL-6 diminished hypothalamic IKKβ and ER stress activation and restored the central insulin and leptin action in an animal model of obesity, the question remains as to how IL-6 promotes these events in the

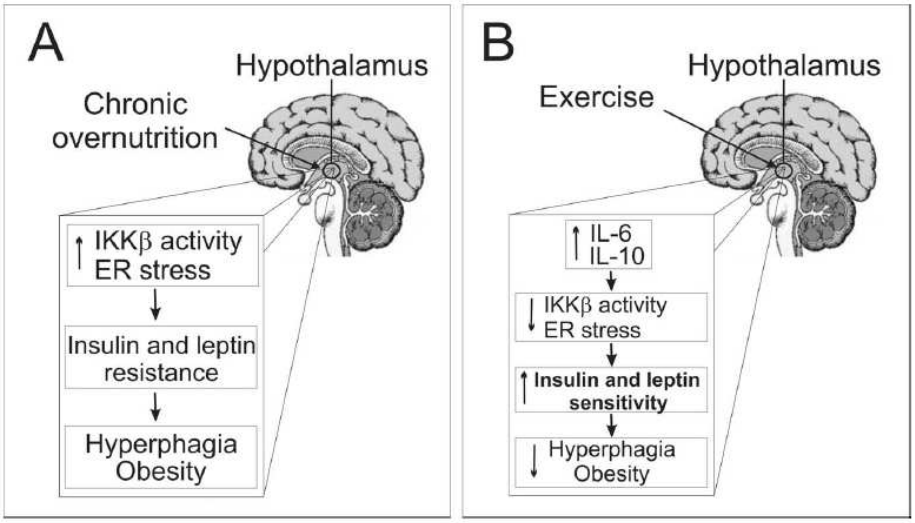

Figure 9. Schematic diagrams of the proposed role of the hypothalamic anti-inflammatory response mediated by exercise. (A) Overnutrition induces hypothalamic IKKβ activation and endoplasmatic reticulum stress, leading to central insulin and leptin resistance, hyperphagia, and obesity. (B) We propose that exercise increases the central anti-inflammatory response, increasing hypothalamic IL-6 and IL-10 expression. This phenomenon is crucial for reducing hypothalamic IKKβ activation and endoplasmatic reticulum stress and turn, restoring insulin and leptin signaling, and reorganizing the set point of nutritional balance.
doi:10.1371/journal.pbio.1000465.g009

hypothalamus. Following exercise, the high circulating levels of IL-6 are followed by an increase in two anti-inflammatory molecules, IL-1ra and IL-10 [25]. Therefore, IL-6 induces an anti-inflammatory environment by inducing the production of IL-1ra and IL-10. In our study, we found that exercise increased the hypothalamic levels of IL-10 but did not change IL-1ra expression in this tissue. Thus, we showed that the anti-inflammatory response mediated by IL-6 involves the increase of IL-10 expression in the hypothalamus.

IL-10 is an important immunoregulatory cytokine with multiple biological effects. In the cytoplasm, it has been demonstrated that IL-10 blocks NF-κB activity at two levels: suppressing IKK activity and NF-κB DNA binding activity [26]. Moreover, IL-10 reduced ER stress in intestinal eptithelial cells, whereas IL-10−/− mice demonstrated that the expression of the ER stress response protein grp-78/BiP was increased in intestinal eptithelial cells under conditions of chronic inflammation [27].

In the CNS, the anti-inflammatory role of IL-10 has been extensively studied in experimental autoimmune encephalomyelitis, an animal model of human multiple sclerosis. The increase in IL-10 expression in the CNS during recovery from brain inflammation and the inability of IL-10 null mice to recover from acute CNS inflammation suggests that the presence of IL-10 within this target organ is required for disease remission [42,43]. However, the role of hypothalamic IL-10 in the control of low-grade inflammation generated during obesity was unknown. Here, we discovered that intrahypothalamic infusion of recombinant IL-10 blocked IKK/NF-κB signaling and ER stress and restored Akt and STAT3 phosphorylation, promoting a re-balance in the energy intake in obese animals. On the other hand, the selective decrease in IL-10 expression in discrete hypothalamic nuclei of obese animals mediated by ASO treatment blunted the effects of both exercise and the intrahypothalamic infusion of recombinant IL-6 in the restoration of central insulin and leptin actions. In addition, we demonstrated that in mice that sustained significantly lower hypothalamic levels of IL-6 and IL-10 after exercise (C3H/HeJ), there was no reduction in pharmacological ER stress activation, in contrast to WT mice. These data are intriguing as IL-10 represents an important cytokine that may reduce both inflammation and ER stress in the hypothalamus. Thus, the modulation of hypothalamic IL-10 expression could be considered the direct target of exercise/IL-6 and constitutes a promising alternative to reduce hypothalamic inflammation and ER stress related to obesity.

The decrease in food intake induced by IL-10 in obese rats is not in accordance with the effects observed in IL-10 KO. It has been reported that mice with combined deficiency of leptin and IL-10 gain less body weight than mice lacking leptin only [44]. However, these discrepancies may be a consequence of methodological differences related to physiological versus genetic approaches and acute versus chronic situation investigated, and most important it may be consequence of IL-10 effects in the regulation of energy expenditure, likewise observed in mice lacking TNF-α receptor [45]; thus, the role of IL-10 in the control of food intake and energy expenditure deserves further exploration.

The long-term reversal effects on body composition, mediated by exercise alone, are controversial. It should be acknowledged that it is often difficult to find long-term reversal effects on body fat in both experimental animals and humans by exercise alone without restrained diet [46]. In the chronic experiments, we observed that the obese animals lost weight during the same period in which a reduction in food intake was observed. After this period, no significant difference was observed in the body weight of exercised animals, although the obese animals presented a significant improvement in metabolic parameters after the chronic exercise protocol.

Since IKKβ/NF-κB inhibition in the CNS represents a potential target therapy to combat obesity and most anti-inflammatory therapies have limited direct effects on IKKβ/NF-κB and a limited capacity for concentration in the CNS, our study provides substantial evidence that physical activity could help to reorganize the set point of nutritional balance and therefore aid in counteracting the energy imbalance induced by overnutrition through the anti-inflammatory response in hypothalamic neurons. Hence, IL-6 and IL-10 are important physiological contributors to the central insulin and leptin action mediated by physical activity, linking it to hypothalamic ER stress and inflammation.

Materials and Methods

Antibodies and Chemicals

Protein A-Sepharose 6 MB and Nitrocellulose paper (Hybond ECL, 0.45 μm) were from Amersham Pharmacia Biotech United Kingdom Ltd. (Buckinghamshire, United Kingdom). Ketamin was from Parke-Davis (São Paulo, SP, Brazil) and diazepam and thiopethal were from Cristália (Itapira, SP, Brazil). Anti-phospho-JAK2 (rabbit polyclonal, AB3805) antibody was from Upstate Biotechnology (Charlottesville, VA, USA). Anti-JAK2 (rabbit polyclonal, SC-278), anti-STAT3 (rabbit polyclonal, SC-483), anti-phospho-IRβ (rabbit polyclonal, SC-25103), anti-IRβ (rabbit polyclonal, SC-711), anti-phospho-IRS-1 (rabbit polyclonal, SC-17199), anti-IRS-1 (rabbit polyclonal, SC-559), anti-IRS-2 (rabbit polyclonal, SC-1556), anti-phosphotyrosine (mouse monoclonal, SC-508), anti-Foxo1 (rabbit polyclonal, SC-11350), anti-IL-1ra (goat polyclonal, SC-8481), anti-TNF-α (rabbit polyclonal, SC-8301), anti-IKKβ (goat polyclonal, SC-34673), anti-PERK (rabbit polyclonal, SC-13073), anti-phospho-PERK (rabbit polyclonal, SC-32577), anti-CHOP (GADD 153) (rabbit polyclonal, SC-575), anti-IL-10 (goat polyclonal, SC-1783), and anti-IL-6 (rabbit polyclonal, SC-7920) antibodies were from Santa Cruz Biotechnology, Inc. Anti-phospho-STAT3 (rabbit polyclonal, #9131), anti-phospho-Akt (rabbit polyclonal, #9271), anti-phospho-Foxo1 (rabbit polyclonal, #9461), anti-beta tubulin (rabbit polyclonal, #2146), anti-phospho-IKKα/β (rabbit polyclonal, #2687), anti-IκBα (rabbit polyclonal, #9242), anti-TLR4 (rabbit polyclonal, #2219), anti-phospho-IRS-1 307 (rabbit polyclonal, #2381), and anti-Akt (rabbit polyclonal, #9272) were from Cell Signalling Technology (Beverly, MA, USA). Leptin, thapsigargin, and recombinant IL-6 and -10 were from Calbiochem (San Diego, CA, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Serum Insulin, Leptin, and IL-6 Quantification

Blood was collected from the cava vein 15 min after the exercise protocols. Plasma was separated by centrifugation (1,100 g) for 15 min at 4 °C and stored at −80 °C until assay. RIA was employed to measure serum insulin. Leptin and IL-6 concentrations were determined using a commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit (Crystal Chem Inc., Chicago, IL). Blood lactate was measured using Accutrend Plus equipment (Roche); sample blood was obtained from the tails every 15 min during the exercise protocols. Serum cholesterol and triglycerides were measured in control and exercised animals after 8 h of fasting using Accutrend Plus equipment (Roche). Serum free fatty acids (FFA) levels were analyzed in rats using the NEFA-kit-U (Wako Chemical GmBH, Neuss, Germany).

Corticosterone levels were determined using urine samples obtained from rats and mice using specific metabolic cage during

24 h after the exercise protocols. The corticosterone level was determined using an EIA kit from Cayman chemical (Ann Arbor, MI).

Animals

Male 4-wk-old Wistar rats were obtained from the University of Campinas Breeding Center. The investigation was approved by the ethics committee and followed the University guidelines for the use of animals in experimental studies and experiments conform to the *Guide for the Care and Use of Laboratory Animals*, published by the U.S. National Institutes of Health (NIH publication no. 85-23 revised 1996). The animals were maintained on 12h:12h artificial light-dark cycles and housed in individual cages. Rats were randomly divided into two groups: control, fed on standard rodent chow (3,948 kcal.Kg^{-1}), and DIO, fed a fat-rich chow (5,358 kcal.Kg^{-1}) *ad libitum* for 3 mo. This diet composition has been previously used [47].

Male (10-wk-old) *ob/ob* mice and their respective controls C57BL/6J background were obtained from The Jackson Laboratory and provided by the University of São Paulo. The mice were bred under specific pathogen-free conditions at the Central Breeding Center of University of Campinas.

Male C3H/HeJ (10-wk-old) mice and their respective controls C3H/HeN were obtained from The Jackson Laboratory and provided by the University of São Paulo. The mice were bred under specific pathogen-free conditions at the Central Breeding Center of the University of Campinas.

Intracerebroventricular Cannulation

The animals were stereotaxically instrumented under intraperitoneal injection of a mix of ketamin (10 mg) and diazepam (0.07 mg) (0.2 ml/100 g body weight) with a chronic 26-gauge stainless steel indwelling guide cannula aseptically placed into the third ventricle at the midline coordinates of 0.5 mm posterior to the bregma and 8.5 mm below the surface of the skull of rats and 1.8 mm posterior to the bregma and 5.0 mm below the surface of the skull of mice.

Exercise Protocols

Animals were acclimated to swimming for 2 d (10 min per day). Water temperature was maintained at 34–35 °C. Rats performed two 3-h exercise bouts, separated by one 45-min rest period. The rats swam in groups of three in plastic barrels of 45 cm in diameter that were filled to a depth of 50 cm. This protocol was conducted between 11:00 a.m. and 6:00 p.m., as previously described [48], and mice performed four 30-min exercise bouts, separated by one 5-min rest period. The mice swam in groups of four in plastic barrels of 40 cm in diameter that were filled to a depth of 20 cm. This protocol was conducted between 3:00 p.m. and 6:00 p.m. Both exercise protocols finished at 6:00 p.m. for evaluation of food intake and analysis of hypothalamic tissue.

The chronic exercise protocol consisted of daily swimming sessions (1 h/d, 5 d/wk, for 4 wk) with an overload (2.0% of the body weight). The hypothalamic tissues and the metabolic parameter were evaluated 36 h after the last exercise session. Rats also performed a single bout of treadmill (Insight LTDA - Ribeirão Preto, SP) running (60 min, speed of 10–15 m/min at a 5% incline) and mice performed a single bout of treadmill running (90 min, speed of 7–10 m/min at a 5% incline).

Intracerebroventricular Treatments

Rats or mice were deprived of food for 2 h with free access to water and received 3 µl of bolus injection into the third ventricle, as follows:

Insulin and leptin treatments. Animals received intrahypothalamic infusion of vehicle, insulin (200 mU), or leptin (10^{-6} M) at 6:00 p.m. to evaluate the food intake or insulin and leptin signaling. Food intake was determined by measuring the difference between the weight of chow given and the weight of chow at the end of a 12-h period.

Recombinant IL-6 and IL-10 treatments. Animals received intrahypothalamic infusion of vehicle, or recombinant IL-6 (50, 100, or 200 ng) or recombinant IL-10 (0.5, 1.0, or 3.0 ng) at 6:00 p.m. to evaluate the food intake. For Western blot analysis, we injected recombinant IL-6 or IL-10 2 h after DMSO or thapsigargin into the third ventricle and the hypothalamus was excised 2 h later.

Thapsigargin treatments. Animals received intrahypothalamic infusion of vehicle, or thapsigargin (3.0 µg). To evaluate the energy intake and for Western blot analysis, thapsigargin was infused 40 min before the exercise protocol and 2 h before the recombinant IL-6 infusion. Immediately after exercise or 2 h after IL-6 infusion, animals received intrahypothalamic infusion of insulin (200 mU) or leptin (10^{-6} M).

IL-6 neutralizing antibody. Animals were randomly selected for treatment with saline, rabbit pre-immune serum (RPIS) or rabbit antiserum against IL-6 (IL-6 Ab) in different doses. IL-6 Ab was injected into the third ventricle of the rats 15 min before the exercise protocol.

ASO IL-10 treatments. Phosphorthioate-modified sense and antisense oligonucleotides (produced by Invitrogen Corp., Carlsbad, CA, USA) were diluted to final concentration of 1 nmol/µl in dilution buffer containing 10 mmol/l Tris–HCl and 1.0 mmol/l EDTA. The oligonucleotides were designed according to the *Mus musculus* IL-10 sequence deposited at the NIH-NCBI (http://www.ncbi.nlm.nih.gov/entrez) under the designation NM 010548 and were composed of 5′-GCC AGT CAG TAA GAG CAG-3′ (sense) and 5′-TGA GAT CTG CAA TGC A-3′ (antisense). Obese Wistar rats were injected into the third ventricle with two daily doses of 3 µl of dilution buffer containing, or not, sense (Sense IL-10) or antisense oligonucleotides (ASO IL-10) for 3 d. For Western blotting analysis, after ASO IL-10 treatment, obese animals were submitted to the exercise protocol or intrahypothalamic infusion of recombinant IL-6. In some experiments, the rats also received intrahypothalamic infusion of insulin (200 mU) or leptin (10^{-6} M) for the determination of food intake and Akt and STAT3 phosphorylation.

Recombinant of TNF-α treatments. Animals received intrahypothalamic infusion of vehicle, or TNF-α (10^{-12}). To evaluate the energy intake and for Western blotting analysis, TNF-α was infused 40 min before the exercise protocol and 2 h before the recombinant IL-6 infusion. Immediately after exercise or 2 h after IL-6 infusion, animals received intrahypothalamic infusion of insulin (200 mU) or leptin (10^{-6} M).

Food Intake Determination

Intrahypothalamic infusions were performed between 5:00 and 6:00 p.m. Thereafter standard chow or high-fat diet was given and food intake was determined by measuring the difference between the weight of chow given and the weight of chow at the end of a 12-h period. Similar studies were carried out in animals after exercise.

Western Blot Analysis

After exercise and/or i.c.v. treatments, the animals were anaesthetized, and the hypothalamus was quickly removed, minced coarsely, and homogenized immediately in a freshly

prepared ice-cold buffer (1% Triton X-100, 100 mmol/l Tris pH 7.4, 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium vanadate, 2 mmol/l phenyl methylsulphonyl fluoride, and 0.1 mg aprotinin) suitable for preserving phosphorylation states of enzymes, and Western blot was performed, as previously described [1].

Nuclear Extract

Foxo1 and STAT-3 nuclear expression were obtained as described [49]. Fragments of hypothalamic tissue from untreated rats or rats treated with insulin or leptin were obtained 30 min after insulin or leptin infusion and were minced and homogenized in 2 vol. of STE buffer (0.32 M sucrose, 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 1 mM DTT, 100 mM sodium fluoride, 100 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 1 mM PMSF, and 0.1 mg aprotinin/ml) at 4 °C with a Polytron homogenizer. The homogenates were centrifuged (1,000×g, 25 min, 4 °C) to obtain pellets. The pellet was washed once and suspended in STE buffer (nuclear fraction). The nuclear fraction was solubilized in Triton buffer [1% (v/v) Triton X-100/150 mM NaCl/10 mM Tris/HCl (pH 7.4)/1 mM EGTA/1 mM EDTA/ 0.2 mM sodium orthovanadate/20 µM leupeptin A/0.2 mM PMSF/50 mM NaF/0.4 nM microcystin LR]. The fraction was centrifuged (15,000 g, 30 min, 4 °C), and the supernatant (nuclear extract) was stored at −80 °C.

Confocal Microscopy

Paraformaldehyde-fixed hypothalami were sectioned (5 µm). The sections were obtained from the hypothalami of six rats per group in the same localization (antero-posterior = −1.78 from bregma) and used in regular single- or double-immunofluorescence staining using DAPI, anti-IL6 receptor alpha (rabbit IgG, SC-13947), anti-IL-10 receptor (rabbit IgG, SC-987), anti-IKKβ (goat IgG, SC-34673), anti-PERK (rabbit IgG, SC-32577), anti-POMC (rabbit IgG, FL-267), and rabbit anti-IRS-1 (rabbit IgG, SC-559) (1:200; Santa Cruz Biotechnology) antibodies. After incubation with the primary antibody, sections were washed and incubated with specific biotinylated anti-rabbit or anti-goat secondary antibodies (1:150 dilution) for 2 h at room temperature, followed by incubation with Streptoavidin reagent (containing avidin-conjugated peroxidase) and color reaction using the DAB substrate kit (Vector Laboratories, Burlingame, CA, USA), according to recommendations of the manufacturer. Analysis and photodocumentation of results were performed using a LSM 510 laser confocal microscope (Zeiss, Jena, Germany). The anatomical correlations were made according to the landmarks given in a stereotaxic atlas [50]. The frequency of positive cells was determined in 100 randomly counted cells using Analysis software (Version 2.4).

mRNA Isolation and Real Time PCR

Hypothalamic total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD, USA), according to the manufacturer's recommendations. Total RNA was rendered genomic DNA free by digestion with Rnase-free Dnase (RQ1, Promega, Madison, WI, USA). Rats were deprived of food for 9 h after for real time PCR analysis. Real time PCR and mRNA isolation were performed using a commercial kit, as follows: IL-6: Rn00561420_m1 IL-10: Rn00563409_m1, POMC: Rn00595020_m1, NPY: Rn00561681_m1, AgRP: Rn01431703_g1, GAPD, #4352338E, for rat and RPS-29 (NCBI: NM012876), sense: 5′-AGGCAA-GATGGGTCACCAGC-3′, antisense: 5′-AGTCGAATCATCC-ATTCAGGTCtG-3′.

Dissection of the Arcuate Nucleus

After 9 h of fasting, rats were killed by decapitation and hypothalamic nuclei were quickly dissected and homogenized in Trizol reagent (Life Technologies, Gaithersburg, MD, USA), according to the manufacturer's recommendations. Later on, each region of the hypothalamus was dissected from 1 mm thick sagittal sections of fresh brain. Arcuate nucleus was dissected from the first sections from the midline of the brain. Coordinates for the arcuate nucleus is ventral part of the medial hypothalamus with anterior and dorsal margin and posterior margin (border with mammilary body).

In Situ Double mRNA Hybridization

For mRNA localization all solutions and materials utilized were RNAse free. The probes were determined and designed using the program Gene Runner 3.05 (Hastings Software, Inc., USA) according to mRNA sequences in NCBI: *POMC* (NM_139326.2), *NPY* (NM_012614.1), *AgRP* (XM_574228.2), *IL6ra* (NM_017020.1), and *IL10ra* (AJ_305049.1). Two probes were synthesized for each mRNA and were 5′-end labeled with Alexa Fluor 488 or 546 by Invitrogen Life Technologies (Carlsbad CA, USA). See details in the supplemental data (Table S1). Frozen sections were air dried for 30 min at 37 °C, fixed using cold acetone for 10 min, and washed twice in PBS for 5 min and twice in 2× SSC for 2 min. The sections were incubated with Proteinase K (20 µg/mL) for 10 min at room temperature and then washed twice for 5 min with 2× SSC. The sections were incubated in 0.1 M triethanolamine pH 8 (TEA Buffer) for 10 min and then with 0.25% acetic anhydride in TEA buffer for 10 min under magnetic stirring and then washed with 2× SSC. The pre-hybridization solution was composed by 50% formamide, 5× SSC, Denhardt's solution (1× final concentration), and completed with DEPC-treated water. The sections were pre-hybridized for 4 h without the probe at 50 °C in humidified chamber with 50% formamide in SSC. The probe mix (including two probes for each mRNA; i.e., IL6ra or IL10ra with POMC, AgRP, or NPY) was composed (for each tissue section) of 20 µL of pre-hybridization solution plus 500 µg/mL of torula RNA, 500 µg/mL of salmon sperm DNA, and 50 ng of riboprobe mix (anti-sense or sense). The mixture was placed over the sections and incubated at 52 °C overnight in a humidified chamber. After 18 h hybridization, the sections were washed four times with 4× SSC buffer for 10 and 5 min in PBS. The sections were visualized in Zeiss 510 confocal microscope.

Statistical Analysis

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

Supporting Information

Figure S1 Serum levels and hypothalamic expression of IL-6. (A) Serum levels of IL-6 and (B) protein expression of IL-6 in the hypothalamic tissue from lean and obese rats under rest condition or after exercise. Data are the means ± SEM. # $p<0.05$ versus respective control at rest; * $p<0.05$ versus respective lean plus exercise; § $p<0.05$ versus control at rest, $n=8$ animals per group.

Found at: doi:10.1371/journal.pbio.1000465.s001 (1.22 MB DOC)

Figure S2 Effects of IL-6 on leptin and insulin action. Intrahypothalamic infusion of recombinant IL-6 improves the anorexigenic effects of insulin (A) or leptin (B) in obese Wistar rats. Data are the means ± SEM. * $p<0.05$ versus obese non-stimulated; ** $p<0.01$ versus obese stimulated with insulin or leptin alone, $n=6–8$ animals per group.
Found at: doi:10.1371/journal.pbio.1000465.s002 (0.88 MB TIF)

Figure S3 IL-6 improves insulin and leptin signaling. Western blots of five independent experiments showing hypothalamic lysates from Wistar rats; (A) Insulin-induced Akt serine phosphorylation and (B) leptin-induce STAT3 tyrosine phosphorylation in lean, obese, obese plus recombinant IL-6, obese plus exercise, and exercise obese pretreated with anti-IL-6 antibody before the exercise protocol. Data are the means ± SEM. # $p<0.05$ versus lean group; * $p<0.05$ versus obese group at rest; § $p<0.01$ versus exercised obese group; $n=6–8$ animals per group.
Found at: doi:10.1371/journal.pbio.1000465.s003 (1.43 MB TIF)

Figure S4 IL-6 suppresses TNF-α induced insulin and leptin resistance. Anorexigenic effects of insulin (A) and leptin (B) in the hypothalamus of lean rats injected with TNF-α, TNF-α plus IL-6, TNF-α plus exercise, and TNF-α in exercised lean animals pretreated with anti-IL-6 antibody before the exercise protocol. Western blots showing hypothalamic lysates from Wistar rats; (C) IKKβ, (D) PERK, (E) IRS-1Ser307, (F) insulin-induced Akt serine phosphorylation, and (G) leptin-induced STAT3 tyrosine phosphorylation and (H) basal levels of Akt and STAT3 phosphorylation in the hypothalamus of lean animals injected with TNF-α, TNF-α plus IL-6, TNF-α plus exercise, and TNF-α in exercised lean animals pretreated with anti-IL-6 antibody before the exercise protocol pretreated with TNF-α. Data are the means ± SEM. # $p<0.05$ versus DMSO group; * $p<0.05$ versus lean plus TNF-α; § $p<0.05$ versus TNF-α plus recombinant IL-6 or TNF-α plus exercised; $n=6–8$ animals per group.
Found at: doi:10.1371/journal.pbio.1000465.s004 (2.44 MB TIF)

Table S1 mRNA and probes sequences used in double mRNA hybridization. The probes were determined and designed according to mRNA sequences in NCBI: POMC (NM_139326.2), NPY (NM_012614.1), AgRP (XM_574228.2), IL6ra (NM_017020.1), and IL10ra (AJ_305049.1). Two probes were synthesized for each mRNA and were 5′-end labeled with Alexa Fluor 488 or 546.
Found at: doi:10.1371/journal.pbio.1000465.s005 (0.03 MB DOC)

Acknowledgments

We thank Mr. Luiz Janeri and Ms. Janine Sabino for the technical assistance and Nicola Conran for the English language editing.

Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: ERR JBC. Performed the experiments: ERR MBF DEC GZR JRP JM CTDS JCM POP DG RMM AGO TMA HFC. Analyzed the data: ERR DEC LAV MJS JBC. Wrote the paper: ERR JBC.

References

1. Carvalheira JB, Ribeiro EB, Araujo EP, Guimaraes RB, Telles MM, et al. (2003) Selective impairment of insulin signalling in the hypothalamus of obese Zucker rats. Diabetologia 46: 1629–1640.
2. Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers MG Jr, et al. (2001) Intracellular signalling. Key enzyme in leptin-induced anorexia. Nature 413: 794–795.
3. Bruning JC, Gautam D, Burks DJ, Gillette J, Schubert M, et al. (2000) Role of brain insulin receptor in control of body weight and reproduction. Science 289: 2122–2125.
4. El-Haschimi K, Pierroz DD, Hileman SM, Bjorbaek C, Flier JS (2000) Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. J Clin Invest 105: 1827–1832.
5. De Souza CT, Araujo EP, Bordin S, Ashimine R, Zollner RL, et al. (2005) Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. Endocrinology 146: 4192–4199.
6. Milanski M, Degasperi G, Coope A, Morari J, Denis R, et al. (2009) Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. J Neurosci 29: 359–370.
7. Zhang X, Zhang G, Zhang H, Karin M, Bai H, et al. (2008) Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity. Cell 135: 61–73.
8. Hayden MS, Ghosh S (2008) Shared principles in NF-kappaB signaling. Cell 132: 344–362.
9. Hosoi T, Sasaki M, Miyahara T, Hashimoto C, Matsuo S, et al. (2008) Endoplasmic reticulum stress induces leptin resistance. Mol Pharmacol 74: 1610–1619.
10. Martinez de Morentin PB, Varela L, Ferno J, Nogueiras R, Dieguez C, et al. (2010) Hypothalamic lipotoxicity and the metabolic syndrome. Biochim Biophys Acta 1801: 350–361.
11. Ozcan L, Ergin AS, Lu A, Chung J, Sarkar S, et al. (2009) Endoplasmic reticulum stress plays a central role in development of leptin resistance. Cell Metab 9: 35–51.
12. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, et al. (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 306: 457–461.
13. Tsiotra PC, Tsigos C (2006) Stress, the endoplasmic reticulum, and insulin resistance. Ann N Y Acad Sci 1083: 63–76.
14. Won JC, Jang PG, Namkoong C, Koh EH, Kim SK, et al. (2009) Central administration of an endoplasmic reticulum stress inducer inhibits the anorexigenic effects of leptin and insulin. Obesity (Silver Spring) 17: 1861–1865.
15. Misra A, Alappan NK, Vikram NK, Goel K, Gupta N, et al. (2008) Effect of supervised progressive resistance-exercise training protocol on insulin sensitivity, glycemia, lipids, and body composition in Asian Indians with type 2 diabetes. Diabetes Care 31: 1282–1287.
16. Tuomilehto J, Lindstrom J, Eriksson JG, Valle TT, Hamalainen H, et al. (2001) Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. N Engl J Med 344: 1343–1350.
17. Petersen AM, Pedersen BK (2005) The anti-inflammatory effect of exercise. J Appl Physiol 98: 1154–1162.
18. Flores MB, Fernandes MF, Ropelle ER, Faria MC, Ueno M, et al. (2006) Exercise improves insulin and leptin sensitivity in hypothalamus of Wistar rats. Diabetes 55: 2554–2561.
19. Wallenius V, Wallenius K, Ahren B, Rudling M, Carlsten H, et al. (2002) Interleukin-6-deficient mice develop mature-onset obesity. Nat Med 8: 75–79.
20. Pedersen BK, Steensberg A, Schjerling P (2001) Muscle-derived interleukin-6: possible biological effects. J Physiol 536: 329–337.
21. Ropelle ER, Fernandes MF, Flores MB, Ueno M, Rocco S, et al. (2008) Central exercise action increases the AMPK and mTOR response to leptin. PLoS ONE 3: e3856. doi:10.1371/journal.pone.0003856.
22. Hu P, Han Z, Couvillon AD, Kaufman RJ, Exton JH (2006) Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. Mol Cell Biol 26: 3071–3084.
23. Moraes JC, Amaral ME, Picardi PK, Calegari VC, Romanatto T, et al. (2006) Inducible-NOS but not neuronal-NOS participate in the acute effect of TNF-alpha on hypothalamic insulin-dependent inhibition of food intake. FEBS Lett 580: 4625–4631.
24. Pedersen BK, Fischer CP (2007) Physiological roles of muscle-derived interleukin-6 in response to exercise. Curr Opin Clin Nutr Metab Care 10: 265–271.
25. Steensberg A, Fischer CP, Keller C, Moller K, Pedersen BK (2003) IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. Am J Physiol Endocrinol Metab 285: E433–E437.
26. Schottelius AJ, Mayo MW, Sartor RB, Baldwin AS Jr (1999) Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. J Biol Chem 274: 31868–31874.
27. Shkoda A, Ruiz PA, Daniel H, Kim SC, Rogler G, et al. (2007) Interleukin-10 blocked endoplasmic reticulum stress in intestinal epithelial cells: impact on chronic inflammation. Gastroenterology 132: 190–207.
28. Bruns B, Maass D, Barber R, Horton J, Carlson D (2008) Alterations in the cardiac inflammatory response to burn trauma in mice lacking a functional Toll-like receptor 4 gene. Shock 30: 740–746.
29. Kobbe P, Kaczorowski DJ, Vodovotz Y, Tzioupis CH, Mollen KP, et al. (2008) Local exposure of bone components to injured soft tissue induces Toll-like receptor 4-dependent systemic inflammation with acute lung injury. Shock 30: 686–691.

30. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, et al. (2007) Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 317: 121–124.
31. Frost RA, Nystrom GJ, Lang CH (2006) Multiple Toll-like receptor ligands induce an IL-6 transcriptional response in skeletal myocytes. Am J Physiol Regul Integr Comp Physiol 290: R773–R784.
32. Patterson CM, Bouret SG, Dunn-Meynell AA, Levin BE (2009) Three weeks of postweaning exercise in DIO rats produces prolonged increases in central leptin sensitivity and signaling. Am J Physiol Regul Integr Comp Physiol 296: R537–R548.
33. Martins C, Robertson MD, Morgan LM (2008) Effects of exercise and restrained eating behaviour on appetite control. Proc Nutr Soc 67: 28–41.
34. Martins C, Truby H, Morgan LM (2007) Short-term appetite control in response to a 6-week exercise programme in sedentary volunteers. Br J Nutr 98: 834–842.
35. Andersson U, Treebak JT, Nielsen JN, Smith KL, Abbott CR, et al. (2005) Exercise in rats does not alter hypothalamic AMP-activated protein kinase activity. Biochem Biophys Res Commun 329: 719–725.
36. Park S, Jang JS, Jun DW, Hong SM (2005) Exercise enhances insulin and leptin signaling in the cerebral cortex and hypothalamus during dexamethasone-induced stress in diabetic rats. Neuroendocrinology 82: 282–293.
37. Kuo LE, Kitlinska JB, Tilan JU, Li L, Baker SB, et al. (2007) Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. Nat Med 13: 803–811.
38. Carey AL, Steinberg GR, Macaulay SL, Thomas WG, Holmes AG, et al. (2006) Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. Diabetes 55: 2688–2697.
39. Wallenius K, Wallenius V, Sunter D, Dickson SL, Jansson JO (2002) Intracerebroventricular interleukin-6 treatment decreases body fat in rats. Biochem Biophys Res Commun 293: 560–565.
40. Sadagurski M, Norquay L, Farhang J, D'Aquino K, Copps K, et al. (2010) Human IL6 enhances leptin action in mice. Diabetologia 53: 525–535.
41. Nybo L, Nielsen B, Pedersen BK, Moller K, Secher NH (2002) Interleukin-6 release from the human brain during prolonged exercise. J Physiol 542: 991–995.
42. Kennedy MK, Torrance DS, Picha KS, Mohler KM (1992) Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. J Immunol 149: 2496–2505.
43. Samoilova EB, Horton JL, Chen Y (1998) Acceleration of experimental autoimmune encephalomyelitis in interleukin-10-deficient mice: roles of interleukin-10 in disease progression and recovery. Cell Immunol 188: 118–124.
44. Siegmund B, Sennello JA, Lehr HA, Batra A, Fedke I, et al. (2004) Development of intestinal inflammation in double IL-10- and leptin-deficient mice. J Leukoc Biol 76: 782–786.
45. Romanatto T, Roman EA, Arruda AP, Denis RG, Solon C, et al. (2009) Deletion of tumor necrosis factor-alpha receptor 1 (TNFR1) protects against diet-induced obesity by means of increased thermogenesis. J Biol Chem 284: 36213–36222.
46. Soderlund A, Fischer A, Johansson T (2009) Physical activity, diet and behaviour modification in the treatment of overweight and obese adults: a systematic review. Perspect Public Health 129: 132–142.
47. Pauli JR, Ropelle ER, Cintra DE, Carvalho-Filho MA, Moraes JC, et al. (2008) Acute physical exercise reverses S-nitrosation of the insulin receptor, insulin receptor substrate 1 and protein kinase B/Akt in diet-induced obese Wistar rats. J Physiol 586: 659–671.
48. Ropelle ER, Pauli JR, Prada PO, de Souza CT, Picardi PK, et al. (2006) Reversal of diet-induced insulin resistance with a single bout of exercise in the rat: the role of PTP1B and IRS-1 serine phosphorylation. J Physiol 577: 997–1007.
49. Ropelle ER, Pauli JR, Prada P, Cintra DE, Rocha GZ, et al. (2009) Inhibition of hypothalamic Foxo1 expression reduced food intake in diet-induced obesity rats. J Physiol 587: 2341–2351.
50. Mai JK, Assheuer J, Paxinos G (1997) Atlas of the human brain. San Diego; London: Academic. viii, 336 p.