ALESSANDRA LIA GASPARETTI GUARILHA

TRANSDUÇÃO DO SINAL DA INSULINA EM ANIMAIS EXPOSTOS AO FRIO – O PAPEL DO CROSS-TALK ENTRE O RECEPTOR β3-ADRENÉRGICO E O RECEPTOR DE INSULINA EM TECIDO ADIPOSO MARROM

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Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutor em Fisiopatologia Médica, Área de concentração em Medicina Experimental

ORIENTADOR: PROF. DR. LÍCIO AUGUSTO VELOSO

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"Ainda que eu tenha o dom de profetizar e conheça todos os mistérios e toda a ciência;
ainda que eu tenha tamanha fé ao ponto de transportar montanhas,
se não tiver amor, nada serei..."

1 Cor. 13,2

SIGLAS

μCi	MicroCuri
^{3}H	Trício
^{125}I	Isótopo de iodo 125
Akt/PKB	Proteína serina/treonina quinase B
AMPc	Adenosina monofosfato cíclica
AMPK	*5'-AMP-activated protein kinase*
Anti-PY	Anti-fosfotirosina
ATP	Adenosina trifosfato
BAT	*Brown adipose tissue* Tecido adiposo marrom
BSA	Soro de albumina bovina
2-DG	2-deoxi-D-[^{3}H]glicose
DTT	Ditiotreitol
EDTA	Ácido etilenodiaminotetracético
ERKs	*Extracellular signal-regulated kinase* Subfamília da MAPK
GLUT-4	Transportador de glicose 4
GPCRs	Receptores acoplados à proteína G
GRB-2	Proteína ligadora do receptor para o fator de crescimento

GTT	Teste de tolerância à glicose
HPLC	*High-performance liquid chromatography*
	Cromatografia líquida de alto desempenho
HOMA	*Homeostasis model assessment*
IR	Receptor de insulina
IRS-1	Substrato 1 do receptor de insulina
IRS-2	Substrato 2 do receptor de insulina
IRSs	Substratos do receptor de insulina
ITT	Teste de tolerância à insulina
kDa	Quilodalton
K*itt*	Constante de decaimento de glicose
MAPK	*Mitogen-activated protein kinase*
ou MAP quinase	Proteína quinase ativadora da mitogênese
MAPKK	*Mitogen-activated protein kinase kinase*
	MAP quinase quinase
mSOS	*Son-of-sevenless*, fator de troca de nucleotídeo guanina
NEFA	*Non-esterified fatty acids*
	Ácidos graxos livres
p21ras	Proteína de 21 kDa da família Ras
PI3-quinase	Fosfatidilinositol 3-quinase
PIP3	Fosfatidilinositol 3,4,5-trifosfato
PMSF	Fluoreto de fenilmetil sulfonila

Raf-1	Proteína da cascata de ativação da MAP quinase
Ras	Proteína originalmente identificada como oncogene, têm participação na regulação do metabolismo e crescimento celular
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
Shc	Molécula adaptadora e substrato do receptor de insulina
Src	Oncogene originalmente definido como produto do sarcoma vírus Rous
Tris	Tri(hidroximetil)-aminometano
TSH	Hormônio tíreo-estimulante
UCP-1	Proteína desacopladora da respiração mitocondrial
WAT	White adipose tissue Tecido adiposo branco

RESUMO

A exposição de animais homeotérmicos ao frio é utilizada como um método reprodutível para se obter um modelo animal de hipoinsulinemia acompanhada por elevada mobilização periférica de glicose. No presente estudo, avaliaram-se as etapas iniciais e intermediárias da via de sinalização da insulina em tecidos periféricos de ratos expostos ao frio. Avaliou-se ainda, a comunicação intracelular entre o receptor β3-adrenérgico e as vias de sinalização da insulina em tecido adiposo marrom de ratos expostos ao frio e tratados, ou não, com compostos agonista ou antagonista β3-adrenérgicos. A exposição de ratos ao frio promoveu a redução da secreção de insulina, acompanhada de um elevado *clearance* de glicose e maior captação de glicose por tecido muscular esquelético, adiposo branco e adiposo marrom. Tais fenômenos foram acompanhados por inibição da ativação da maior parte dos componentes da via de sinalização da insulina em tecido muscular esquelético e adiposo branco; por estimulação da maior parte dos componentes da via de sinalização da insulina em tecido adiposo marrom; e por efeitos variados (estímulo, inibição e não-modulação) de componentes da via de sinalização da insulina em fígado. Por fim, este estudo demonstrou que a exposição ao frio ativa a sinalização β3-adrenérgica em tecido adiposo marrom. Tal ativação leva à modulação da atividade de vários componentes da via de sinalização da insulina neste tecido. Entretanto, fatores independentes da sinalização β3-adrenérgica parecem contribuir para a complexa regulação do sinal da insulina observada em tecido adiposo marrom de ratos expostos ao frio. Em conclusão, o presente estudo revelou alguns dos intrincados mecanismos pelos quais a exposição ao frio controla a atividade da insulina em animais homeotérmicos, podendo favorecer a identificação de potenciais alvos para a ação terapêutica em doenças onde a resistência à insulina desempenha papel central.

ABSTRACT

Cold exposure provides a reproducible model of improved glucose turnover accompanied by reduced blood levels of insulin. In the present study, the initial and intermediate steps of the insulin-signaling pathway in peripheral tissues of rats exposed to cold environment were evaluated. Also, the intracellular connection between insulin and β3-adrenergic signaling in brown adipose tissue of cold exposed rats treated, or not, with β3-adrenergic agonist or antagonist compounds were evaluated. During cold exposure, insulin secretion was significantly impaired, while whole body glucose clearance rates were significantly improved. This was accompanied by an increased glucose uptake by skeletal muscle, white adipose tissue and brown adipose tissue. These phenomena were paralleled by an apparent molecular resistance to insulin in skeletal muscle and white adipose tissue; by improved molecular response to insulin in brown adipose tissue; and by ambiguous effects (stimulation, inhibition and not modulation) of regulation of the insulin-signaling pathway in liver. Finally, cold exposure activated the β3-adrenergic signaling in brown adipose tissue. It leads to modulation of activity of several components of the insulin signal transduction pathway in this tissue. However, β3-adrenergic receptor independent mechanisms seem to contribute to the complex regulation of the insulin signaling observed in brown adipose tissue of rats exposed to cold. In conclusion, the present study revealed some of the complex mechanisms that participate in the cold-exposure-induced control of the insulin action in homeothermic animals. These results may favour the identification of novel potential targets for therapeutics in diabetes and related disorders.

1. INTRODUÇÃO GERAL

Obesidade, diabetes mellitus e doenças associadas a estes distúrbios estão entre os mais importantes problemas de saúde pública na atualidade (KOPELMAN, 2000; DEITEL, 2002). Resistência à ação da insulina participa da gênese do diabetes mellitus e parece desempenhar importante papel na associação entre diabetes, obesidade e doenças afins. Desta forma, nas últimas duas décadas grandes esforços têm sido empenhados na tentativa de se caracterizar os mecanismos por meio dos quais se instala a resistência à insulina.

Em músculo e tecido adiposo a insulina estimula a captação, o armazenamento e a utilização de glicose por intermédio da ativação da translocação dos transportadores de glicose (GLUT-4) para a membrana celular. Por outro lado, em fígado a captação de glicose é independente da ação da insulina, mas a gliconeogênese é inibida por este hormônio.

A insulina inicia suas ações celulares por meio de sua ligação a um receptor específico na membrana plasmática (FREYCHET et al., 1971; CUATRECASAS, 1972). O receptor de insulina (IR) é uma glicoproteína heterotetramérica constituída por duas subunidades α e duas subunidades β ligadas por pontes dissulfeto (MASSAGUE et al., 1981; KASUGA et al., 1982a). A subunidade α é totalmente extracelular e contém o sítio de ligação da insulina. A subunidade β é responsável pela transmissão do sinal, sendo uma proteína de localização transmembrânica que possui um sítio catalítico com atividade tirosina quinase, capaz de se autofosforilar e fosforilar outros substratos em aminoácidos tirosina (KASUGA et al., 1982b; VIRKAMAKI et al. 1999). Ao se ligar à subunidade α, a insulina promove uma mudança conformacional do receptor e a atividade quinase da subunidade β é estimulada; isto leva à transferência de grupos fosfato da adenosina trifosfato (ATP) para aminoácidos tirosina do receptor, promovendo um aumento da atividade quinase e a fosforilação de substratos protéicos intracelulares (KASUGA et al., 1982b; WHITE et al., 1984).

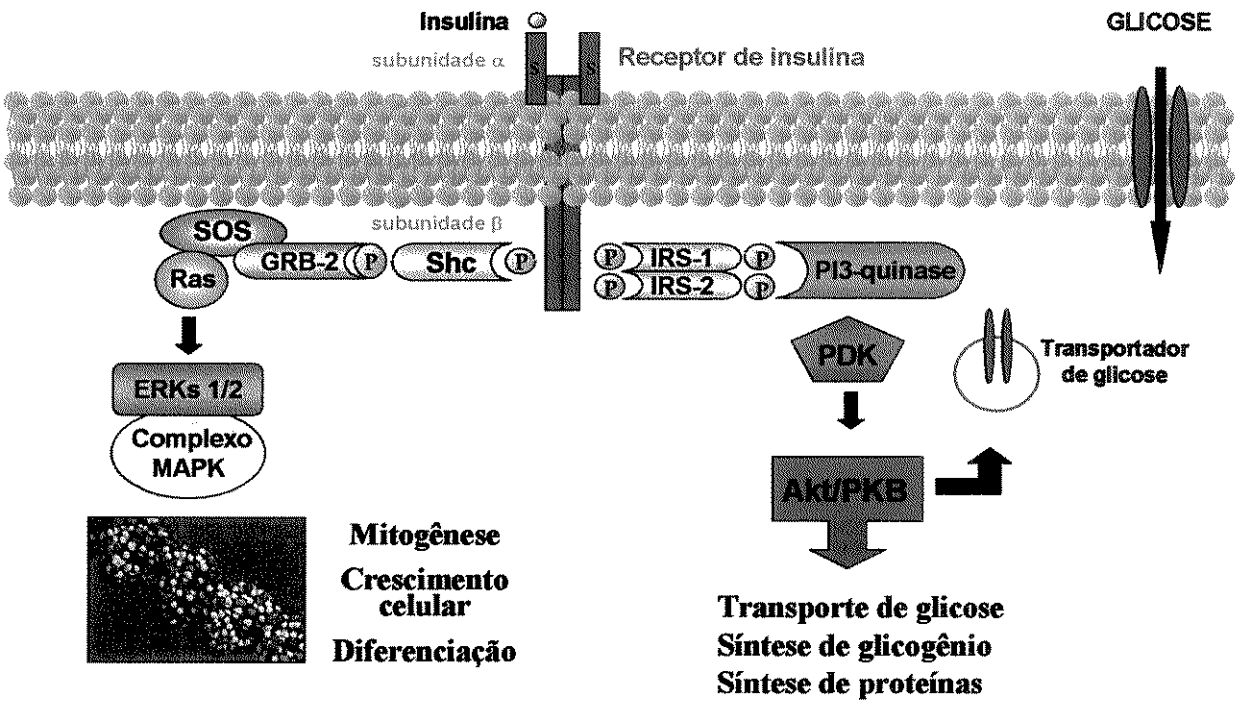
Dentre os substratos do receptor de insulina têm se estudado com maior atenção os substratos 1 e 2 (IRS-1 e IRS-2, respectivamente), a proteína adaptadora Shc (Sh, pela homologia com o oncogene viral Src, e c pela similaridade com proteína do colágeno) e outras proteínas que, de forma direta ou indireta, têm sua atividade modulada pela insulina

como GRB-2 (proteína ligadora do receptor para o fator de crescimento), fosfatidilinositol 3-quinase (PI3-quinase), proteína serina-quinase B (Akt/PKB) e elementos da via MAP quinase (MAPK, *mitogen activated protein kinase*) (WHITE, 1997).

Em estudos com cultura de células e em tecidos animais, o IRS-1 e o IRS-2 fosforilados associam-se à enzima PI3-quinase, ativando-a (FOLLI et al., 1992; SAAD et al., 1993). A ativação da PI3-quinase aumenta os níveis teciduais de fosfatidilinositol-3, 4, 5-trifosfato, que é um intermediário essencial na ativação da serina-quinase Akt/PKB, estimulando o transporte de glicose, síntese de glicogênio e síntese protéica (CHEATHAM et al., 1994; KOHN et al., 1996; TANTI et al., 1997; KROOK et al., 1998).

As proteínas IRS-1, IRS-2 e Shc, quando fosforiladas em tirosina, se ligam e ativam a GRB-2, e é por essa via que a insulina ativa a MAPK. Para exercer esse efeito a GRB-2 age como uma molécula adaptadora que ativa a via p21ras/MAPK, ligando-se ao fator permutador de guanina chamado mSOS (*son-of-sevenless*). Assim, a interação do complexo GRB-2/mSOS aos substratos do receptor de insulina pode mediar a estimulação da p21ras pela insulina. A proteína Ras se liga à Raf-1, a qual fosforila e ativa a MAPKK, que finalmente ativará a MAPK, resultando em um sinal de crescimento celular para a célula alvo (SKOLNIK et al., 1993; WHITE e KAHN, 1994; JIANG et al., 1999). A MAPK possui diversas subfamílias; entre elas está a subfamília da ERK (1/2) (*extracellular signal-regulated kinases 1/2)*, proteínas de 44 e 42 kDa, respectivamente (PEARSON et al., 2001).

Fica claro que os IRS-1 e 2 são proteínas de grande importância no processo de transmissão do sinal da insulina, localizadas estrategicamente na fase inicial desta via e, atuando como ancoradouros/ ativadores de proteínas como PI3-quinase e GRB-2, ativam diversas enzimas como a Akt e MAPK (ERKs). Portanto, o estudo da regulação do IRS-1 e IRS-2, de suas associações e da ativação da Akt e ERKs em tecidos de modelos animais que controlam diferentemente as ações da insulina, têm contribuido para um melhor entendimento dos mecanismos moleculares pós-receptores de alteração de sensibilidade à insulina.



Transmissão do sinal da insulina. De acordo com a descrição no texto.

Dentre os modelos animais nos quais há alteração da via de sinalização da insulina desencadeando resistência insulínica, pode-se citar o modelo de obesidade induzida pelo tratamento com glutamato monossódico (HIRATA et al., 2003), o jejum prolongado (72 horas) (ALMIRA e REDDY, 1979; PENICAUD et al., 1985; SAAD et al., 1992; FOLLI et al., 1993; ROJAS et al., 2001), e o tratamento com dexametasona (ROJAS et al., 2003), entre outros.

Além destes modelos clássicos, existem duas situações que promovem importantes alterações metabólicas periféricas em mamíferos. Uma delas é a hipotermia, quando a temperatura corporal de animais experimentais cai gradativamente até um ponto de equilíbrio ao redor de 15-18°C. A hipotermia pode ser obtida por anestesia por meio de uma mistura de 80% hélio e 20% oxigênio e temperatura de 0±1°C (STEFFEN, 1988), por administração intraperitoneal de etanol e temperatura de 4°C (HUTTUNEN et al., 1998) ou até mesmo indução farmacológica com halotano (HOO-PARIS et al., 1988). A outra

situação é a exposição de animais à temperatura de 4°C (BING et al., 1998). Apesar de ambos os modelos serem de exposição à baixa temperatura, importantes diferenças são observadas entre eles.

A indução de hipotermia leva a uma rápida queda das concentrações sanguíneas de insulina acompanhada por redução dos estoques hepáticos de glicogênio e hiperglicemia, provavelmente por aumento da glicogenólise (STEFFEN, 1988). Além disso, o tratamento de ratos hipotérmicos com insulina exógena revela que há resistência periférica ao hormônio oferecendo proteção ao sistema nervoso central, que utiliza glicose como substrato energético, enquanto células de órgãos periféricos utilizam estoques lipídicos (HOO-PARIS et al., 1988).

Por outro lado, animais expostos à temperatura de 4°C apresentam queda rápida das concentrações sanguíneas de insulina e leptina (TORSONI et al., 2003) o que é acompanhado por aumento de ingestão alimentar (OHTANI et al., 1999; TORSONI et al., 2003), por aumento das concentrações sanguíneas de catecolaminas (DULOO et al., 1988; GABALDON et al., 1995), bem como aumento transitório das concentrações sanguíneas de ácidos graxos livres (PEREIRA-DA-SILVA et al., 2003, TORSONI et al., 2003), hormônio tireo-estimulante (TSH) e corticosterona (HEFCO et al., 1975; SMITH, 1984). O elevado tônus simpático gerado durante a exposição ao frio estimula a lipólise no tecido adiposo branco (WAT) e a termogênese no tecido adiposo marrom (BAT) (VALLERAND e JACOBS, 1992; LOWELL e BACHMAN, 2003). Hipoleptinemia e hipoinsulinemia associadas à termogênese elevada, como se observa neste modelo, se contrapõe à associação entre hipoleptinemia e hipoinsulinemia do jejum em animais em temperatura ambiente, onde há redução da termogênese.

A exposição de animais homeotérmicos ao frio leva a uma melhora na captação de glicose apesar da reduzida concentração sanguínea de insulina e redução na secreção de insulina basal ou estimulada pela glicose (VALLERAND et al., 1983, 1987; SMITH, 1984; SHIBATA et al., 1989; DE SOUZA et al., 2003). Ambos os mecanismos, dependentes e independentes da insulina são supostos participantes deste modelo (GOTTESMAN et al., 1983; LAVELLE-JONES et al., 1987) e uma modulação tecido-específica de captação de glicose têm sido descrita (VALLERAND et al., 1987). Estes parâmetros metabólicos e

bioquímicos refletem uma adaptação ao frio para uma otimização do gasto energético por meio do controle da ingestão alimentar, aumento da produção endógena de calor e controle do fluxo de nutrientes para órgãos e tecidos.

Considerando-se que mecanismos que promovem melhora na captação de glicose, seja de forma dependente ou independente da ação da insulina, têm grande interesse para a ciência clínica, acredita-se que o estudo do modelo de exposição de animais ao frio possa evidenciar novos potenciais alvos para a ação terapêutica em diabetes e doenças afins. **Partindo dessa premissa, o objetivo inicial deste estudo foi realizar uma completa caracterização de parâmetros metabólicos, bioquímicos e hormonais, bem como caracterizar etapas iniciais e intermediárias da via de sinalização da insulina em fígado, músculo esquelético, tecido adiposo branco e tecido adiposo marrom de ratos expostos à baixa temperatura.** Para tanto, foi avaliada a expressão e o grau de fosforilação das proteínas IR, IRS-1, IRS-2, Akt e ERK, estimulados por insulina, em tecidos periféricos de ratos expostos ao frio.

Um dos responsáveis pela regulação da termogênese é o tecido adiposo marrom. Além de ser alvo para a ação da insulina e expressar os principais elementos da via de sinalização deste hormônio, o BAT é regulado pelo sistema nervoso simpático, o qual desempenha um importante papel na patogênese da resistência à insulina, de doenças metabólicas associadas e doenças vasculares, tais como diabetes do tipo 2, obesidade, dislipidemia e hipertensão (REAVEN et al., 1996).

O BAT é regulado pelas catecolaminas que atuam principalmente em adrenoceptores β_3 (REHNMARK et al., 1990; ROHLFS et al., 1995), modulando a termogênese por meio de uma proteína desacopladora da respiração mitocondrial chamada UCP-1 (ARCH e KAUMANN, 1993; COLLINS et al., 1994; EL HADRI et al., 1996; LOWELL e FLIER, 1997; VALVERDE et al., 1997; PORRAS et al., 1998). A UCP-1 está presente em tecido adiposo marrom de roedores, onde seus níveis são aumentados durante a exposição ao frio (KLAUS et al., 1991), ou após estimulação adrenérgica (LI et al., 1997), ao passo que estão diminuídos durante o jejum (CHAMPIGNY e RICQUIER, 1990). O receptor β_3–adrenérgico quando ativado por catecolaminas, acopla-se à proteína G,

estimula a adenilato ciclase, e conseqüentemente promove o aumento das concentrações intracelulares do segundo mensageiro AMPc, resultando em estimulação da lipólise, inibição da lipogênese (LAFONTAN e BERLAN, 1993) e aumento da termogênese (HIMMS-HAGEN, 1990; VAN SPRONSEN et al., 1993). A ativação da expressão da UCP-1 promove um *bypass* na cadeia mitocondrial de elétrons utilizando parte da diferença de gradiente eletroquímico que se forma entre as membranas interna e externa da mitocôndria para produzir calor em detrimento da síntese de ATP. Desta forma, a necessidade de substratos energéticos é aumentada. Acredita-se que uma melhor ação insulínica em BAT durante a exposição ao frio cumpra a função de acelerar a captação de glicose e desta forma prover fonte de energia para a produção de calor. Assim, ambos os receptores, o de insulina e o β_3–adrenérgico, participam da modulação da termogênese.

Estudos visando desenvolver uma nova classe de drogas que, pelo menos parcialmente, mimetizem os efeitos da exposição ao frio melhorando a mobilização de glicose e, por intermédio da ativação da termogênese, produza efeitos anti-obesidade têm sido alvo de investigações. É o caso dos agonistas β_3–adrenérgicos, os quais, quando utilizados em animais experimentais melhoram a homeostase da glicose e parcialmente inibem a obesidade induzida pela dieta (ROCHET et al., 1988; COLLINS et al., 1997).

Entretanto, ao contrário do esperado, em um estudo prévio utilizando adipócitos marrons isolados foi demonstrado que a estimulação de receptores β_3–adrenérgicos com o agonista CL316243 promoveu a redução da fosforilação em tirosina de receptores de insulina e dos substratos do receptor (IRS-2 em menor extensão que IRS-1) estimulados pela insulina (KLEIN et al., 1999), refletindo na redução da atividade quinase do receptor e redução na captação de glicose (ISSAD et al., 1995; KLEIN et al., 1999). Com base nos conceitos previamente expostos, não fica claro porque em adipócitos isolados o estímulo β_3–adrenérgico promoveu redução da captação de glicose, enquanto que trabalhos utilizando animais *in vivo* durante a exposição ao frio com conseqüente descarga adrenérgica demonstrou melhora na captação de glicose.

Assim, visto que as características mais marcantes dos animais expostos ao frio são o aumento transitório das concentrações sanguíneas de catecolaminas e uma melhora na captação de glicose; e, que os sinais adrenérgicos gerados durante a exposição ao frio devam participar do controle do fluxo de glicose pela modulação da ação da insulina e pela ativação de mecanismos de captação de glicose dependentes e independentes de insulina (CUNNINGHAM et al., 1985; SHIBATA et al., 1989; LIU et al., 1994); e, finalmente, sabendo-se que os efeitos da exposição ao frio no BAT, principal alvo da termogênese, são creditados à estimulação β_3–adrenérgica, fica claro o interesse em se investigar a participação do receptor β_3–adrenérgico na captação de glicose induzida pelo frio. **Neste contexto, este estudo também teve como objetivo investigar a inter-relação entre o receptor β3-adrenérgico e o receptor de insulina em tecido adiposo marrom de ratos expostos ao frio.** Para tanto, foi avaliada a expressão e o grau de fosforilação das proteínas IR, IRS-1, IRS-2, Akt e ERK, bem como a associação dos substratos IRS-1 e 2 com a PI3-quinase estimulados por insulina após tratamento com agonista ou antagonista β_3–adrenérgico de ratos expostos ao frio.

2. OBJETIVOS

Os objetivos do presente estudo foram:

ARTIGO I

- Avaliar parâmetros metabólicos, bioquímicos e hormonais em animais expostos ao frio.

- Investigar a conexão entre a regulação tecido-específica da homeostase da glicose modulada pelo frio e os eventos moleculares, iniciais e intermediários, da via de sinalização da insulina em tecidos periféricos. Para tanto, foram avaliados os níveis e graus de fosforilação do receptor de insulina, do IRS-1, do IRS-2, e a subseqüente ativação da Akt e das ERKs (1/2) após estímulo agudo com insulina em tecido hepático, muscular, adiposo branco e marrom de ratos expostos ao frio.

ARTIGO II

- Investigar a participação do receptor β3-adrenérgico na captação de glicose induzida pelo frio e na transdução do sinal da insulina em tecido adiposo marrom por intermédio do tratamento de animais controle ou expostos ao frio com compostos agonista ou antagonista β3-adrenérgicos. Para tanto, foram avaliados os níveis e graus de fosforilação do receptor de insulina, do IRS-1, do IRS-2, bem como a interação dos IRSs (1 e 2) com a PI3-quinase, e a subseqüente ativação da Akt e das ERKs (1/2) após estímulo agudo com insulina em tecido adiposo marrom de ratos.

3. MATERIAL E MÉTODOS

3.1. Animais experimentais e caracterização metabólica, bioquímica e hormonal

Foram utilizados ratos machos, da linhagem Wistar-Hannover (*Rattus norvegicus*) com oito semanas de idade, pesando aproximadamente 250 gramas, fornecidos pelo Biotério Central da Unicamp (CEMIB). Após o desmame, os animais receberam, como alimentação, ração comercial para roedores (Nuvilab CR-1, da Nuvital) *ad libitum.*

Os animais foram mantidos em gaiolas individuais e divididos em grupos mantidos em temperatura ambiente e expostos ao frio. A exposição ao frio ocorreu em câmara fria à temperatura de 4±1°C por até oito dias. Ratos da mesma idade e peso foram mantidos em temperatura ambiente de 23±1°C por igual período e utilizados como grupo controle. Ciclos de 12 horas luz e 12 horas de escuro, recebendo água e ração comercial *ad libitum* foram aplicados em ambos os grupos.

Na segunda parte dos estudos, ambos os grupos, controle e expostos ao frio, foram tratados com injeções intraperitoneais de salina ou agonista β3-adrenérgico BRL37344 ((±)-(R*,R*)-[4-[2-[[2-(3-Chlorophenyl)-2-hydroxyethyl] amino] propyl] phenoxy]-acetic acid sodium) ou antagonista β3-adrenérgico SR59230A (3-(2-Ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronaphth-1-ylamino]-(2S)-2-propanol oxalate). As drogas foram administradas durante oito dias na dose de 150 nmol/dose de BRL37344 em 200 µl (duas vezes ao dia) e 85 nmol/dose de SR59230A em 200 µl (duas vezes ao dia) ou 200 µl de salina para o grupo controle (duas vezes ao dia).

A avaliação da temperatura corporal, ingestão alimentar, peso corpóreo e a coleta de amostras de sangue para determinação de parâmetros bioquímicos e hormonais foi sempre realizada por volta das 10:00 hs, após jejum de 2 horas. Tais medidas foram realizadas nos tempos 0 e 2 horas e diariamente por oito dias após início da exposição ao frio (4°C). A temperatura corpórea foi medida utilizando-se um termômetro retal de alta sensibilidade (Thermistor – Hanna Instruments).

3.1.1. Determinação da glicose plasmática:

A dosagem da glicose plasmática foi realizada pelo método enzimático colorimétrico de glicose oxidase-peroxidase (TRINDER, 1969). Os resultados foram expressos em mg/dl.

3.1.2. Determinação da insulina:

A insulina plasmática foi avaliada por radioimunoensaio e a curva padrão foi determinada com insulina de rato (SCOTT et al., 1981). Os resultados foram expressos em ng/ml.

3.1.3. Determinação da leptina:

A leptina das amostras de soro foi dosada utilizando-se o kit comercial de ELISA segundo a indicação do fabricante (Diagnostic System Laboratories Inc, Webster, Texas, USA). Os resultados foram expressos em pg/ml.

3.1.4. Determinação de TSH:

O hormônio estimulante da tireóide das amostras de soro foi dosado utilizando-se kit de radioimunoensaio conforme a descrição do fabricante (Amersham Pharmacia Biotech – BIOTRAK). Os resultados foram expressos em ng/ml.

3.1.5. Determinação de corticosterona:

Os níveis de corticosterona das amostras de soro foram obtidos utilizando-se kit de radioimunoensaio conforme a descrição do fabricante (Amersham Pharmacia Biotech – BIOTRAK). Os resultados foram expressos em ng/ml.

3.1.6. Determinação de ácidos graxos livres:

Os ácidos graxos livres das amostras de soro foram dosados utilizando-se o kit comercial de ELISA segundo a indicação do fabricante (NEFA C – Wako Chemicals GmbH). Os resultados foram expressos em mM.

3.1.7. Determinação de catecolaminas:

Amostras de sangue para obtenção de plasma foram coletadas no oitavo dia do protocolo experimental. Os níveis de catecolaminas plasmáticas foram obtidos por HPLC de fase reversa (CASSIS et al., 1998). Os resultados foram expressos em pg/ml.

3.2. Determinação do glicogênio hepático e muscular

No oitavo dia do protocolo experimental, fragmentos de fígado e músculo gastrocnêmio foram coletados, pesados e digeridos em solução de KOH 30% sob aquecimento em banho-maria a 100°C para determinação colorimétrica de glicogênio (PIMENTA et al., 1989).

3.3. Teste de tolerância intraperitoneal à glicose (GTT)

Foi realizado após 12 horas de jejum, ao final do protocolo experimental. Por meio de um corte na extremidade da cauda do animal efetuou-se uma primeira coleta de sangue para dosagem de glicose, o que equivaleu ao tempo zero do teste. Em seguida, foi realizada a injeção intraperitoneal de uma solução de glicose 50% (2 g/Kg de peso corporal), sendo a seguir coletadas as amostras de sangue nos tempos 30, 60, 90 e 120 minutos, para as dosagens de glicose. Para a dosagem de insulina utilizou-se plasma diluído em solução fisiológica (cloreto de sódio 0,9%) nos mesmos tempos acima citados.

3.4. Teste de tolerância à insulina (ITT)

Foi realizado após 6 horas de jejum, ao final do protocolo experimental. Por meio de um corte na extremidade da cauda do animal efetuou-se uma primeira coleta de sangue para dosagem de glicose, o que equivaleu ao tempo zero do teste. Foi realizada a injeção de 2 U de insulina regular na veia da cauda do animal e amostras de sangue foram coletadas nos tempos 4, 8, 12 e 16 minutos para determinação da glicose sérica. A taxa de remoção de glicose (*Kitt*), durante o teste de tolerância à insulina foi calculada utilizando-se a fórmula ($0{,}693/t_{1/2}$). A glicose sérica ($t_{1/2}$) foi calculada pela curva de análise dos mínimos quadrados dos teores séricos de glicose após a administração da insulina, quando as concentrações de glicose decaíram linearmente (BONORA et al., 1987).

3.5. Avaliação da ação da insulina pelo modelo homeostático HOMA

O índice HOMA foi calculado por intermédio da fórmula: insulina basal/ 22,5exp (-In glicose basal) segundo MATTHEWS et al. (1985). Os níveis basais de insulina e glicose foram obtidos no oitavo dia do protocolo experimental.

3.6. Determinação da composição corporal

A composição corpórea foi obtida por meio de análise das carcaças (DOBUSH et al., 1985; KUMAR et al., 2002).

3.7. Secreção estática de insulina induzida por glicose

Grupos contendo cinco ilhotas foram isolados pelo método da colagenase (MALAISSE-LAGAE e MALAISSE, 1984; ARAÚJO et al., 2002) e pré-incubados por 30 minutos a 37°C em solução de Krebs-bicarbonato, suplementada com BSA e equilibradas sob gaseamento constante com carbogênio (95% O_2/ 5% CO_2). A seguir, as ilhotas foram

incubadas durante 1 hora em Krebs-bicarbonato contendo 2,8 mM de glicose (baixa concentração) e 16,7 mM de glicose (concentração supra-fisiológica). A solução de incubação foi coletada isenta de ilhotas e armazenada a –20°C para posterior dosagem da secreção de insulina por radioimunoensaio (SCOTT et al., 1981).

3.8. Captação de 2-deoxi-D-[^{3}H]glicose (2-DG)

Foi realizada após 12 horas de jejum, ao final do protocolo experimental. Sob anestesia, foi realizada a abertura da cavidade abdominal para injeção de 6 μCi 2-DG com ou sem 0,1 U de insulina em tampão fosfato com 0,1% de albumina pela veia cava do animal. Após 16 minutos, coletaram-se fragmentos de fígado, músculo, tecido adiposo branco e tecido adiposo marrom, os quais foram pesados e colocados em solução solubilizadora de tecido NCS-II (200 mg de tecido para 1 ml de solução solubilizadora). A radioatividade do ^{3}H do sobrenadante foi determinada por contagem em líquido de cintilação.

3.9. Imunoprecipitação e *Immunoblotting*

3.9.1. Extração dos tecidos

Para o estudo das etapas iniciais e intermediárias da ação insulínica, os animais foram anestesiados intraperitonealmente com tiopental sódico (50 mg/kg peso), e submetidos à extração dos tecidos, logo após a perda dos reflexos corneano e caudal.

Inicialmente foi aberta a cavidade abdominal e injetada solução fisiológica (grupo negativo) ou insulina regular (grupo positivo - 0,2 ml, 10^{-6} M ou 1,5 U) pela veia porta do animal. Após tempos pré-estabelecidos da injeção de insulina, foram coletados fragmentos de tecidos periféricos (fígado, músculo gastrocnêmio, tecido adiposo branco e tecido adiposo marrom), os quais foram colocados imediatamente em tubo tipo *falcon* contendo tampão de extração, mantidos em gelo. Os tecidos foram homogeneizados durante 20 segundos com processador do tipo "polytron PTA 20S (modelo PT 10/35; Brinkmann Instruments, Westbury, NY)", operado em velocidade máxima. Durante e após

o procedimento, o material homogeneizado foi mantido em gelo para evitar a desfosforilação do receptor de insulina e seus substratos.

No final da extração, foi adicionado Triton X-100 1% em todas as amostras e mantidas em gelo. Após 40 minutos, os materiais extraídos e homogeneizados foram submetidos à centrifugação. Utilizou-se a velocidade de 12.000 rpm por 20 minutos a 4°C para remover o material insolúvel. O sobrenadante foi utilizado para as etapas seguintes: uma parte foi utilizada para determinar a concentração protéica de cada amostra pelo método colorimétrico de biureto (BRADFORD, 1976); outra parte foi utilizada para avaliação do extrato total, ou seja, separação das proteínas em gel de poliacrilamida (SDS-PAGE), com tampão de Laemmli (LAEMMLI, 1970) acrescido de DTT 200 mM na proporção de 5:1 e finalmente outra parte foi utilizada para imunoprecipitação com anticorpos específicos.

3.9.2. *Immunoblotting* ou *Westernblotting*

Alíquotas contendo 200 µg de proteína por amostra foram aplicadas em gel SDS-PAGE. No mesmo gel foi aplicada uma amostra padrão de proteínas, ou seja, o marcador de peso molecular com pesos moleculares conhecidos: miosina (205 kDa), beta galactosidase (116 kDa), albumina sérica bovina (80 kDa) e ovalbumina (49 kDa). As proteínas apareciam sob coloração azul no gel de eletroforese e na membrana de nitrocelulose, permitindo a orientação quanto ao peso molecular das bandas observadas.

A eletroforese foi realizada em cuba de minigel da *Bio Rad* (Mini-Protean), com solução tampão para eletroforese, previamente diluída. O SDS-PAGE foi submetido inicialmente a 30 volts até a passagem da linha demarcada pela fase de empilhamento (*stacking*) e 120 volts até o final do gel de resolução (*resolving*). A seguir, as proteínas separadas no SDS-PAGE foram transferidas para uma membrana de nitrocelulose, utilizando-se o equipamento de eletrotransferência de minigel da *Bio Rad* com solução tampão para transferência mantido em voltagem constante de 120 volts por 2 horas (TOWBIN et al., 1979), sob refrigeração contínua por gelo.

As membranas de nitrocelulose contendo as proteínas transferidas foram incubadas em solução bloqueadora por 2 horas, à temperatura ambiente, para diminuir a ligação inespecífica de proteínas. A seguir, as membranas foram lavadas com solução basal (3 sessões de 10 minutos) e incubadas com anticorpo diluído em solução tampão por 4 horas, à temperatura ambiente sob agitação constante, ou durante uma noite a 4°C. Foram novamente lavadas com solução basal e incubadas posteriormente em solução com proteína A marcada com ^{125}I, durante 2 horas à temperatura ambiente. O excesso de proteína A foi lavado com solução basal e então as membranas foram expostas ao filme de RX (Kodak XAR - Rochester, NY) com intensificador (Cronex Lightning Plus - DuPont, Wilmington, DE) em cassete mantido a -80°C. Após 12 - 48 horas, os filmes foram revelados na forma convencional.

A intensidade das bandas foi determinada pela leitura das auto-radiografias reveladas por densitometria ótica, utilizando um *scanner* (HP 3400) e o programa *Scion Image* (Scion Corporation software).

3.9.3. Imunoprecipitação

Volumes das amostras com a mesma concentração protéica (determinada pelo método colorimétrico de biureto) foram utilizadas para imunoprecipitação com anticorpo anti-receptor de insulina, anti-IRS-1 e anti-IRS-2. As amostras foram colocadas sob incubação durante 12 - 14 horas a 4°C, sob agitação contínua. Em seguida acrescentou-se proteína A-Shepharose 6MB em todas as amostras para precipitação do complexo antígeno/anticorpo, sendo mantidas sob agitação contínua por mais duas horas. Após nova centrifugação por 15 minutos na velocidade de 12.000 rpm a 4°C, o sobrenadante foi descartado e o material precipitado foi lavado três vezes com a solução tampão específica para lavagem.

As proteínas precipitadas foram a seguir tratadas com tampão de Laemmli (LAEMMLI, 1970) contendo 100 mM de DTT, aquecidas em água fervente por 5 minutos e centrifugadas por 1 minuto. As proteínas foram então submetidas à eletroforese em

SDS-PAGE e transferidas para uma membrana de nitrocelulose, seguindo as etapas descritas no item 3.9.2. (*immunoblotting* ou *westernblotting).* Nestes experimentos de imunoprecipitação, as membranas foram submetidas ao *blotting* com anticorpo anti-fosfotirosina ou anti-PI3-quinase.

3.10. Fracionamento subcelular

Os animais foram anestesiados intraperitonealmente com tiopental sódico (50 mg/kg peso), e submetidos à extração dos tecidos, logo após a perda dos reflexos corneano e caudal.

Inicialmente foi aberta a cavidade abdominal e injetada solução fisiológica (grupo negativo) ou insulina regular (grupo positivo - 0,2 ml, 10^{-6} M ou 1,5 U) na veia porta do animal. Após tempos pré-estabelecidos da injeção de insulina, foram coletados fragmentos de tecidos periféricos (músculo gastrocnêmio, tecido adiposo branco e tecido adiposo marrom), os quais foram imediatamente homogeneizados com processador do tipo "polytron" em tubos *falcon* contendo solução tampão de extração, mantido todo o tempo no gelo. A seguir, as amostras foram submetidas à centrifugação (1.000 X g, 25 minutos, 4°C) para se obter a separação das frações.

O sobrenadante resultante da centrifugação foi retirado e ultracentrifugado (100.000 X g, 60 minutos, 4°C) para se obter o sobrenadante que corresponde à fração citosólica, o qual foi retirado e armazenado em gelo. O "pellet" obtido desta última centrifugação foi ressuspendido em solução tampão para fracionamento subcelular (0,32 M sacarose, 20 mM tris-HCl (pH 7,4), 2 mM EDTA, 1 mM DTT, 100 mM NaF, 100 mM pirofosfato de sódio, 100 mM Na_3VO_4, 1 mM PMSF e 0,1 mg/ml aprotinina) mais 1% de Nonidet P-40, mantido em gelo por 20 minutos e ultracentrifugado (100.000 X g, 10 minutos, 4°C), obtendo-se, assim, o sobrenadante que corresponde à fração de membrana. Parte do sobrenadante da fração citosólica e de membrana foi submetido à determinação do conteúdo de proteínas totais pelo método fotocolorimétrico de Biureto (BRADFORD, 1976), enquanto que a outra parte foi submetida ao *immunoblotting* ou *westernblotting* (item 3.9.2.) conforme descrito anteriormente.

3.11. Análise estatística

Os resultados foram expressos como média ± erro padrão da média (X ± E.P.M.). Para comparação de dois grupos, foi utilizado o Teste *t* de Student para dados não pareados. Quando necessário foi utilizada Análise de variância (ANOVA), seguida pelo teste de Bonferroni para comparações múltiplas de variáveis independentes. Foi adotado o nível de significância de 5 % ($p<0,05$).

4. CAPÍTULOS

4.1.Artigo I

"Cold exposure induces tissue-specific modulation of the insulin-signalling pathway in *Rattus norvegicus*".

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Cold exposure induces tissue-specific modulation of the insulin-signalling pathway in *Rattus norvegicus*

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Cold exposure provides a reproducible model of improved glucose turnover accompanied by reduced steady state and glucose-induced insulin levels. In the present report we performed immunoprecipitation and immunoblot studies to evaluate the initial and intermediate steps of the insulin-signalling pathway in white and brown adipose tissues, liver and skeletal muscle of rats exposed to cold. Basal and glucose-induced insulin secretion were significantly impaired, while glucose clearance rates during a glucose tolerance test and the constant for glucose decay during a 15 min insulin tolerance test were increased, indicating a significantly improved glucose turnover and insulin sensitivity in rats exposed to cold. Evaluation of protein levels and insulin-induced tyrosine (insulin receptor, insulin receptor substrates (IRS)-1 and -2, ERK (extracellular signal-related kinase)) or serine (Akt; protein kinase B) phosphorylation of proteins of the insulin signalling cascade revealed a tissue-specific pattern of regulation of the molecular events triggered by insulin such that in white adipose tissue and skeletal muscle an impaired molecular response to insulin was detected, while in brown adipose tissue an enhanced response to insulin was evident. In muscle and white and brown adipose tissues, increased 2-deoxy-D-glucose (2-DG) uptake was detected. Thus, during cold exposure there is a tissue-specific regulation of the insulin-signalling pathway, which seems to favour heat-producing brown adipose tissue. Nevertheless, muscle and white adipose tissue are able to take up large amounts of glucose, even in the face of an apparent molecular resistance to insulin.

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Exposure of homoeothermic animals to a cold environment leads to improved glucose clearance rates in spite of reduced blood insulin concentrations and basal or glucose-stimulated insulin secretion (Vallerand *et al.* 1983, 1987; Smith, 1984; Shibata *et al.* 1989). These changes of biochemical and metabolic parameters reflect adaptation to a novel environment and are responsible for an optimization of energy expenditure. Besides modulation of insulin and glucose levels, cold exposure leads to increased food ingestion (Ohtani *et al.* 1999; Torsoni *et al.* 2003), lower blood leptin levels (Torsoni *et al.* 2003), higher blood catecholamine levels (Dulloo *et al.* 1988; Gabaldon *et al.* 1995) and a transitory increase in blood thyroid-stimulating hormone (TSH), non-esterified fatty acids (NEFA) and corticosterone levels (Hefco *et al.* 1975; Smith, 1984; Torsoni *et al.* 2003). Since mechanisms that promote improved glucose tolerance are of potential therapeutic interest in diabetes mellitus, several studies have attempted to characterize the effects of cold exposure upon the modulation of glucose homeostasis. Both insulin-dependent and -independent mechanisms are supposed to participate in this process (Gottesman *et al.* 1983; Lavelle-Jones *et al.* 1987), and tissue-specific modulation of glucose uptake has been reported (Vallerand *et al.* 1983).

Insulin signals through a heterotetrameric transmembrane receptor belonging to the family of receptors that bear intrinsic tyrosine kinase activity (Virkamaki *et al.* 1999). In skeletal muscle and in white and brown adipose tissues (WAT and BAT), glucose uptake is mostly dependent on insulin activation of its signalling pathway (Pessin & Saltiel, 2000). On the other hand, in the liver, glucose uptake is independent of insulin action but glucose output is inhibited by this hormone (Michael *et al.* 2000). To date, several intracellular branches of the insulin-signalling pathway have been characterized at the molecular level (Saltiel & Kahn, 2001). Most studies agree that activation of the pathway IRSs–phosphatidylinositol 3(PI3)-kinase–Akt1 is a necessary event in order to achieve full stimulation of the glucose transport apparatus in muscle and fat (Summers *et al.* 1999). On the other hand the same IRSs–PI3-kinase–Akt1 pathway, in association with activation of ERK, seems to be required to promote inhibition of hepatic gluconeogenesis (Michael *et al.* 2000).

In the present study the IR–IRS1,2–PI3-kinase–Akt1 and IR–IRS1,2–ERK pathways were evaluated in muscle, liver, WAT and BAT of rats exposed to a cold environment for 8 days, in an attempt to characterize the connection between tissue-specific regulation of glucose homeostasis modulated by cold and the molecular events of the insulin-signalling pathway.

METHODS

Antibodies, chemicals and buffers

Reagents for SDS-polyacrylamide gel electrophoresis and immunoblotting were from Bio-Rad (Richmond, CA, USA). Hepes, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol, and bovine serum albumin (fraction V) were from Sigma (St Louis, MO, USA). Protein A-Sepharose 6MB was from Pharmacia (Uppsala, Sweden), ^{125}I-protein A was from ICN Biomedicals (Costa Mesa, CA, USA), 2-deoxy-D-[^{3}H]glucose was from New England Nuclear Corp. (Boston, MA, USA), and nitrocellulose paper (BA85, 0.2 μm) was from Amersham (Aylesbury, UK). Sodium thiopental (Amytal) and human recombinant insulin (Humulin R) were from Lilly (Indianapolis, IN, USA). Polyclonal anti-phosphotyrosine antibodies were raised in rabbits and affinity-purified on phosphotyramine columns. Anti-IR, anti-IRS1, anti-IRS2, anti-GLUT-4, anti-pERK (α pERK/Tyr 204, detecting pERK42 and pERK44) and anti-phospho [Ser^{473}]Akt1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-p85–PI3-kinase antiserum was from UBI (Lake Placid, NY, USA). Enzymatic colorimetric assay for the quantification of non-esterified-fatty-acids (NEFA) was from Wako Chemicals USA, Inc. (Richmond, VA, USA), leptin detection kit was from Linco Research Inc., (St Charles, MO, USA). Corticosterone and TSH radioimmunoassay (RIA) kits were from Amersham Pharmacia Biotech – BIOTRAK (Aylesbury, UK). Insulin was determined by RIA (Scott *et al.* 1981).

Animals and cold exposure protocols

Male Wistar rats (*Rattus norvegicus*) (8 weeks old, 200–300 g) obtained from the University of Campinas Animal Breeding Center were used in the experiments. The investigation followed the University guidelines for the use of animals in experimental studies and conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85–23 revised 1996). The animals were maintained on 12 h:12 h artificial light–dark cycles and housed in individual cages. After the acclimatizing period (3 days), the animals were randomly divided into two groups: cold-exposed (4 ± 1 °C, 8 days) and thermo-neutrality-maintained animals (23 ± 1 °C; control). The animals were allowed free access to standard rodent chow and water *ad libitum*. For tissue extraction (at day 8 of the experimental protocol), rats were anaesthetized by intraperitoneal injection of sodium amobarbital (15 mg (kg body weight)$^{-1}$), and the experiments were performed after the loss of corneal and pedal reflexes. Following experimental procedures the rats were killed under anaesthesia following the recommendations of the NIH publication No. 85–23.

Metabolic, hormone and biochemical measurements

Measurements of food intake, rectal temperature and body weight (during the light cycle) were obtained at time 0 and 2 h from the beginning of the experimental period, and daily during the eight experimental days in control and cold-exposed rats. Rectal temperature was measured with a Thermistor high precision digital thermometer (Hanna Instruments, Inc., Woonsocket, RI, USA) inserted 1.5 cm from the anus. Blood samples were always obtained from rats fasted for 2 h. Plasma glucose was measured by the glucose oxidase method in samples collected from the tail at time 0, and 2 h and daily during the experimental period (Trinder, 1969). NEFA was determined by ELISA according to the manufacturer's directions, in samples collected at time 0 and 2h, and on days 2, 4 and 8 of the experimental period. Insulin was detected by RIA, utilizing a guinea-pig anti-rat insulin antibody and rat insulin as standard (Scott *et al.* 1981), on samples collected at time 0 and 2 h, and on days 2, 4 and 8 of the experimental period. Serum leptin concentrations were measured by enzyme-linked immunosorbent assay (ELISA) on samples collected at time 0 and 2 h, and on days 2, 4 and 8 of the experimental period. Corticosterone and TSH were measured by RIA, according to the manufacturer's specifications, on samples collected at time 0 and 2 h, and on days 2 and 8 of the experimental period.

Catecholamine measurements

Plasma catecholamine levels were determined by reverse-phase HPLC according to a method previously described (Cassis *et al.* 1998). Plasma samples were collected at day 8 of the experimental protocol.

Glycogen measurements

After 8 days of exposure to either 23 ± 1 °C or 4 ± 1 °C, liver and gastrocnemius muscle fragments were collected (post-anaesthesia) and digested in pre-warmed KOH solution (30 %) for glycogen measurements as previously described (Pimenta *et al.* 1989).

Oral glucose tolerance test (GTT)

An oral GTT was performed on experimental day 8, after an overnight fast; the rats were anaesthetized as described above. After the collection of an unchallenged sample (time 0), a solution of 20 % glucose (2g (kg body weight)$^{-1}$) was administered into the stomach of the rats through a gastric catheter. Blood samples were collected at 30, 60, 90 and 120 min from the tail tip, for determinations of glucose and insulin concentrations.

Insulin tolerance test (ITT)

An intravenous (I.V.) ITT was performed on experimental day 8. Food was withdrawn 6 h before the test and the rats were anaesthetized as described above. Insulin (6 μg) was injected through the tail vein and blood samples were collected at 0, 4, 8, 12 and 16 min from the tail tip for serum glucose determination. The constant rate for glucose disappearance (K_{itt}) was calculated using the formula $0.693/t_{\frac{1}{2}}$. The half-time of glucose decay, $t_{\frac{1}{2}}$, was calculated from the slope of the least-square analysis of plasma glucose concentrations during the linear decay phase (Bonora *et al.* 1987).

Evaluation of insulin action by homeostatic model analysis (HOMA)

Homeostatic model analysis (HOMA) was calculated employing the formula insulin/22.5exp(–ln glucose) (Matthews *et al.* 1985), using insulin and glucose levels determined at day 8 of the experimental protocol.

Body composition

Whole body composition at experimental day 8 was determined post mortem following a method previously described (Kumar *et al.* 2002) with minor modifications. The carcass was weighted and placed within a drying stove (50 ± 5 °C) until it reached a stable weight. Carcass water content was calculated as the difference between the initial and final weights. The dried carcass was broken

into small fragments, which were wrapped in in filter paper and placed within a Soxlet extractor. The fragments were then washed over 2 days with petroleum ether. Body fat content was determined as the difference between the dried and fat-free weights.

Glucose-induced insulin secretion

To measure insulin secretion, groups of five islets isolated by the collagenase method (Araujo *et al.* 2002) were pre-incubated for 30 min at 37 °C in Krebs bicarbonate medium (NaCl 115 mM, KCl 5.0 mM, $CaCl_2$ 2.56 mM, $MgCl_2$ 1.0 mM, $NaHCO_3$ 24 mM and glucose 5.6 mM), supplemented with BSA (3 g l^{-1}) and equilibrated with a mixture of O_2 and CO_2 (95:5, v/v); pH 7.4 (Lacy & Kostianovsky, 1967). The solution was then replaced by fresh buffer containing low (2.8 mM) or supra-physiological (16.7 mM) concentrations of glucose, and the islets were incubated for 1 h longer. The insulin content in the supernatant was measured by RIA (Scott *et al.* 1981).

2-deoxy-D-glucose uptake studies

The *in vivo* tissue uptake of 2-deoxy-D-glucose (2-DG) at experimental day 8 was measured according to the procedure described by Turinsky (1983) with minor modifications. The rats were anaesthetized and then injected with 6 μCi of 2-deoxy-D-[^{3}H], sucrose with or without 0.1 U insulin in 0.4 ml isotonic phosphate buffer (pH 7.4) with 0.1% defatted bovine serum albumin, through the portal vein. After 16 min, slices of skeletal muscle (gastrocnemius), interscapular brown adipose tissue, epididimal white adipose tissue and liver were quickly removed, weighed, and solubilized in NCS-II Tissue Solubilizer (Amersham, Little Chalfont, Bucks, UK). The radioactivity of ^{3}H in the resulting supernatant was measured in a liquid scintillation fluid (ACS-II Amersham-Japan, Tokyo), using a scintillation counter (Aloka, Model LSC-1000, Kyoto). The results were expressed as counts min^{-1} (mg tissue weight)$^{-1}$). Cellular uptake of 2-DG was calculated as the difference between the total tissue radioactivity and the amount of radioactivity present in the tissue extracellular space. The cellular radioactivity was then converted to picomoles of 2-DG using the specific activity, and the results were expressed per milligram of dry tissue.

Tissue extraction, immunoblotting and immunoprecipitation

The abdominal cavity of anaesthetized rats was opened and the rats received an infusion of insulin (0.2 ml, 10^{-6} M) or saline (0.2 ml) through the cava vein. After different intervals (described under Results), fragments (3.0 mm × 3.0 mm × 3.0 mm) of BAT, WAT, liver and skeletal muscle were excised and immediately homogenized in solubilization buffer at 4 °C (1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM phenylmethylsulfonic fluoride (PMSF) and 0.1 mg aprotinin ml^{-1}) with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA) operated at maximum speed for 30 s. Insoluble material was removed by centrifugation for 20 min at 9000 *g* in a 70.Ti rotor (Beckman) at 4 °C. The protein concentration of the supernatants was determined by the Bradford dye binding method. Aliquots of the resulting supernatants containing 5.0 mg of total protein were used for immunoprecipitation with antibodies against IR, IRS1 and IRS2 at 4 °C overnight, followed by SDS-PAGE, transfer to nitrocellulose membranes and blotting with antiphosphotyrosine, anti-IR, anti-IRS1, anti-IRS-2 or anti-p85–PI3 kinase. In direct immunoblot experiments 0.2 mg of protein extracts obtained from each tissue were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-IR, IRS1, IRS2, anti-phospho-Akt or anti-phospho-ERK antibodies as described (Saad *et al.* 1993).

Subcellular fractionation

To characterize the expression and subcellular localization of GLUT-4, a subcellular fractionation protocol was employed as described previously (Mizukami *et al.* 1997), with minor modifications. Fragments of BAT, WAT and skeletal muscle obtained from rats treated or not with insulin (0.2 ml, 10^{-6} M, tissue obtained 15 min after insulin infusion) according to the protocols described above, were minced and homogenized in 2 volumes of STE buffer at 4 °C (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, 0.1 mg aprotinin ml^{-1}) in a Polytron homogenizer. The homogenates were centrifuged (1000 *g*, 25 min, 4 °C) to obtain pellets. The pellet was 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, 0.1 mg aprotinin ml^{-1}), kept on ice for 30 min and centrifuged (15 000 *g*, 30 min, 4 °C) to obtain the nuclear fraction. The supernatant was centrifuged (100 000 *g*, 60 min, 4 °C) to obtain the cytosol fraction and the pellet, which was suspended in STE buffer plus 1% Nonidet P-40, kept on ice for 20 min and centrifuged (100 000 *g*, 20 min) to obtain the membrane fraction. The fractions were treated with Laemmli buffer with 100 mM dithiothreitol, heated in a boiling water bath for 5 min, and aliquots (200 μg of protein) were subjected to SDS-PAGE and Western blotting with anti-GLUT-4 antibodies as described (Mizukami *et al.* 1997).

Data presentation and statistical analysis

All numerical results are expressed as the means ± S.E.M. of the indicated number (*n*) of experiments. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the ScionCorp software (Scion Image). Student's *t* test for unpaired samples and analysis of variance (ANOVA) for multiple comparisons were used for statistical analysis as appropriate. The *post hoc* test was employed when required. The level of significance was set at $P < 0.05$.

RESULTS

Metabolic characterization of cold-exposed rats

Figure 1 depicts a comprehensive evaluation of the metabolic parameters studied in cold-exposed and control rats. Cold exposure promoted an early fall in body weight (Fig. 1*A*), which gradually tended to recover from experimental day 5 on. The slope of weight gain–time curve is less steep in cold-exposed than in control rats. Daily food intake was immediately stimulated by cold exposure (Fig. 1*B*). From day 3 on there was a significant difference in food consumption between the groups. Body temperature (Fig. 1*C*) presented an early fall (at 2 h) in cold-exposed rats but rapidly accommodated, returning to the control level. The same occurred with blood glucose (Fig. 1*D*), which presented a rapid, non-significant fall at 2 h followed by prompt recovery. On the other hand, insulin (Fig. 1*E*) and leptin (Fig. 1*F*) were significantly lower in cold-exposed rats throughout the experimental period. Blood TSH (Fig. 1*G*) and corticosterone (Fig. 1*H*) levels were similar

between experimental groups throughout the study period, while NEFA levels were constantly higher in cold-exposed rats. However at none of the time points analysed, not even in the *post hoc* analysis, was a statistically significant difference detected (Fig. 1*I*). Finally, the noradrenaline (norepinephrine) level at experimental day 8 was significantly increased in cold-exposed rats compared to control (7280 ± 354 *vs.* 3875 ± 288 pg ml^{-1}, respectively, $n = 8$, $P < 0.05$).

Glucose turnover and insulin secretion in cold-exposed rats

Several studies have demonstrated that although cold-exposed rats are hypoinsulinaemic, they mobilize glucose with greater efficiency than their respective controls (Vallerand *et al.* 1983, 1987). In the present study, rats exposed to cold for 8 days were able to promote a much more efficient glucose uptake during a GTT, even producing significantly lower levels of insulin (Fig. 2*A* and *B*). Increased insulin sensitivity of cold-exposed rats was further confirmed by an I.V. ITT, which showed a significant increase of ~50 % in K_{itt} (Fig. 2*B*, inset), and a reduced HOMA value (Fig. 2 *C*).

To investigate if the low insulin level was due to a primary defect of insulin secretion by the β-cells, pancreatic islets were isolated from control and cold-exposed rats and freshly prepared for insulin secretion studies. As depicted in Fig. 3*A*, basal and glucose-stimulated insulin secretion were significantly lower in islets isolated from cold-

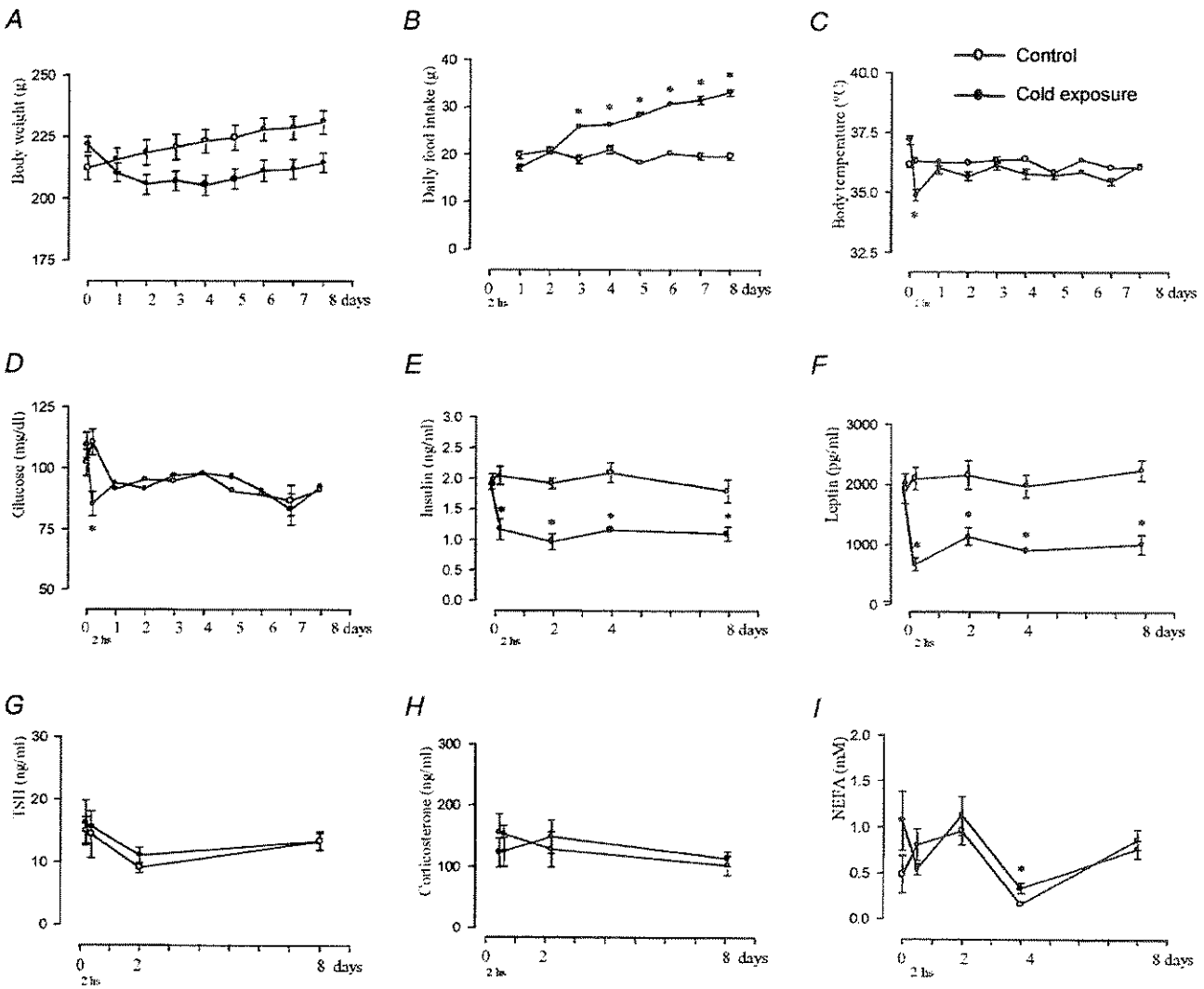

Figure 1. Metabolic characterization of rats exposed to cold

Body weight (*A*), daily food intake (*B*), body temperature (*C*), plasma glucose (*D*), serum insulin (*E*), serum leptin (*F*), serum TSH (*G*), serum corticosterone (*H*) and serum NEFA (*I*) concentrations were determined in rats exposed to 4 °C (●) or maintained at room temperature (○) according to the methods described in the text. Results are expressed as means ± S.E.M.; $n = 12$ for *A*–*D* and *I*; $n = 8$ for *E*–*H*. * $P < 0.05$ *vs.* control.

exposed rats, confirming that direct exposure of rats to cold affects the functional response of the insulin-producing organ. However, as shown in Fig. 3*B*, following a glucose stimulus, pancreatic islets from cold-exposed rats presented a significantly higher percentage response over basal than pancreatic islets from control rats.

Tissue-specific glucose uptake and energy depots in rats exposed to cold

Glucose uptake by skeletal muscle and WAT is highly stimulated by insulin. BAT also presents the same characteristics as WAT, possessing all the molecular machinery needed for insulin signalling, and expressing insulin-sensitive GLUT-4 (Valverde *et al.* 1998; Kawashita *et al.* 2002). On the other hand, liver glucose uptake is independent of insulin action; however, the pancreatic hormone tightly regulates hepatic gluconeogenesis. Since cold exposure leads to high glucose turnover in spite of low basal and stimulated insulin levels, we decided to evaluate tissue-specific glucose uptake, as well as whole-body fat and glycogen contents, in insulin-sensitive tissues. Figure 4 shows that insulin-induced 2-DG uptake was significantly

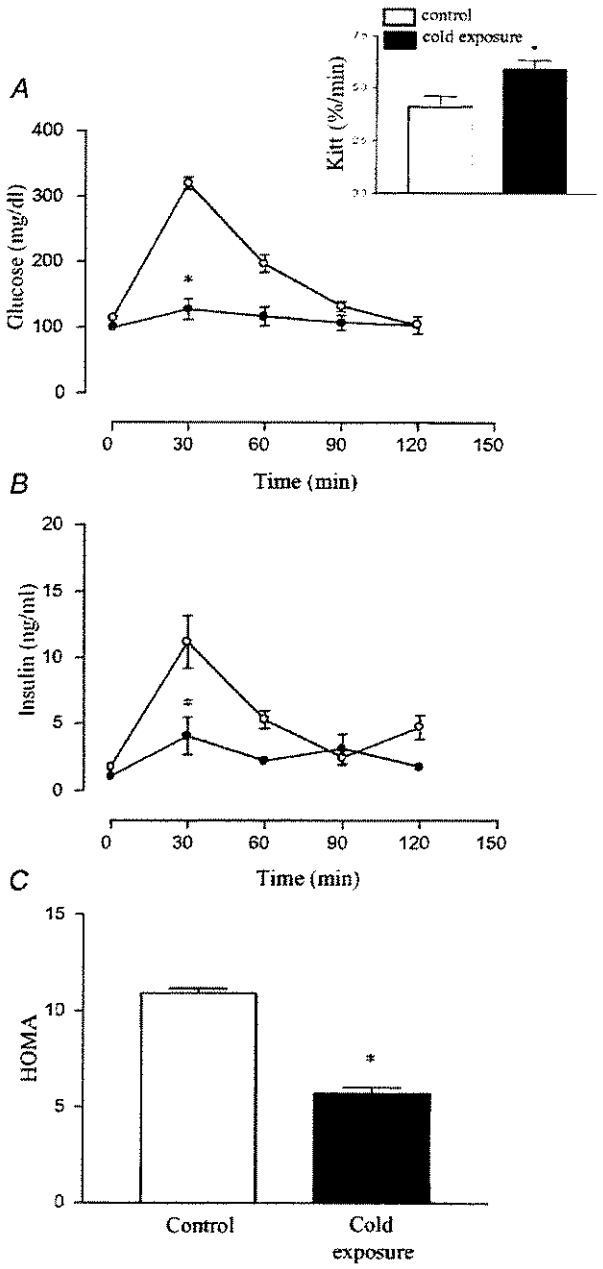

Figure 2. Effect of 8 days cold exposure on glucose metabolism

Glucose (*A*) and insulin (*B*) concentrations during I.P. GTT, and glucose disappearance rate (*A*, inset) in control and cold-exposed rats. The constant rate for plasma glucose disappearance (K_{itt}) was calculated as described in Methods. HOMA values (*C*) for control and cold-exposed rats were calculated as previously described (Matthews *et al.* 1985). Results are representative of the mean ± S.E.M.; $n = 8$ for *A–C*; $n = 12$ for *A*, inset. * $P < 0.05$ *vs.* control.

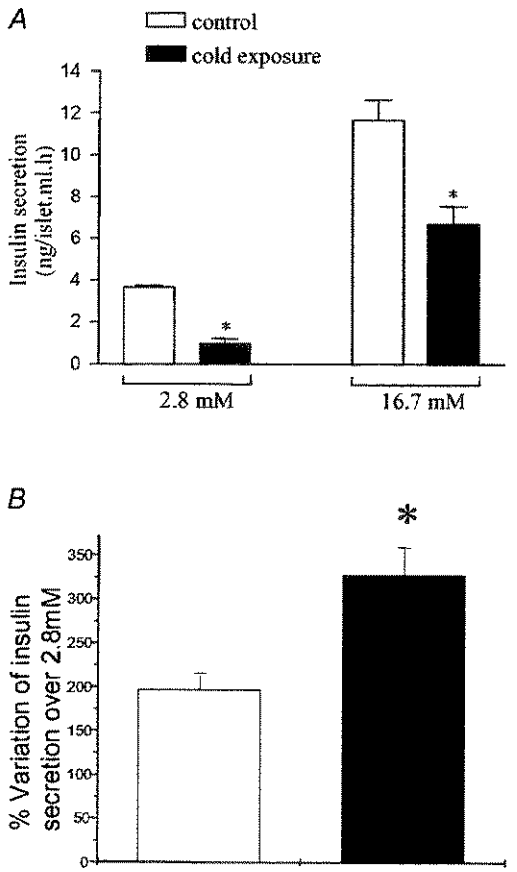

Figure 3. Static insulin secretion studies

Insulin secretion (*A*) was calculated from the accumulation of insulin in supernatants of 5 islets/well isolated from cold-exposed or control rats and maintained in medium containing either 2.8 or 16.7 mM glucose. The percentage increment (*B*) of glucose-induced insulin secretion was obtained from the difference in secretion between islets exposed to 2.8 and 16.7 mM glucose in each experimental group. Values are representative of means ± S.E.M.; $n = 6$ wells/group. * $P < 0.05$ *vs.* control.

increased in BAT, WAT and skeletal muscle of cold-exposed rats, while in the liver glucose uptake was similar in cold-exposed and control rats. Moreover, cold exposure produced no changes on muscle glycogen content (Fig. 5*B*) but led to a significant increase of liver glycogen stocks (Fig. 5*A*). Finally, whole body fat content was dramatically reduced after 8 days of cold exposure (Fig. 5*C*).

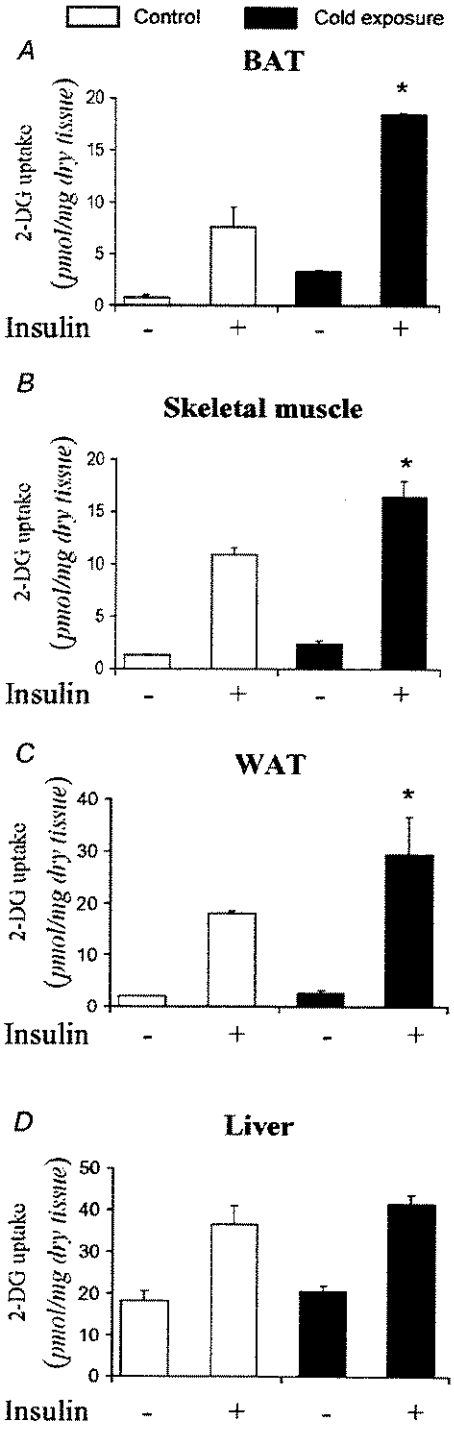

Figure 4. Tissue-specific glucose uptake

2-[^{3}H]-Deoxyglucose uptake in brown adipose tissue (BAT) (*A*), skeletal muscle (*B*), white adipose tissue (WAT) (*C*) and liver (*D*) of control and cold-exposed insulin animals was determined as described in the text. Results are expressed as means ± S.E.M.; $n = 5$. * $P < 0.05$ *vs.* control + insulin.

Effects of cold exposure upon the insulin signalling pathway

The results presented above suggested that both insulin-dependent and -independent mechanisms participate in the homeostasis of glucose in cold-exposed rats. In these animals, glucose flow into BAT, WAT and skeletal muscle was significantly increased while glucose flow into liver

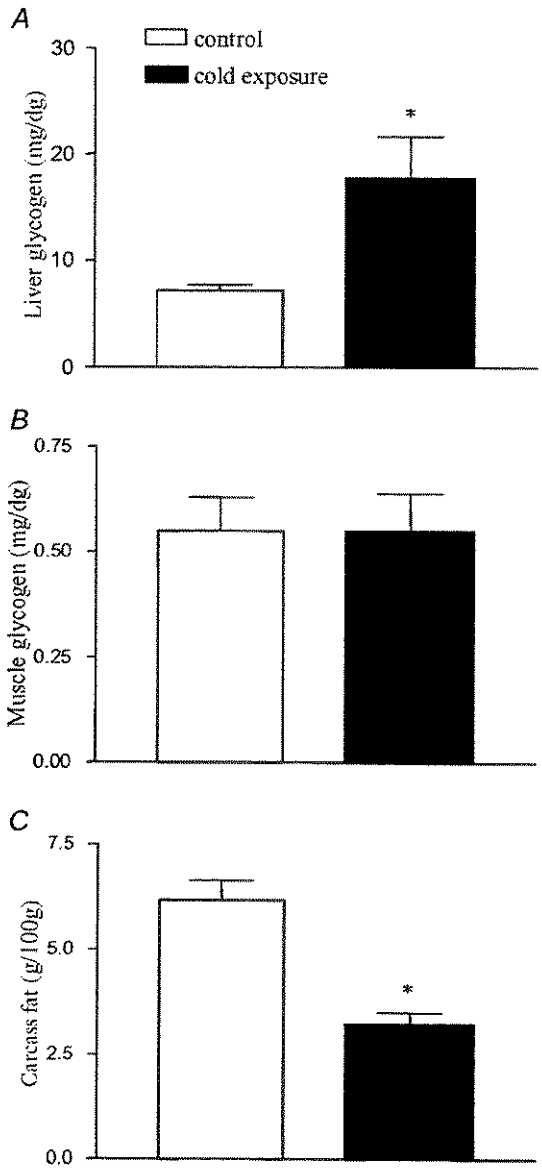

Figure 5. Tissue glycogen and fat content

Hepatic (*A*) and muscular (*B*) glycogen concentrations, and percentage of body fat (*C*) were determined in cold-exposed and control rats. Results are expressed as means ± S.E.M., $n = 6$. * $P < 0.05$ *vs.* control.

was unaffected. On the other hand, body fat was significantly consumed, while liver glycogen content was increased. In view of these findings we decided for evaluating the initial and intermediate steps of two branches of the insulin-signalling cascade in BAT, WAT, skeletal muscle and liver of rats exposed to cold.

In BAT (Fig. 6) cold exposure led to no modulation of IR (Fig. 6*A*) and IRS1 (Fig. 6*C*) protein expression. However, the IRS2 protein level was significantly increased in cold-exposed rats (Fig. 6*E*). Following insulin stimulation tyrosine phosphorylation of IR (Fig. 6*B*), IRS1 (Fig. 6*D*) and IRS2 (Fig. 6*F*) was significantly higher in cold-exposed rats than in controls. On the other hand, insulin-induced serine phosphorylation of Akt (Fig. 6*G*) and tyrosine phosphorylation of ERK (Fig. 6*H*) were unaffected by cold exposure. The insulin-induced associations of p85–PI3-kinase with IRS1 and IRS2 were significantly incremented in cold-exposed rats (not shown).

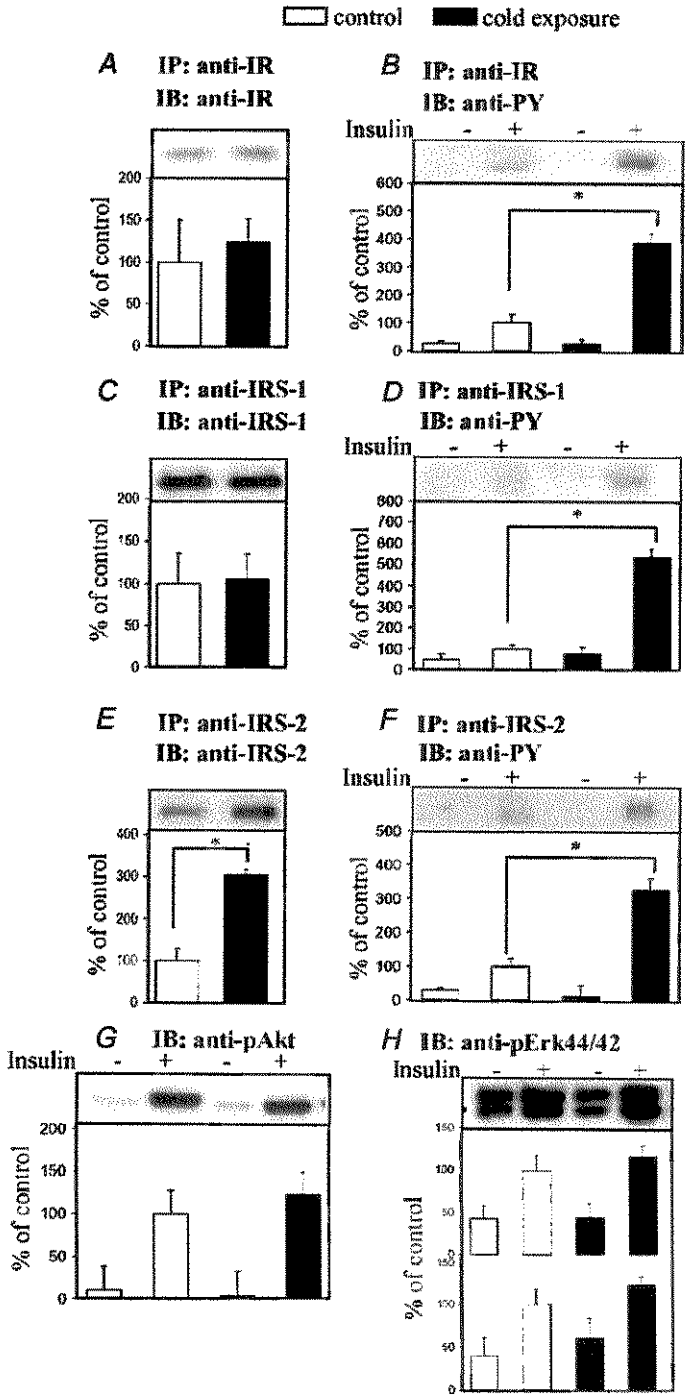

Figure 6. Insulin signal transduction in brown adipose tissue

The protein levels of IR (*A*), IRS1 (*C*), and IRS2 (*E*) were determined in brown adipose tissue (BAT) of control and cold-exposed rats. Samples (200 μg) of total protein extracts from each tissue were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with anti-IR (*A*), anti-IRS1 (*C*) or anti-IRS2 (*E*) antibodies. Tyrosine phosphorylation of IR (*B*), IRS1 (*D*) and IRS-2 (*F*) was evaluated in brown adipose tissue protein extracts by immunoprecipitation (IP) with anti-IR (*B*), anti-IRS1 (*D*) and anti-IRS-2 (*F*) antibodies, and immunoblotting with anti-phosphotyrosine (PY) antibodies. Serine^{473} phosphorylation of Akt (*G*) and tyrosine phosphorylation of ERK (*H*) were determined by blotting of total protein extracts, separated by SDS-PAGE and transferred to nitrocellulose membranes. Data are presented as means ± S.E.M., $n = 6$. * $P < 0.05$ *vs.* control.

In WAT (Fig. 7) significant reductions of IR (Fig. 7*A*), IRS1 (Fig. 7*C*) and IRS2 (Fig. 7*E*) protein expression were detected in cold-exposed rats. Acutely injected insulin led to significantly lower level of tyrosine phosphorylation of IR (Fig. 7*B*) and IRS1 (Fig. 7*D*) and a tendency towards lower levels of tyrosine phosphorylation of IRS2 (Fig. 7*F*) in cold-exposed animals. Both serine phosphorylation of Akt (Fig. 7*G*) and tyrosine phosphorylation of ERK (Fig. 7*H*), following insulin treatment, were significantly reduced in cold-exposed rats compared to control rats.

In skeletal muscle (Fig. 8) protein expression of IRS1 (Fig. 8*C*) and IRS2 (Fig. 8*E*) was significantly reduced by cold exposure, while the protein level of IR (Fig. 8*A*) was not affected by this condition. Nevertheless, insulin-

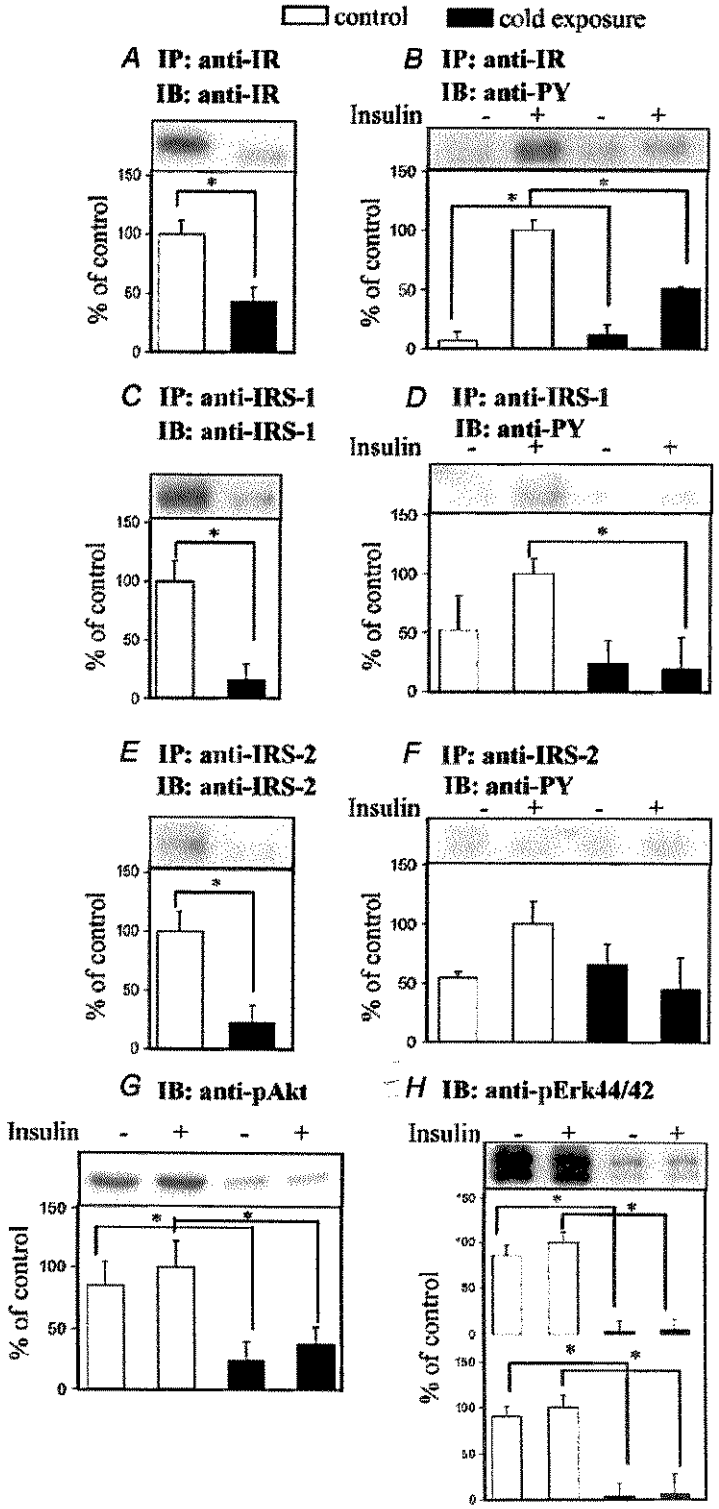

Figure 7. Insulin signal transduction in white adipose tissue

The protein levels of IR (*A*), IRS1 (*C*), IRS2 (*E*) were determined in white adipose tissue (WAT) of control and cold-exposed rats. Samples (200 μg) of total protein extracts from each tissue were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with anti-IR (*A*), anti-IRS1 (*C*) or anti-IRS2 (*E*) antibodies. Tyrosine phosphorylation of IR (*B*), IRS1 (*D*) and IRS-2 (*F*) was evaluated in white adipose tissue protein extracts by immunoprecipitation (IP) with anti-IR (*B*), anti-IRS1 (*D*) and anti-IRS-2 (*F*) antibodies, and immunoblotting with anti-phosphotyrosine (PY) antibodies. Serine^{473} phosphorylation of Akt (*G*) and tyrosine phosphorylation of ERK (*H*) were determined by blotting of total protein extracts, separated by SDS-PAGE and transferred to nitrocellulose membranes. Data are presented as means ± S.E.M., $n = 6$. * $P < 0.05$ *vs.* control.

induced tyrosine phosphorylation of IR (Fig. 8*B*) and IRS1 (Fig. 8*D*) were significantly lower in cold-exposed rats while the opposite occurred with IRS2 (Fig. 8*F*). The insulin-induced serine phosphorylation of Akt (Fig. 8*G*) was significantly reduced in cold-exposed rats while no difference between experimental groups was detected in insulin-induced tyrosine phosphorylation of ERK (Fig. 8*H*).

Finally, in the liver (Fig. 9) the exposure of rats to cold for 8 days exerted no effect upon IR (Fig. 9*A*), IRS1 (Fig. 9*C*) and IRS2 (Fig. 9*E*) protein expression. In spite of this, insulin-induced tyrosine phosphorylation of IR (Fig. 9*B*) and IRS2 (Fig. 9*F*) was significantly increased, while IRS1 (Fig. 9*D*) tended to increase in cold-exposed rats as compared to controls. Cold exposure promoted a significant fall in insulin-induced serine phosphorylation of Akt

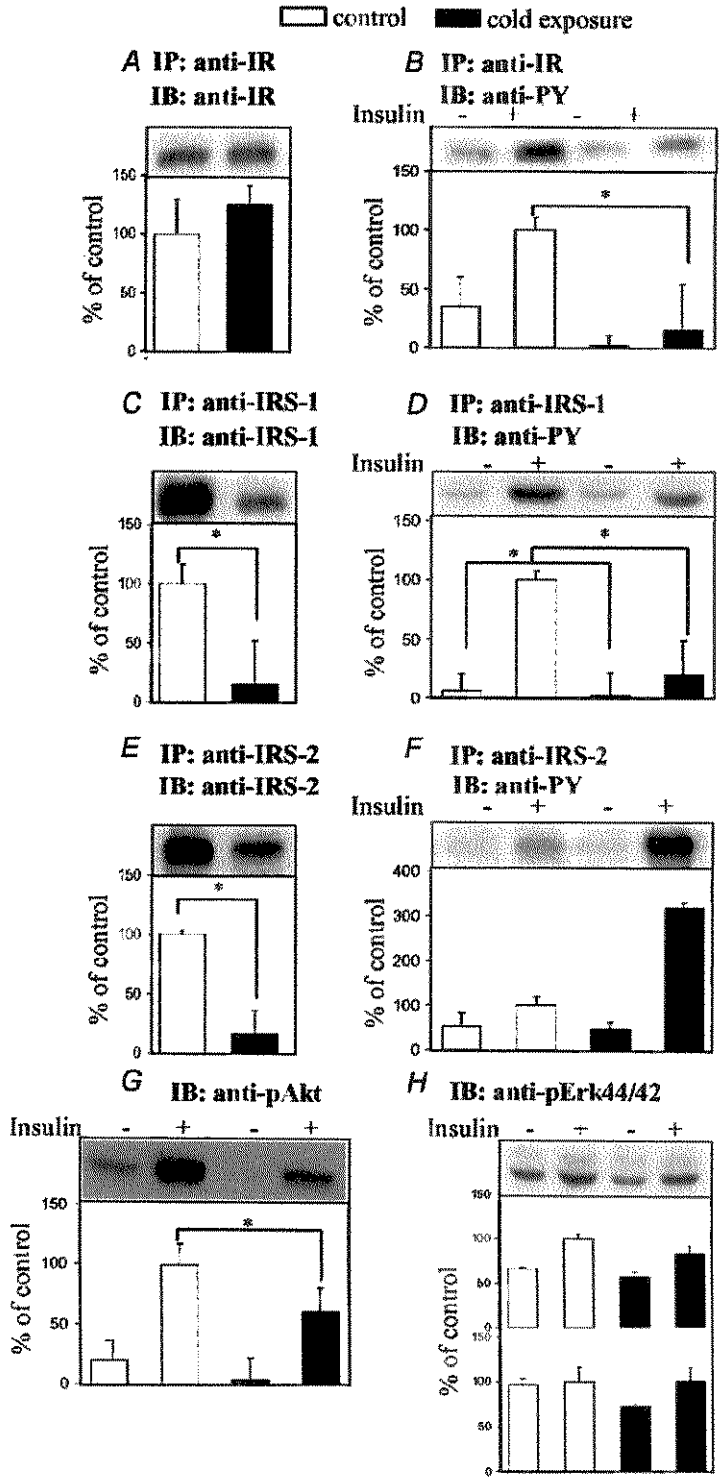

Figure 8. Insulin signal transduction in skeletal muscle

The protein levels of IR (*A*), IRS1 (*C*), IRS2 (*E*) were determined in skeletal muscle of control and cold-exposed rats. Samples (200 μg) of total protein extracts from each tissue were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with anti-IR (*A*), anti-IRS1 (*C*) or anti-IRS2 (*E*) antibodies. Tyrosine phosphorylation of IR (*B*), IRS1 (*D*) and IRS-2 (*F*) was evaluated in skeletal muscle protein extracts by immunoprecipitation (IP) with anti-IR (*B*), anti-IRS1 (*D*) and anti-IRS-2 (*F*) antibodies, and immunoblotting with anti-phosphotyrosine (PY) antibodies. Serine^{473} phosphorylation of Akt (*G*) and tyrosine phosphorylation of ERK (*H*) were determined by blotting of total protein extracts, separated by SDS-PAGE and transferred to nitrocellulose membranes. Data are presented as means ± S.E.M., $n = 6$. * $P < 0.05$ *vs.* control.

(Fig. 9*G*), and exerted no modulation upon insulin-induced tyrosine phosphorylation of ERK (Fig. 9*H*).

Effects of cold exposure upon GLUT-4 expression

The effect of cold exposure upon GLUT-4 expression and translocation to cell membrane was evaluated in subcellular fractions of BAT, WAT and skeletal muscle from insulin-treated and non-insulin-treated, cold-exposed and control rats. As depicted in Fig. 10, cold exposure induced an increase of GLUT-4 expression in whole tissue extracts from BAT, WAT and skeletal muscle (Fig. 10*A–C*, left-hand bar graphs). In control rats, treatment with insulin promoted a significant increase of GLUT-4 in the membrane fraction, which was accompanied by a proportional decrease of GLUT-4 in the cytosol fraction, in all tissues evaluated (Fig. 10*A–C*, middle bar graphs). Conversely, in cold-

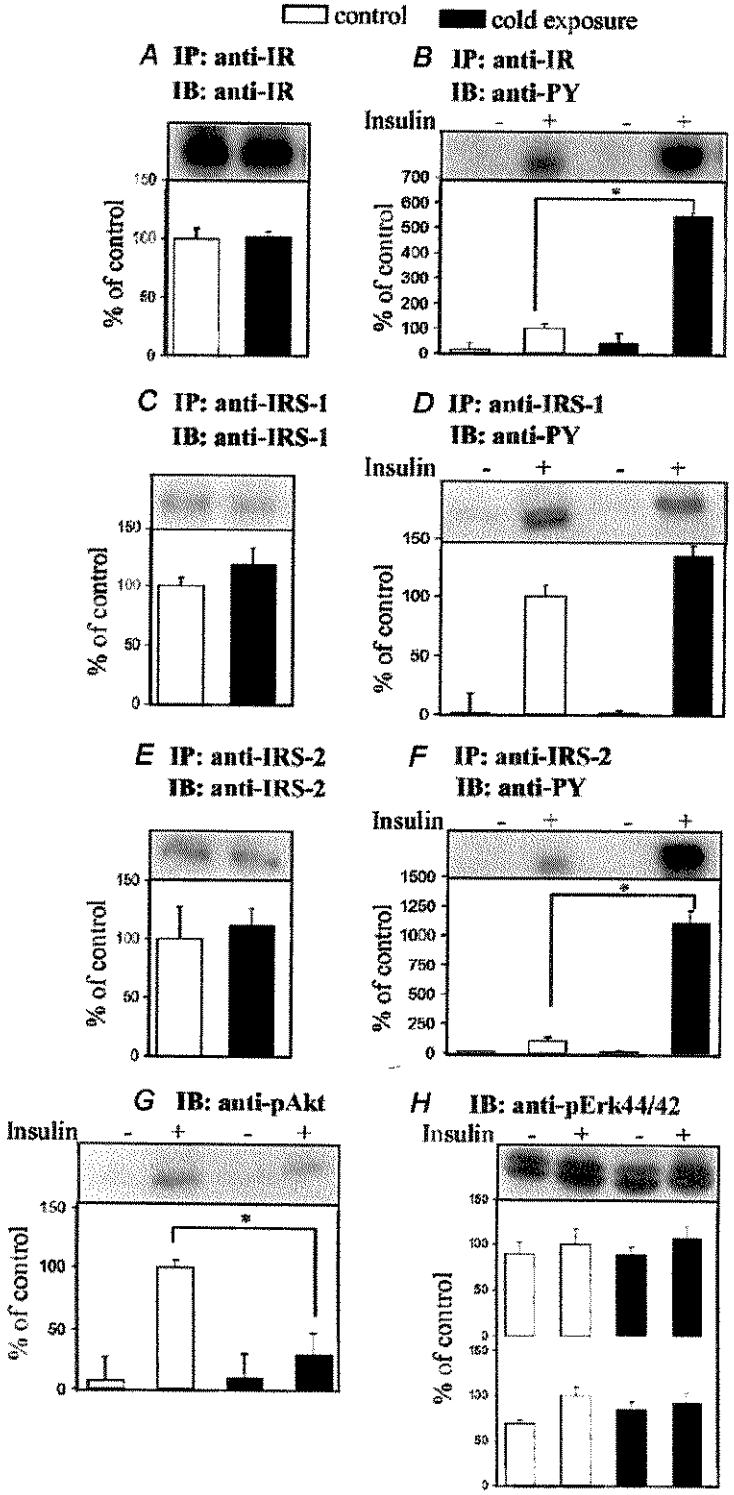

Figure 9. Insulin signal transduction in liver

The protein levels of IR (*A*), IRS1 (*C*), IRS2 (*E*) was determined in liver of control and cold-exposed rats. Samples (200 μg) of total protein extracts from each tissue were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted (IB) with anti-IR (*A*), anti-IRS1 (*C*) or anti-IRS2 (*E*) antibodies. Tyrosine phosphorylation of IR (*B*), IRS1 (*D*) and IRS-2 (*F*) was evaluated in liver protein extracts by immunoprecipitation (IP) with anti-IR (*B*), anti-IRS1 (*D*) and anti-IRS-2 (*F*) antibodies, and immunoblotting with anti-phosphotyrosine (PY) antibodies. Serine^{473} phosphorylation of Akt (*G*) and tyrosine phosphorylation of ERK (*H*) were determined by blotting of total protein extracts, separated by SDS-PAGE and transferred to nitrocellulose membranes. Data are presented as means ± S.E.M., $n = 6$. * $P < 0.05$ *vs.* control.

exposed rats insulin exerted no significant effect on GLUT-4 translocation to the membrane fraction (Fig. 10*A–C*, right-hand bar graphs). In fact, in cold-exposed rats, the GLUT-4 concentration in the membrane fraction was higher than GLUT-4 expression in the cytosolic fraction, even in the absence of an insulin stimulus. Thus, cold exposure induces an increase of whole tissue GLUT-4 expression that is mostly due to an increase in the GLUT-4 concentration in the membrane fraction, and is independent of insulin action.

DISCUSSION

Although much work has been done to characterize the molecular mechanisms of insulin resistance (Saad, 1994; Saltiel & Kahn, 2001; White, 2002), an ever-growing interest

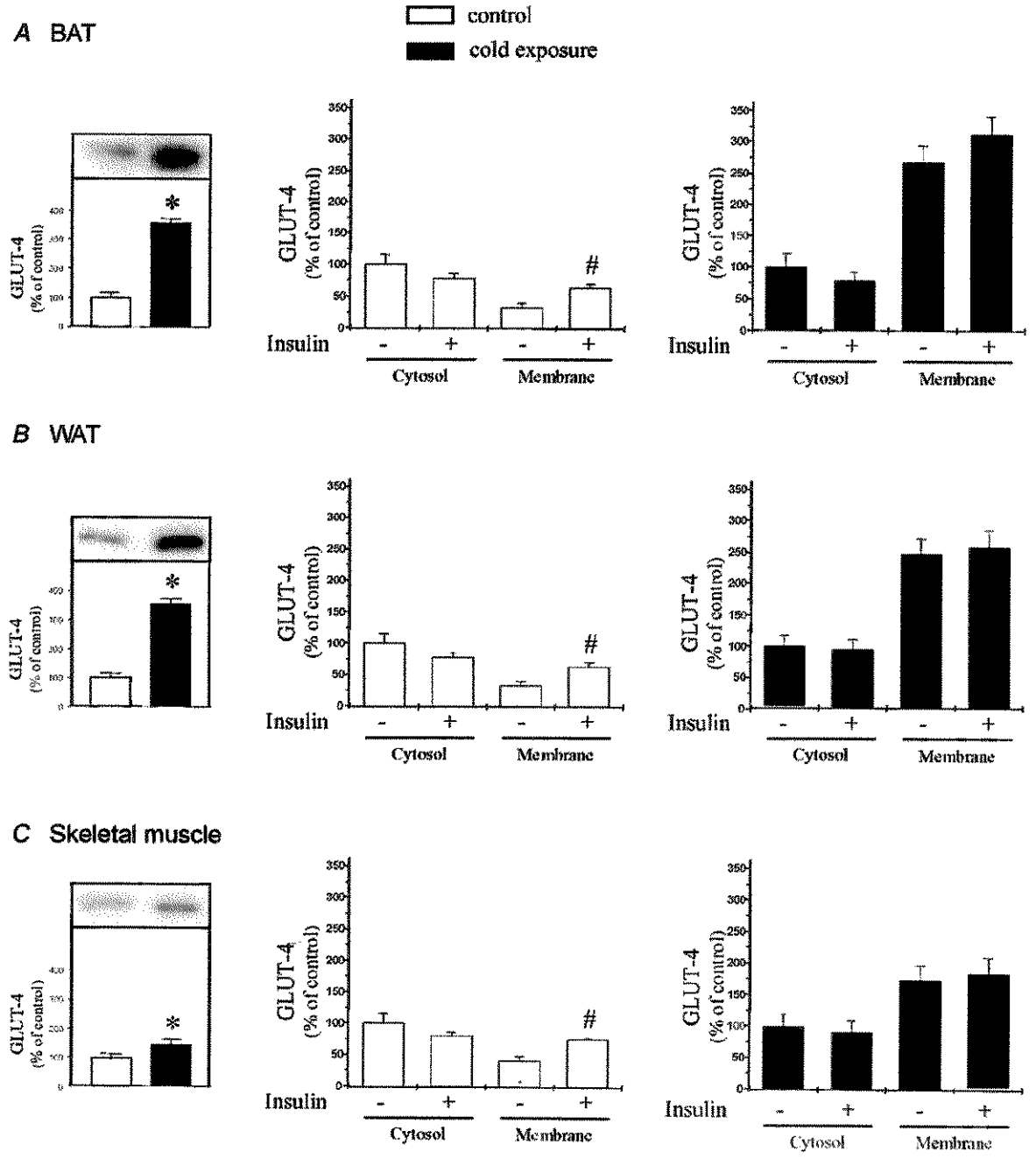

Figure 10. GLUT-4 expression and subcellular distribution

The protein levels of GLUT-4 were determined by SDS-PAGE and immunoblot of whole tissue extracts (*A–C* left-hand panels) and in subcellular fractions (middle and right-hand panels) of cytosol and membrane of BAT (*A*), WAT (*B*) and skeletal muscle (*C*). Data are presented as means ± S.E.M., $n = 6$. * $P < 0.05$ *vs.* control. # $P < 0.05$ *vs.* membrane without (−) insulin.

is focused on the mechanisms that allow for improved glucose clearance rates. The most obvious reason for this interest is the promise of finding new molecular targets for drug therapy to be used in diabetes mellitus and related diseases.

Exposure of homoeothermic animals to a cold environment leads to a unique situation characterized by low insulin secretion accompanied by increased glucose mobilization and an evident improvement of the biological response to insulin action (Smith & Davidson, 1982; Vallerand *et al.* 1987). According to several studies, the main factor responsible for the improved glucose turnover observed in cold-exposed animals is not an enhanced response to insulin, but an increase in glucose uptake driven by insulin-independent mechanisms (Smith & Davidson, 1982; Cunningham *et al.* 1985; Vallerand *et al.* 1987; Dulloo *et al.* 1988; Shibata *et al.* 1989). These facts have enhanced the interest in the characterization of the mechanisms that lead to improved glucose mobilization in cold-exposed animals and at least one new class of drug seems to partially mimic the effects of cold exposure upon glucose homeostasis. β3-Adrenergic compounds such as CL-316,243 (Jost *et al.* 2002) and BRL 26830A (Rochet *et al.* 1988) stimulate glucose uptake by BAT and increase glucose clearance rates when used in humans and animal models of glucose intolerance. Cold exposure increases sympathetic tonus and this effect may play a central role in many of the physiological adaptations observed in the present model. For example, high sympathetic tonus reduces insulin secretion by pancreatic islets (Gilon & Henquin, 2001), augments BAT metabolic activity (Scarpace *et al.* 1996; Puigserver *et al.* 1998) and promotes an increase in glucose uptake by WAT (Moreno-Aliaga *et al.* 2002). Besides this, some other phenomena may contribute for this effect. Shivering thermogenesis is known to participate in body temperature control during acute exposure to cold (Smith & Davidson, 1982). Skeletal muscle glycogen is a major source of energy for shivering thermogenesis and blood glucose clearance is stimulated through this mechanism (Martineau & Jacobs, 1988). However, following thermal adaptation, such as in the model herein employed, shivering thermogenesis almost vanishes, and no longer participates in glucose homeostasis (Smith & Davidson, 1982). Other mechanisms that may participate to a certain degree in the control of glucose uptake in cold-exposed rats are blood levels of NEFA, hormones other than insulin, and body composition (Martineau & Jacobs, 1989; Haman *et al.* 2002).

Although several studies have evaluated the effects of cold exposure upon glucose homeostasis, insulin secretion, and other aspects related to energy metabolism in animals and humans (Vallerand *et al.* 1988, 1987; Liu *et al.* 1999; Haman *et al.* 2002), no previous research has investigated insulin signal transduction in tissues of cold-exposed animals. In the present report we have characterized some of the most important metabolic parameters of an animal model of exposure to cold, in parallel with a study of two branches of the insulin-signalling cascade in BAT, WAT, liver and skeletal muscle.

The animal model employed in our study matches most of the previous descriptions of the metabolic characteristics of rats exposed to cold (Cunningham *et al.* 1985; Vallerand *et al.* 1987; Torsoni *et al.* 2003), and the data obtained support the fact that cold-exposed animals are able to promote an increased turnover of glucose, even in the presence of low levels of insulin, and that much of this effect is certainly independent of the action of this hormone. Based on the 2-DG uptake data, we observed, as expected, that a more intense effect of cold exposure upon glucose mobilization occurred in BAT. In this tissue, there is a clear improvement of the initial steps of the insulin-signalling pathway. Nevertheless, in more distal steps of the pathway, specifically, insulin-induced serine phosphorylation of Akt and tyrosine phosphorylation of ERK, only a non-significant tendency towards augmentation promoted by cold exposure is seen. Considering that both Akt-dependent and -independent mechanisms participate in insulin-induced glucose uptake by insulin-sensitive tissues (Saltiel & Kahn, 2001), and that cold exposure promotes glucose uptake by insulin-independent mechanisms, the apparent positive regulation of the initial steps of insulin signalling in BAT may account for a fraction but not the whole of the increased BAT glucose uptake detected in this model.

In both WAT and skeletal muscle there is a significant positive effect of cold exposure upon glucose uptake. This effect may be observed either in non-insulin-stimulated animals, or following an acute insulin stimulus. However, insulin-signalling events in both tissues display a characteristic pattern of impaired signal transduction. The only molecular event that did not present negative regulation after cold exposure was insulin-induced IRS2 tyrosine phosphorylation in skeletal muscle. In spite of this fact, and even considering that the insulin-induced p85–PI3-kinase association with IRS2 was incremented by cold exposure as well (not shown), the more distal event of insulin-induced serine phosphorylation of Akt was negatively regulated by cold in muscle. Thus, as a whole it seems that in skeletal muscle and WAT there is a powerful effect of cold exposure upon insulin-dependent and -independent glucose uptake such that even in the face of clear negative regulation of the molecular steps of insulin signal transduction, higher glucose uptake occurs. The effect of cold exposure upon GLUT-4 expression and its subcellular distribution provides further support for an insulin-independent mechanism leading to higher glucose

uptake in WAT and skeletal muscle. According to the present study and to some previous observations (Shimizu *et al.* 1993; Lin *et al.* 1998), cold exposure promotes an increase in GLUT-4 expression, which is mostly due to an increased concentration of this transporter in the membrane fraction, independent of insulin action. There are, however, at least two physiological events that are traditionally under the influence of insulin signalling, which present clear signs of reduced insulin responsiveness in these tissues. These are lipolysis in WAT and glycogen accumulation in the skeletal muscle. In the present model a significant reduction of fat mass and a tendency for NEFA levels in the blood to increase were detected in cold-exposed rats, while the muscle glycogen content was similar in control and cold-exposed rats. Therefore, it seems that in WAT and skeletal muscle of cold-exposed rats, at least some of the tissue-specific functions regulated by insulin follow the expected pattern for a tissue with molecular resistance to insulin action.

In the liver, cold exposure led to a dichotomized effect concerning the functional response to insulin and the molecular activation of its signal transduction pathway. Thus, insulin-induced 2-DG uptake was not modified, while the liver glycogen content was significantly increased in cold-exposed rats. On the other hand, the insulin-induced tyrosine phosphorylation of IR and IRS2 was significantly increased, and tyrosine phosphorylation of IRS1 was noticeably but non-significantly increased by cold exposure, whilst insulin-induced serine phosphorylation of Akt was significantly reduced in cold-exposed rats. ERK tyrosine phosphorylation was similar in control and cold-exposed rats.

In conclusion, cold-exposed rats are able to mobilize glucose more efficiently than controls, even presenting a clear pattern of molecular resistance to insulin in at least two tissues that act as important targets for insulin action, WAT and skeletal muscle. It is interesting to notice that in certain models of insulin resistance a similar pattern of modulation of insulin signal transduction leads to impaired glucose uptake (Saad, 1994); thus, molecular impairment of the insulin signalling machinery may co-exist with normal or increased glucose uptake. The effects of cold upon insulin signalling are tissue specific and within every tissue cold effects seem to be function-specific, in such a way that they may positively influence some responses controlled by insulin and negatively influences others. Since increased sympathetic tonus is a major characteristic of cold-exposed rats we believe that further characterization of molecular cross-talk between insulin and adrenergic receptors (Klein *et al.* 1999; Paez-Espinosa *et al.* 2001) may prove helpful in advancing the understanding of glucose homeostasis in cold-exposed animals and disclosing new potential targets for therapeutics in diabetes mellitus.

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4.2. Artigo II

"β- Adrenergic-dependent and – independent mechanisms participate in cold-induced modulation of insulin signal transduction in brown adipose tissue of rats."

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ENDOCRINOLOGY

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β3-Adrenergic-dependent and -independent mechanisms participate in cold-induced modulation of insulin signal transduction in brown adipose tissue of rats

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Abstract During cold exposure, homeothermic animals mobilize glucose with higher efficiency than at thermoneutrality. An interaction between the insulin signal transduction machinery and high sympathetic tonus is thought to play an important role in this phenomenon. In the present study, rats were exposed to cold during 8 days and treated, or not, with a β3-adrenergic agonist, BRL37344 sodium 4-2-2-(3-chlorophenyl)-2-hydroxyethyl amino propyl phenoxy-acetic acid sodium (BRL37344), or antagonist, SR59230A 3-(2-ethylphenoxy)-[(1S)-1,2,3,4-tetrahydronaphth-1-ylamino]-(2S)-2-propanol oxalate (SR59230A), to evaluate the cross-talk between insulin and β3-adrenergic intracellular signaling in brown adipose tissue. The drugs did not modify food ingestion, body temperature, and body weight in control and cold-exposed rats. Treatment of control rats with BRL37344 led to higher insulin-induced tyrosine phosphorylation of the insulin receptors, insulin receptor substrate (IRS)-1 and ERK, higher insulin-induced IRS-1/PI3-kinase association, and higher [Ser^{473}] phosphorylation of Akt. Cold exposure alone promoted higher insulin-induced tyrosine phosphorylation of the insulin receptors, IRS-1, IRS-2, and ERK, and higher insulin-induced IRS-1 and IRS-2/PI3-kinase association. Except for the regulation of ERK, SR59230A abolished all the cold-induced effects upon the insulin signal transduction pathway. However, this antagonist only partially inhibited the cold-induced increase of glucose uptake. Thus, the sympathetic tonus generated during cold-exposure acts, in brown adipose tissue, through the β3-adrenergic receptor and modulates insulin signal transduction, with the exception of ERK. However, insulin-independent mechanisms other than β3-adrenergic activation participate in cold-induced glucose uptake in brown adipose tissue of rats.

Keywords Akt · β 3-Adrenergic receptor · ERK · Glucose · Insulin

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Introduction

The characterization of insulin-independent mechanisms that play a role in glucose uptake is of major interest for unveiling novel potential targets for therapeutics in diabetes mellitus. Cold exposure of homeothermic animals leads to improved glucose uptake in spite of an apparent resistance to insulin action [8, 29, 30]. During cold exposure, a high adrenergic tonus stimulates lipolysis in white adipose tissue and thermogenesis in brown adipose tissue (BAT) [7, 15, 20, 28]. The adrenergic signals generated during cold exposure may also participate in the control of glucose flow by modulating insulin action and perhaps activating insulin-independent mechanisms of glucose uptake [4, 13, 17, 23]. In a recent series of studies, we have provided evidence that cold exposure induces tissue-specific modulation of the insulin signal transduction [6, 8, 25]. In BAT, cold exposure leads to improved glucose uptake accompanied by enhanced insulin-induced tyrosine phosphorylation of the insulin receptor (IR) and its main intracellular substrates (S), IRS-1 and IRS-2 [8]. However, in this particular situation, no modulation of [Ser^{473}] phosphorylation of Akt was observed, suggesting that Akt-independent mechanisms may play a role in BAT glucose uptake during cold exposure [8].

In isolated, SV40-imortalized brown adipocytes, β3-adrenergic stimulation with CL316243 did not improve basal glucose uptake and led to a hampering of the insulin signal transduction and action, apparently through a PKA/PKC-dependent mechanism [10]. Since most of the effects of cold exposure on BAT are credited

to β3-adrenergic stimulation, it is unclear why, in isolated brown adipocytes, the β3-adrenergic stimulus opposes insulin action, while in living animals cold exposure promotes enhanced glucose uptake and improvement of the initial steps of the insulin signal transduction in BAT [8]. Therefore, the objective of the present study was to evaluate the participation of β3-adrenergic receptor activity in cold-induced glucose uptake and insulin signal transduction in BAT of living rats, using in vivo treatment protocols with the β3-adrenergic agonist, BRL37344 sodium 4-2-2-(3-chlorophenyl)-2-hydroxyethyl amino propyl phenoxy-acetic acid sodium (BRL37344), and the β3-adrenergic antagonist, SR59230A 3-(2-ethylphenoxy)-[(1S)-1,2,3,4-tetrahydronaphth-1-ylamino]-(2S)-2-propanol oxalate (SR59230A).

Materials and methods

Antibodies, chemicals, and buffers

Reagents for SDS-polyacrylamide gel electrophoresis and immunoblotting were from Bio-Rad (Richmond, Calif., USA). HEPES, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol, bovine serum albumin (fraction V), selective β3-adrenoceptor agonist BRL37344, and selective β3-adrenoceptor antagonist SR59230A were from Sigma-Aldrich (St. Louis, Mo., USA). Protein A-Sepharose 6 MB was from Pharmacia (Uppsala, Sweden), [^{125}I]-protein A was from ICN Biomedicals (Costa Mesa, Calif., USA), 2-deoxy-D-[^{3}H]glucose was from New England Nuclear (Boston, Mass., USA), and nitrocellulose paper (BA85, 0.2 μm) was from Amersham (Aylesbury, UK). Sodium thiopental and human recombinant insulin were from Novo Nordisk (Belo Horizonte, Brazil). Polyclonal anti-phosphotyrosine antibodies were raised in rabbits and affinity-purified on phosphotyramine columns. Anti-IR (rabbit polyclonal, sc-711), anti-phospho-ERK (Tyr 204, detecting pERK42 and pERK44; goat polyclonal, sc-7976), anti-phospho-Akt (Ser 473, rabbit polyclonal, sc-7985-R), anti-IR substrate-1 (IRS-1) (rabbit polyclonal, sc-560), anti-IR substrate-2 (IRS-2) (goat polyclonal, sc-9299), anti-Akt (goat polyclonal, sc-1618), anti-ERK2 (rabbit polyclonal, detecting both ERK42 and ERK44, sc-153), anti-β3-adrenergic receptor (goat polyclonal, sc-1473), and anti-GLUT-4 (goat polyclonal, sc-1608) were from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Rabbit anti-p85/phosphatidylinositol 3 kinase (PI3 kinase) antiserum was from UBI (Lake Placid, N.Y., USA).

Animals, treatments, and cold-exposure protocols

Male Wistar rats (*Rattus norvegicus*, 8 weeks old, 200–300 g) obtained from the University of Campinas Animal Breeding Center were used in the experiments. The investigation followed the University guidelines for the use of animals in experimental studies and conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health [(NIH) publication no. 85-23, revised 1996]. The animals were maintained on 12:12 artificial light-dark cycles and housed in individual cages. After the acclimatizing period (3 days), the animals were randomly divided into six groups and treated during 8 days, according to one of the following protocols: cold-exposed (4 ± 1°C) treated with saline, cold-exposed treated with BRL37344, cold-exposed treated with SR59230A, thermoneutrality-maintained (23 ± 1°C) treated with saline, thermoneutrality-maintained treated with BRL37344, and thermoneutrality-maintained treated with SR59230A. The drugs were administered via intraperitoneal injection twice a day for 8 days at the following doses: 150 nmol/dose in 200 μl for BRL37344, 85 nmol/dose in 200 μl SR59230A, or 200 μl saline/dose. The animals were allowed free access to standard rodent chow and water ad libitum. For tissue extraction, rats were anesthetized by intraperitoneal injection of sodium amobarbital (15 mg/kg body weight), and the experiments were performed after the loss of corneal and pedal reflexes. Following experimental procedures, the rats were killed under anesthesia following the recommendations of the NIH publication no. 85-23.

Metabolic, hormone, and biochemical measurements

Measurements of food intake, rectal temperature, and body weight were obtained daily in control and cold-exposed rats treated with saline, BRL37344, or SR59230A. Rectal temperature was measured with a Thermistor high-precision digital thermometer (Hanna Instruments, Woonsocket, R.I., USA) inserted 1.5 cm from the anus. Glucose was determined by the glucose oxidase method, as previously described [26]. Serum insulin was measured by radioimmunoassay [22].

Intraperitoneal glucose tolerance test

An intraperitoneal glucose tolerance test (ipGTT) was performed at the end of the experimental period. After an overnight fast, the rats were anesthetized as described above. After collection of an unchallenged sample (time 0), a solution of 20% glucose (2.0 g/kg body weight) was administered into the peritoneal cavity. Blood samples were collected from the tail at 30, 60, 90, and 120 min for determination of glucose and insulin concentrations.

2-D glucose uptake studies

The tissue uptake of 2-D glucose (2-DG) was measured in vivo according to a method previously described [27], with minor modifications [8]. The rats were anesthetized

and then injected with 6.0 μCi of 2-deoxy-D-[^{3}H] glucose, [^{14}C] sucrose, and 0.1 U insulin in 0.4 ml isotonic phosphate buffer, pH 7.4, with 0.1% defatted bovine serum albumin, through the portal vein. After 16 min, slices of interscapular BAT were quickly removed and solubilized in NCS-II Tissue Solubilizer (Amersham, Bucks, England). The radioactivity of [^{3}H] in the resulting supernatant was measured in a liquid scintillation fluid (ACS-II Amersham-Japan, Tokyo), using a scintillation counter (Aloka, Model LSC-1000, Kyoto). The results were expressed as cpm/mg tissue/weight. Cellular uptake of 2-DG was calculated as the difference between the total tissue radioactivity and the amount of radioactivity present in the tissue extracellular space. The cellular radioactivity was then converted to picomoles of 2-DG, using the specific activity, and the results were expressed per milligram of dry tissue.

Tissue extraction, immunoblotting, and immunoprecipitation

The abdominal cavity of each anesthetized rats was opened, and insulin (0.2 ml, 10^{-6}M) or saline (0.2 ml) was injected through the cava vein. After different intervals (described in the legends of the Figures), fragments (3.0×3.0×3.0 mm) of BAT were excised and immediately homogenized in solubilization buffer at 4°C [1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM PMSF, and 0.1 mg aprotinin/ml] with a Polytron PTA 20S generator (model PT 10/35, Brinkmann Instruments, Westbury, N.Y.) operated at maximum speed for 30 s. Insoluble material was removed by centrifugation for 20 min at 11,000 rpm in a 70.Ti rotor (Beckman) at 4°C. The protein concentration of the supernatants was determined by the Bradford dye binding method. Aliquots of the resulting supernatants containing 5.0 mg of total protein were used for immunoprecipitation with antibodies against IR, IRS-1, and IRS-2 at 4°C overnight, followed by SDS/PAGE, transfer to nitrocellulose membranes, and blotting with anti-phosphotyrosine, anti-IR, anti-IRS-1, anti-IRS-2, or anti-p85/PI3-kinase. In direct immunoblot experiments, 0.2 mg protein extract obtained from each tissue was separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-IR, -IRS-1, -IRS-2, -Akt, -ERK, -GLUT-4, -phospho-Akt, or -phospho-ERK antibodies as described [31].

Data presentation and statistical analysis

All numerical results are expressed as the mean ± SEM of the indicated number of experiments. The results of blots for specific protein amount measurements are presented as direct comparisons of bands in autoradiographs and quantified by densitometry, using the ScionCorp software (Scion Image). The results of blots for determination of protein phosphorylation and protein–protein interaction are presented as a stoichiometric relation to the total amount of the specific protein. The mean densitometric values obtained from control rats treated with insulin are set as 100%. Student's t-test for unpaired samples and analysis of variance for multiple comparisons were used for statistical analysis as appropriate. The level of significance was set at $P < 0.05$.

Results

Biological consequences of treatment with BRL37344 and SR59230A

Two daily doses of the compounds BRL37344 and SR59230A during 8 days promoted no significant changes in body-weight variation, food intake, and body temperature within control or cold-exposed groups (Fig. 1). As expected [8], cold exposure, per se, induced body-weight loss during the first two experimental days, while food ingestion was significantly increased (19.8 ± 0.4 vs 29.6 ± 0.5 g/day, $n = 8$, $P < 0.05$, for control and cold-exposed rats, respectively). As a whole, rats of cold-exposure groups were lighter at experimental days 5, 6, and 7 and had higher daily food intake at days 4, 5, and 6, as compared to rats from control groups.

Glucose and insulin levels during the treatment with the β3 active compounds

At the end of the experimental period, there were no differences in the fasting blood glucose levels between the groups. However, during an ipGTT, there was a significant ($P < 0.05$) reduction of glucose levels in control rats treated with BRL37344 (Fig. 2a) and a significant ($P < 0.05$) increase of glucose levels in cold-exposed rats treated with SR59230A (Fig. 2b). Fasting blood insulin levels were lower in cold-exposed rats (1.4 ± 0.4 ng/ml vs 3.3 ± 0.9 ng/ml for cold-exposed and control, respectively, $n = 5$, $P < 0.05$), but no significant effect was exerted by any of the drugs.

The effects of the β3 active compounds on the early and intermediate steps of the insulin signal transduction pathway in BAT

Neither cold exposure nor the treatment with BRL37344 or SR59230A promoted significant changes in protein amounts of IR in BAT (Fig. 3a). The treatment of control rats with BRL37344 induced a significant increase of insulin-stimulated tyrosine phosphorylation of IR (Fig. 3b). However, this increase (two-fold) was of a smaller magnitude than the one induced by cold exposure alone (4.2-fold, Fig. 3b, control/BRL37344/+insulin vs cold-exposed/+insulin,

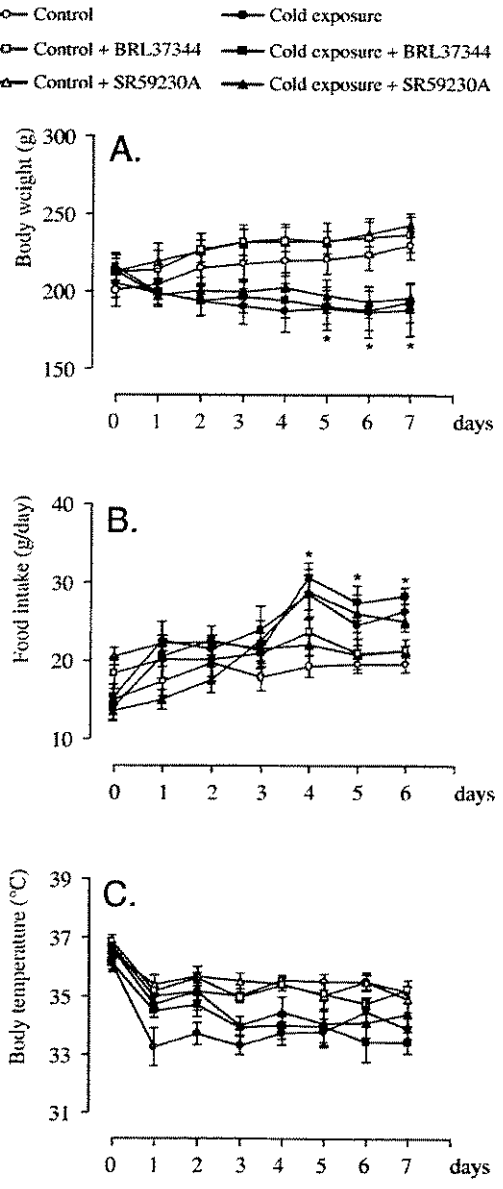

Fig. 1 Metabolic characterization of rats exposed to cold. Body weight (**a**), daily food intake (**b**), and body temperature (**c**) were determined in control (*empty symbols*) and cold-exposed rats (*filled symbols*) treated, or not, with BRL37344 or SR59230A, according to the methods described in the text. Results are expressed as mean ± SEM, $n = 8$, and $P < 0.05$ vs control. Cold-exposed and cold-exposed rats treated with BRL37344 or SR59230A were different from control at days 5, 6, and 7 (**a**) and at days 4, 5, and 6 (**b**)

$P < 0.05$). The administration of SR59230A to control rats induced no modulation of basal and insulin-induced tyrosine phosphorylation of the IR. When cold-exposed rats were treated with SR59230A, a significant fall in the insulin-induced tyrosine phosphorylation of IR was observed (Fig. 3b). Finally, the treatment of cold-exposed rats with BRL37344 induced a significant fall in the insulin-induced tyrosine phosphorylation of IR as compared to cold-exposed rats not submitted to any drug treatment (Fig. 3b).

Both BRL37344 and SR59230A treatments led to a significant fall in IRS-1 protein expression in BAT of cold-exposed rats (Fig. 4a). BRL37344, but not SR59230A, induced a significant increase in insulin-induced tyrosine phosphorylation of IRS-1 in control rats (Fig. 4b). In cold-exposed rats, the treatment with either BRL37344 or SR59230A led to a significant fall in insulin-induced tyrosine phosphorylation of IRS-1, as compared to the effect of cold exposure alone (Fig. 4b). All the effects of the $\beta 3$ active compounds upon insulin signal transduction through IRS-1 were also observed on IRS-1/p85/PI3-kinase association. Thus, BRL37344 was capable of inducing a significant increase of insulin-induced IRS-1/p85/PI3-kinase association in BAT of control rats (Fig. 4c). Both BRL37344 and SR59230A led to a significant fall in insulin-induced IRS-1/p85/PI3-kinase association in BAT of cold-exposed rats (Fig. 4c).

Cold exposure promoted a significant increase of IRS-2 expression in BAT (Fig. 5a). This effect was completely abolished by treatment with BRL37344 or SR59230A (Fig. 5a). BRL37344 and SR59230A treatments did not modulate basal or insulin-induced tyrosine phosphorylation of IRS-2 and the association of IRS-2 with PI3-kinase in BAT of control rats (Fig. 5b, c). However, both drugs significantly reduced insulin-induced tyrosine phosphorylation of IRS-2 and IRS-2/p85/PI3-kinase association in BAT of cold-exposed rats (Fig. 5b, c).

Insulin induces the [Ser^{473}] phosphorylation of Akt through IRS-1 or IRS-2 engagement of PI3-kinase. Cold-exposure and treatment with $\beta 3$-adrenergic-active compounds did not modulate Akt protein expression in BAT (Fig. 6a). Nevertheless, BRL37344, but not SR59230A, was capable of significantly increasing insulin-induced [Ser^{473}] phosphorylation of Akt in control rats (Fig. 6b). In cold-exposed rats, SR59230A, but not BRL37344, significantly reduced insulin-induced [Ser^{473}] phosphorylation of Akt (Fig. 6b). Cold exposure per se did not promote any further increase of insulin-induced [Ser^{473}] phosphorylation of Akt, as compared to the insulin-treated control (Fig. 6b).

The p42 and p44 isoforms of ERK may be stimulated by insulin through at least three different intermediates: IRS-1, IRS-2, and Shc [21]. ERK may also be directly stimulated by adrenergic signals acting through Gi protein and c-Src [1]. In the present study, cold-exposure and treatment with $\beta 3$-adrenergic-active compounds did not modulate ERK protein expression in BAT (Fig. 6c). Nevertheless, BRL37344, but not SR59230A, was able to significantly increase insulin-induced, but not basal, tyrosine phosphorylation of ERK (42 kDa and 44 kDa) in control rats (Fig. 6d). In contrast to the other proteins herein evaluated, neither BRL37344 nor SR59230A was able to modulate insulin-induced ERK tyrosine phosphorylation in cold-exposed rats (Fig. 6d).

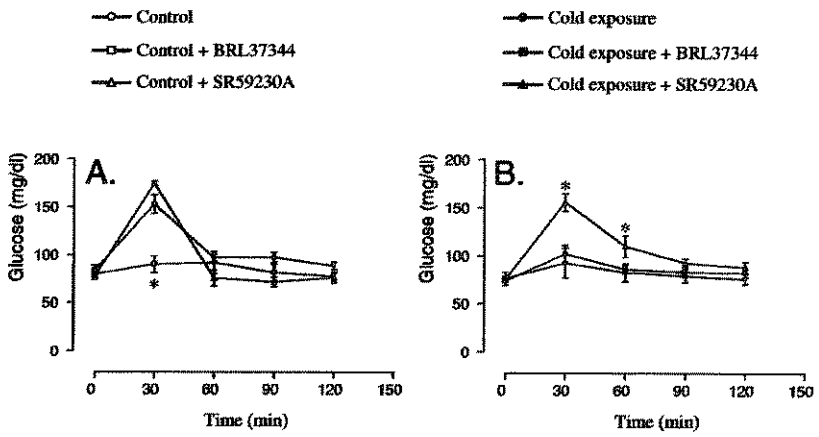

Fig. 2 Glucose levels during the intraperitoneal glucose tolerance test. After an overnight fast, the rats were anesthetized and an unchallenged blood sample (time 0) was collected. A solution of 20% glucose (2.0 g/kg body weight) was administered into the peritoneal cavity. Blood samples were collected from the tail at 30, 60, 90, and 120 min for determination of glucose concentrations [$n=5$, $P<0.05$ vs control (**a**) or vs cold-exposed (**b**)]

Regulation of β3-adrenergic receptor and GLUT-4 expression in BAT

Cold exposure and treatment with BRL37344 or SR59230A promoted significant increase of β3-adrenergic receptor protein expression in BAT (3.7-fold, 4.1-fold, and 3.9-fold, respectively, Fig. 7a). Both BRL37344 and SR59230A produced a tendency (not significant) to reduce β3-adrenergic receptor expression in BAT of cold-exposed rats (Fig. 7a). Concerning the expression of GLUT-4, significant increases of expression were detected in cold-exposed (3.1-fold), BRL37344- (2.3-fold), and SR59230A- (2.2-fold) treated rats (Fig. 7b). Both compounds significantly inhibited the effect of cold-exposure to increase GLUT-4 expression (Fig. 7b).

β3-adrenergic antagonism partially inhibits cold-induced glucose uptake by BAT

The effect of β3-adrenergic stimulus upon BAT glucose uptake was evaluated in cold-exposed rats treated or not with BRL37344 and SR59230A. As depicted in Fig. 8, the exposure to cold promoted a significant increase (2.4-fold, $P<0.05$) of insulin-induced glucose uptake by BAT; treatment with BRL37344 did not modulate this effect; however, treatment with the β3 antagonist SR59230A was capable of reducing significantly, but not completely, glucose uptake by this tissue.

Discussion

In the present study, the role of β3-adrenergic signaling upon the activity of the insulin signal transduction machinery was evaluated in BAT of rats exposed to cold. The molecular cross-talk between adrenergic and insulin signaling systems has been known for quite some

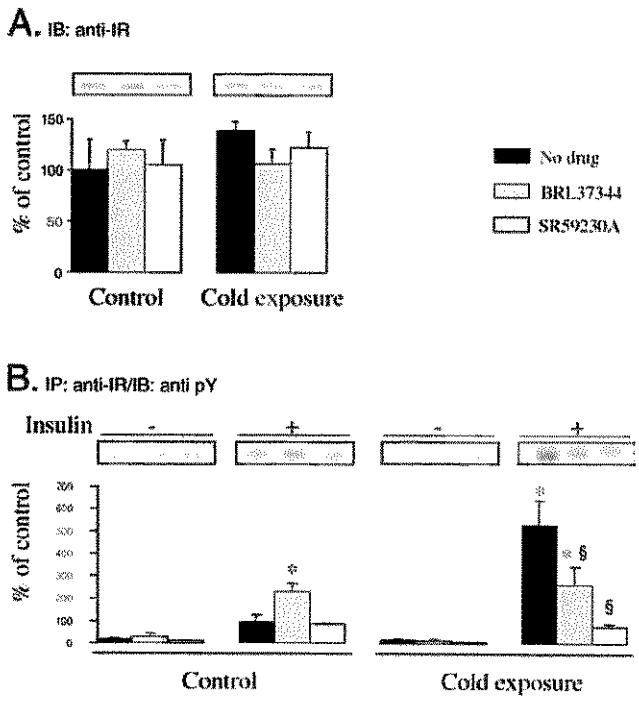

Fig. 3 Insulin receptor (IR) expression and tyrosine phosphorylation. The protein levels of the IR (**a**) were evaluated by direct immunoblot of brown adipose tissue (BAT) total protein extracts obtained from control and cold-exposed rats treated, or not, with BRL37344 or SR59230A. For this, BAT was homogenized, and proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted (*IB*) with anti-insulin receptor (*anti-IR*) antibodies. For evaluation of insulin-induced tyrosine phosphorylation (**b**), rats from each group were acutely treated i.v. with saline (−) or insulin (+) (0.2 ml, 10^{-6} M), and BAT fragments were obtained, homogenized, and used in immunoprecipitation (*IP*) experiments with anti-IR antibodies. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-phosphotyrosine antibodies (*anti-pY*). Data are presented as the mean ± SEM, $n=6$, and * $P<0.05$ vs control treated with insulin; § $P<0.05$ vs cold-exposed treated with insulin

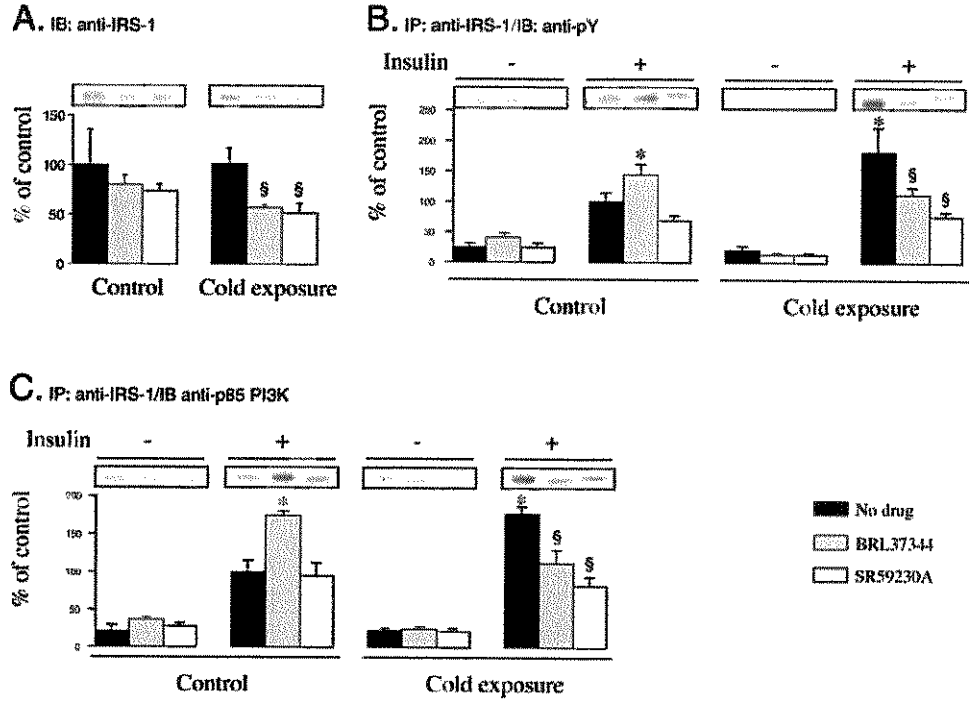

Fig. 4 IR substrate-1 (*IRS-1*) expression, tyrosine phosphorylation and association with phosphatidylinositol 3 (PI3) kinase. The protein levels of the IRS-1 (**a**) were evaluated by direct immunoblot of BAT total protein extracts obtained from control and cold-exposed rats treated, or not, with BRL37344 or SR59230A. For this, BAT was homogenized, and proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted (*IB*) with anti-IR substrate-1 (*anti-IRS-1*) antibodies. For evaluation of insulin-induced tyrosine phosphorylation (**b**), rats from each group were acutely treated i.v. with saline (−) or insulin (+) (0.2 ml, 10^{-6} M), and BAT fragments were obtained, homogenized, and used in IP experiments with anti-IRS-1 antibodies. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-pY antibodies. For evaluation of IRS-1 association with PI3 kinase (**c**), nitrocellulose membranes harboring IRS-1 immunoprecipitates were blotted with anti-p85/PI3 kinase antibodies (*anti-p85 PI3K*). Data are presented as the mean ± SEM, $n=6$, and * $P<0.05$ vs control treated with insulin; § $P<0.05$ vs cold-exposed treated with insulin

time [9]. Most clinical and experimental data concerning this issue suggest that adrenergic stimulus exerts a negative regulatory role upon insulin action [9, 24]. However, since the characterization of the β3-adrenergic receptor, novel and stimulating concepts on this interaction were revealed [12, 16, 34]. In this study, we observed that treatment of rats during 8 days with selective β3-adrenergic agonist or antagonist compounds did not modify food ingestion, body weight, and body temperature. The fasting blood levels of glucose and insulin were not modified by the treatments; however, glucose levels during a GTT were reduced by the β3-adrenergic agonist in control rats and increased by the β3-adrenergic antagonist in cold-exposed rats. In a previous study, the treatment of obese, diabetic ZDF rats during 14 days with the β3-adrenergic agonist CL 316243 caused a significant weight loss accompanied by reduction of food ingestion [14]. In the same study, no significant modification of body weight and food ingestion was detected in the lean, control group. In addition, no significant modulation of basal insulin and only marginal reduction of basal glucose levels were observed [14]. All these findings are in agreement with the results of the present study and demonstrate that most pharmacological effects of β3 agonists are detectable at the clinical level only in obese and diabetic animals.

Next, we evaluated the effects of the β3-adrenergic active compounds upon insulin signal transduction in BAT. The compounds did not modify IR, Akt, and ERK expression in control and cold-exposed rats, but significantly reduced IRS-1 and IRS-2 protein expression in cold-exposed animals. Since IRS proteins may be engaged by signal transduction through several G-protein coupled receptors [2, 24, 32], and since the rate of synthesis and degradation of proteins is directly influenced by the proteins' usage, we believe that the high adrenergic tonus generated during cold-exposure might prime IRS-1 and IRS-2. When the animals are treated with the β3-adrenergic active compounds, a modification on synthesis or degradation process may occur. That seems to be the case for the β3-adrenergic receptor as well. Cold-exposure alone or the treatment of control rats with both the β3-adrenergic-active compounds induced a significant increase of the receptor expression. However, in cold-exposed rats, BRL37344 and SR59230A tended to inhibit the effect of cold-exposure upon the receptor expression.

Next we observed that, as a rule, in control rats, the treatment with BRL37344 led to higher insulin-induced

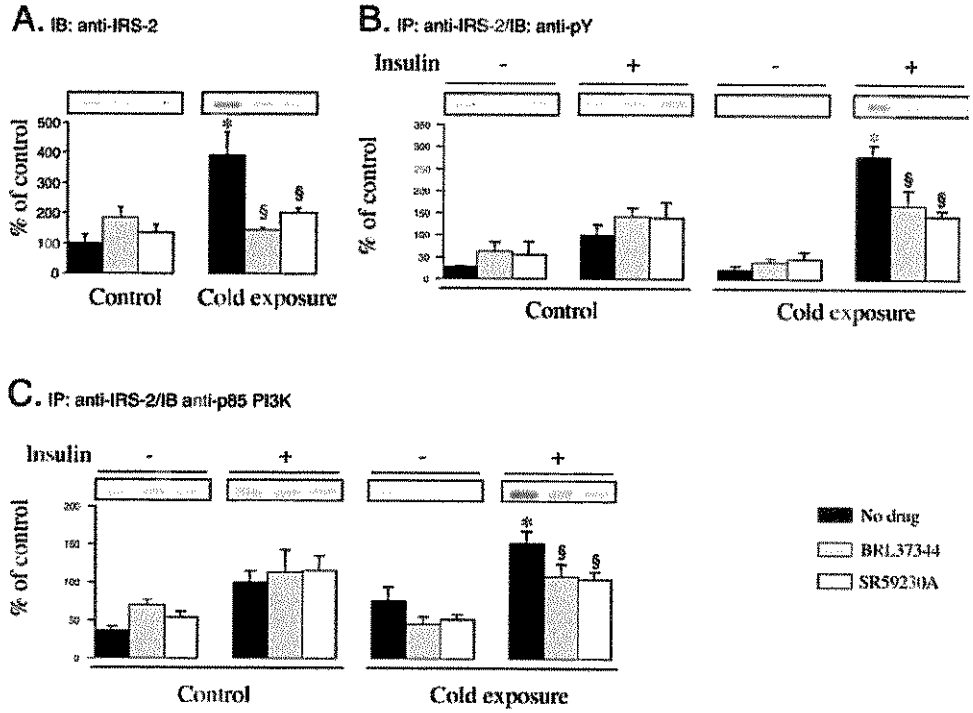

Fig. 5 IRS-2 expression, tyrosine phosphorylation, and association with PI3 kinase. The protein levels of IRS-2 (a) were evaluated by direct immunoblot of BAT total protein extracts obtained from control and cold-exposed rats treated, or not, with BRL37344 or SR59230A. For this, BAT was homogenized, and proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-IRS-2 antibodies. For evaluation of insulin-induced tyrosine phosphorylation (b), rats from each group were acutely treated i.v. with saline (–) or insulin (+) (0.2 ml, 10^{-6} M), and BAT fragments were obtained, homogenized, and used in IP experiments with anti-IRS-2 antibodies. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-pY antibodies. For evaluation of IRS-2 association with PI3 kinase (c), nitrocellulose membranes harboring IRS-2 immunoprecipitates were blotted with anti-p85/PI3K. Data are presented as the mean ± SEM, $n = 6$, and* $P < 0.05$ vs control treated with insulin; § $P < 0.05$ vs cold-exposed treated with insulin

tyrosine phosphorylation of IR and IRS-1 but exerted no effect on IRS-2. Increased insulin-induced IRS-1 association with PI3-kinase accompanied the higher insulin-induced tyrosine phosphorylation of IRS-1 in control rats. On the other hand, SR59230A exerted no effect on insulin-induced events in BAT of control rats. In cold-exposed rats, both BRL37344 and SR59230A promoted significant inhibition of insulin-induced tyrosine phosphorylation of IR, IRS-1, and IRS-2, and of insulin-induced association of IRS-1 and IRS-2 with PI3-kinase. BRL37344 was also effective to enhance insulin-induced serine phosphorylation of Akt and tyrosine phosphorylation of ERK. However, in cold-exposed rats, insulin-induced serine phosphorylation of Akt was only marginally inhibited by SR59230A. No effect of the compounds was observed on insulin-induced ERK phosphorylation in cold-exposed rats. The fact that SR59230A reversed the cold-induced sensitization to the insulin signal strongly suggests that this effect is, at least in part, mediated by a β3-adrenergic dependent mechanism. Conversely, we believe that the inhibitory effect observed with BRL37344 may be due to signal desensitization.

The chronic treatment with the β3 agonist BRL37344 sensitizes insulin signaling through all except one (IRS-2) protein tested in BAT of control rats. These data oppose the findings reported by Klein and coworkers [10] in isolated, immortalized brown adipocytes and suggest that either cultured adipocytes may lose some of its original characteristics, or perhaps, other neural phenomena present in the undisrupted tissue may modulate the β3-adrenergic signal. Of course, since β3 receptors are expressed in other tissues, most importantly in white adipose tissue [12], we cannot exclude the possibility that indirect mechanisms might play a role in the effect induced by BRL37344. Among indirect factors, β3-adrenergic-induced, or cold-induced regulation of production of adipokines or cytokines known to exert a regulatory role upon insulin signaling has been reported [18, 19]. Moreover, even cold-induced hypoinsulinemia may participate in this complex regulation.

The β3-adrenergic stimulus promoted an impressive sensitizing effect upon the insulin-induced tyrosine phosphorylation of ERK and serine phosphorylation of Akt in control rats. Concerning ERK, a similar phenomenon has been previously described as a consequence of insulin and β2-adrenergic cross-talk [33]. Nevertheless, this is the first time that such effect is reported for insulin and β3-adrenergic cross-talk. It is

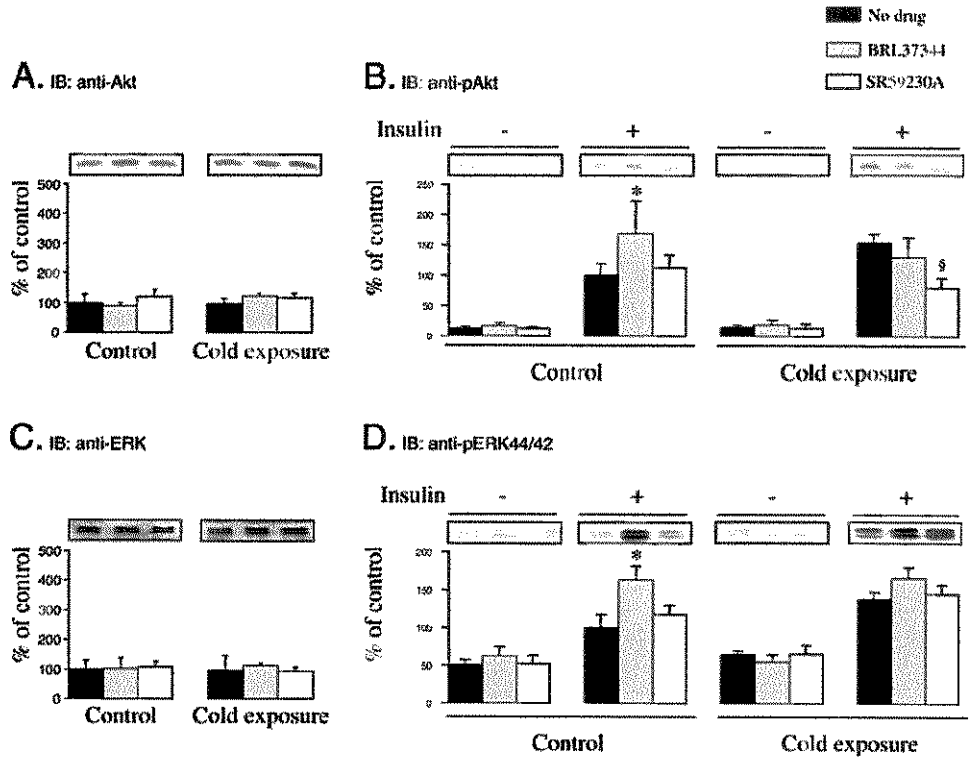

Fig. 6 Akt and ERK expression, [Ser^{473}] phosphorylation of Akt, and tyrosine phosphorylation of ERK. The protein levels of Akt (**a**) and ERK (**c**) were evaluated by direct immunoblot of BAT total protein extracts obtained from control and cold-exposed rats treated, or not, with BRL37344 or SR59230A. For this, BAT was homogenized, and proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-Akt (**a**) or anti-ERK (**c**) antibodies. For evaluation of insulin-induced [Ser^{473}] phosphorylation of Akt (**b**) and tyrosine phosphorylation of the 42-kDa and 44-kDa isoforms of ERK (**d**), rats from each group were acutely treated i.v. with saline (–) or insulin (+) (0.2 ml, 10^{-6} M). BAT fragments were obtained, homogenized, separated by SDS-PAGE, transferred to nitrocellulose membranes, and used in IB experiments with anti-phospho-[Ser^{473}] Akt (*anti-pAkt*) (**b**) or anti-phospho-ERK (*anti-pERK 44/42*) (**d**) antibodies. Data are presented as the mean ± SEM, $n = 6$, and * $P < 0.05$ vs control treated with insulin; § $P < 0.05$ vs cold-exposed treated with insulin

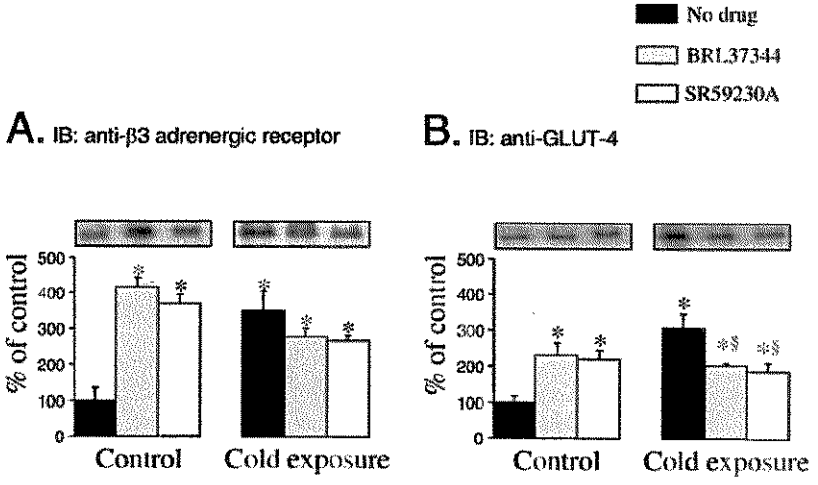

Fig. 7 β3-Adrenergic receptor and GLUT-4 expression in BAT. The protein levels of the β3-adrenergic receptor (**a**) and GLUT-4 (**b**) were evaluated by direct immunoblot of BAT total protein extracts obtained from control and cold-exposed rats treated, or not, with BRL37344 or SR59230A. For this, BAT was homogenized, and protein was separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-β3-adrenergic receptor (**a**) or anti-GLUT-4 (**b**) antibodies. Data are presented as the mean ± SEM $n = 6$, and * $P < 0.05$ vs control; § $P < 0.05$ vs cold-exposed

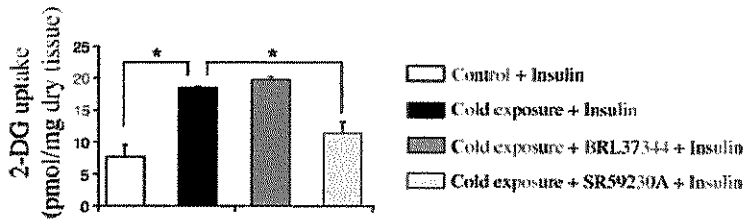

Fig. 8 Insulin-stimulated glucose uptake by brown adipose. 2-[^{3}H] deoxyglucose uptake in BAT of insulin-stimulated, cold-exposed rats, treated, or not, with BRL37344 or SR59230A and compared to control, insulin-stimulated rats. The technical procedure is described under "Materials and methods." Results are expressed as mean ± SEM: $n = 5$, * $P < 0.05$

interesting to notice that, in cold-exposed rats, differently of the inhibitory effect of the β3-adrenergic compounds upon insulin signal transduction through the IRSs and Akt, no effect was observed for ERK. This might be due to the multiplicity and robustness of the mechanisms that lead to ERK activation, which can be exemplified, in BAT, by the different regulation of UCP-1 expression, by insulin and β3-adrenergic stimulus [11].

To evaluate if the molecular phenomena described above would be translated into functional events controlled by insulin, we measured GLUT-4 protein expression and in vivo insulin-induced glucose uptake by BAT. Cold-exposure and the treatment with BRL37344 or SR59230A promoted a significant increase of GLUT-4 expression. However, both compounds significantly inhibited cold-induced hyperexpression of GLUT-4. Once more, a cold-induced desensitization of the β3-adrenergic signal seems to operate. Finally, cold-exposure significantly increased insulin-induced glucose uptake. This effect was not modulated by BRL37344 but was partially inhibited by SR59230A. In isolated brown adipocytes, β3-adrenergic stimulation inhibits insulin-induced glucose uptake [10]. However, in living rats, β3-adrenergic stimulus is known to increase glucose uptake [5] and, according to our data, it becomes clear that only part of the cold-dependent increase of glucose uptake by BAT is due to β3-adrenergic stimulation.

In conclusion, the present data evidence a positive cross-talk between the insulin and β3-adrenergic signaling systems in BAT of rats. This cross-talk responds to part of the effect of cold upon insulin signaling in BAT. The remaining of the effect of cold may be delivered by other adrenergic receptors, as recently demonstrated [3], and perhaps by other circulating factors.

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5. DISCUSSÃO GERAL

Diabetes mellitus tipo 2 é uma doença crônica e progressiva que associada a condições como hipertensão, aterosclerose, falência renal, neuropatias e outras, é responsável por elevados índices de mortalidade em várias regiões do mundo (ZIMMET et al., 2001). Resistência à insulina e falência secundária das células beta-pancreáticas desempenham um importante papel no desenvolvimento destas doenças (MATHIS et al., 2001; SALTIEL e KAHN, 2001). Entretanto, os eventos que levam aos defeitos de sinalização e secreção da insulina, bem como os detalhes sobre mecanismos moleculares que participam na patogênese do diabetes não estão completamente elucidados.

Diversos grupos têm caracterizado os mecanismos moleculares de resistência à insulina em diferentes modelos animais e em humanos com diabetes ou intolerância à glicose. Diferentes defeitos de sinalização foram identificados, tais como parcial ou completa diminuição da expressão de algum componente protéico da sinalização da insulina (FLIER, 1983; CARVALHO et al., 1996; SAAD et al., 1997; MAUVAIS-JARVIS et al., 2002; BERTELLI et al., 2003), redução da fosforilação em tirosina (ou serina para Akt) de proteínas que participam da cascata de sinalização da insulina (SAAD et al., 1993; VELLOSO et al., 1996), aumento da atividade catalítica das fosfatases tirosina atuando na via de sinalização da insulina (TANTI et al., 1991; HAUGUEL-DE MOUZON et al., 1993), aumento da atividade serina quinase de algumas enzimas chave (WHITE, 2002), além de defeitos genéticos que interferem com o funcionamento adequado de elementos da sinalização insulínica (ALMIND et al., 1996; PEDERSEN, 1999).

Assim, muito têm sido feito para caracterizar os mecanismos moleculares por meio dos quais se instala a resistência à insulina (SAAD, 1994; SALTIEL e KAHN, 2001; WHITE, 2002), e principalmente para entender os mecanismos que levam a uma melhora da captação de glicose com a promessa de encontrar novos alvos moleculares para ação de drogas com potencial uso terapêutico em diabetes mellitus e distúrbios associados.

A exposição de animais homeotérmicos ao frio leva a uma situação única, caracterizada por diminuição da secreção da insulina basal ou induzida por glicose, acompanhada de uma mobilização aumentada de glicose e uma melhora na resposta à ação da insulina (SMITH e DAVIDSON, 1982; VALLERAND et al., 1983, 1987). De acordo com estudos prévios, o principal fator responsável pela melhora na captação da glicose

observada no modelo animal de exposição ao frio ocorre por mecanismos independentes da insulina (SMITH e DAVIDSON, 1982; CUNNINGHAM et al., 1985; VALLERAND et al., 1987; DULLOO et al., 1988; SHIBATA et al., 1989).

A exposição de animais ao frio promove aumento do tônus simpático e este efeito deve ter uma função central nas adaptações fisiológicas observadas neste modelo. Por sua vez, o elevado tônus simpático promove redução da secreção de insulina pelas ilhotas pancreáticas (GILON e HENQUIN, 2001), aumento da atividade metabólica do BAT (SCARPACE et al., 1996; PUIGSERVER et al., 1998) e aumento na captação de glicose pelo WAT (MORENO-ALIAGA et al., 2002). Além destes, outros fenômenos podem contribuir para este efeito. Por exemplo, a termogênese por tremor participa do controle da temperatura corporal durante a exposição aguda ao frio (SMITH e DAVIDSON, 1982) e o glicogênio muscular é a sua maior fonte de energia, estimulando assim a mobilização de glicose por este mecanismo (MARTINEAU e JACOBS, 1988). Entretanto, no modelo animal utilizado neste trabalho ocorre uma adaptação térmica, na qual o tremor quase desaparece e não participa da homeostase da glicose (SMITH e DAVIDSON, 1982). Outros fenômenos que podem participar deste controle de mobilização de glicose em ratos expostos ao frio são os níveis sanguíneos de NEFA, outros hormônios além da insulina e composição corporal (MARTINEAU e JACOBS, 1989; HAMAN et al., 2002).

Muitos estudos têm avaliado os efeitos da exposição ao frio sobre a homeostase da glicose, secreção de insulina e outros aspectos relacionados ao metabolismo energético em animais e humanos (VALLERAND et al., 1987, 1988; LIU et al., 1999; HAMAN et al., 2002), entretanto a sinalização da insulina em tecidos de animais expostos ao frio não foi ainda investigada. Assim, neste trabalho foram caracterizados alguns parâmetros metabólicos do modelo animal de exposição ao frio, além das etapas iniciais e intermediárias da via de sinalização da insulina em tecido adiposo, músculo esquelético e fígado.

O modelo animal de exposição ao frio utilizado neste trabalho apresenta as mesmas características metabólicas descritas em estudos prévios (CUNNINGHAM et al., 1985; VALLERAND et al., 1983, 1987; TORSONI et al., 2003), as quais reforçam o

conceito de que animais expostos ao frio são capazes de promover aumento da captação da glicose em paralelo a baixos níveis de insulina, provavelmente por mecanismos independentes da insulina.

Em WAT e músculo esquelético ocorreu um significativo efeito positivo da exposição ao frio sobre a captação de glicose. Entretanto, a via de sinalização da insulina em ambos tecidos apresentou um padrão de regulação negativa, com exceção da fosforilação em tirosina do IRS-2 e de sua associação com a PI3-quinase (dado não mostrado) em músculo esquelético de animais expostos ao frio. Assim, parece que em músculo esquelético e WAT existe um efeito da exposição ao frio sobre mecanismos de captação de glicose majoritariamente independentes da insulina, de forma que, mesmo diante de uma regulação negativa da sinalização da insulina, ocorre um aumento da mobilização de glicose neste modelo. O efeito da exposição ao frio sobre a expressão do GLUT-4 e de sua distribuição subcelular reitera o fato de que mecanismos independentes da insulina levam a um aumento da captação de glicose em WAT e músculo esquelético. De acordo com o presente estudo e de prévias observações (SHIMIZU et al., 1993; LIN et al., 1998), a exposição ao frio promoveu aumento da expressão do GLUT-4, com subseqüente aumento da concentração deste transportador na fração de membrana, independente da ação da insulina. Existem dois eventos fisiológicos que são tradicionalmente influenciados pela sinalização da insulina, os quais apresentam claros sinais de reduzida responsividade da insulina nestes tecidos, a magnitude da lipólise no WAT e a concentração de glicogênio no músculo esquelético. No presente modelo foi observada uma redução significativa de massa gorda e uma tendência de aumento nos níveis sanguíneos de NEFA em ratos expostos ao frio, enquanto que os conteúdos de glicogênio muscular foram semelhantes em ambos ratos controle e expostos ao frio. Assim, algumas, mas nem todas as funções reguladas pela insulina em WAT e músculo esquelético de animais expostos ao frio, seguem o padrão esperado para um tecido com resistência molecular à ação da insulina.

Em fígado de animais expostos ao frio foi observado um padrão de dicotomia de ambos os efeitos de resposta funcional e transdução do sinal da insulina. Não foi observada diferença na captação de glicose, enquanto que os conteúdos de glicogênio

hepático foram significativamente aumentados em ratos expostos ao frio. As fosforilações em tirosina do IR e IRS-2 após estímulo agudo com insulina foram significativamente aumentadas, enquanto que a fosforilação em tirosina do IRS-1 apresentou tendência a aumento pela exposição ao frio. Entretanto, a fosforilação em serina da Akt foi significativamente reduzida em ratos expostos ao frio e a fosforilação em tirosina da ERK não foi alterada.

Baseado nos resultados de captação de glicose, o efeito mais intenso da exposição ao frio sobre a mobilização da glicose ocorreu no BAT. Além disso, neste tecido ocorreu uma melhora nas etapas iniciais da via de sinalização da insulina, enquanto que, nas etapas posteriores da via, especificamente na fosforilação em serina da Akt e em tirosina da ERK, apesar da tendência, não foi observado aumento destes parâmetros pela exposição ao frio. Considerando-se que mecanismos dependentes e independentes da Akt participam da captação de glicose induzida pela insulina em tecidos sensíveis à insulina (SALTIEL e KAHN, 2001) e que a exposição ao frio promove captação de glicose por mecanismos majoritariamente independentes da insulina, a aparente regulação positiva observada nas etapas iniciais da sinalização da insulina no BAT deve corresponder a uma fração, mas não ao total deste aumento de captação observada no BAT deste modelo.

Considerando-se dados da literatura que descrevem vias de captação de glicose independentes da insulina, tais como captação de glicose via CAP/Cbl (SALTIEL e KAHN, 2001) que é dependente do IR, mas independente da PI3-quinase; captação de glicose via AMPK (*5'-AMP-activated protein kinase*, OLIVEIRA et al., 2004); e captação de glicose via PKCs (CHERNOGUBOVA et al., 2004), sugere-se a participação de algumas destas vias no modelo animal de exposição ao frio. De acordo com OLIVEIRA et al. (2004), animais expostos ao frio apresentaram melhora na captação de glicose pelo músculo esquelético por meio da via AMPK, isso mesmo diante de prejudicada sinalização da insulina. Visto que o WAT de animais expostos ao frio apresentou o mesmo padrão de resposta funcional e molecular que o músculo esquelético, é possível que a via da AMPK também contribua para a melhora da captação de glicose independente da insulina neste modelo animal. Entretanto, em BAT de animais expostos ao frio foi observado aumento da captação de glicose e melhora somente do início da via de sinalização da insulina. Tendo

como base estudo prévio que demonstrou captação de glicose via PKCs convencionais e atípicas a partir da PI3-quinase (CHERNOGUBOVA et al., 2004), é plausível que no BAT deste modelo animal possa estar ocorrendo a ativação de PKA, via estimulação de receptor adrenérgico, que por sua vez recruta PI3-quinase, ativando PKCs. Além disso, sabendo-se da existência de *cross-talk* entre receptores acoplados à proteína G e a via de sinalização da insulina (HADCOCK et al., 1992; VELLOSO et al., 1996; CARVALHO et al., 2003), é possível que a via da insulina seja modulada positivamente até a PI3-quinase e esta quinase uma vez ativada, estimula PKCs, promovendo captação de glicose. É possível ainda que, ambas vias (via PKA e via da insulina) ativem sinergicamente a PI3-quinase, ativando PKCs e promovendo assim, captação de glicose. Frente a estas observações, constata-se que uma completa investigação das prováveis vias moduladoras da captação de glicose independente da insulina possa trazer importantes contribuições na compreensão da homeostase da glicose neste modelo animal.

O BAT é um tecido especializado que participa ativamente na produção de calor e gasto energético pelo processo de termogênese, por meio da proteína UCP-1, desviando os prótons da produção de ATP para favorecer a produção de calor (BING et al., 1998; JEZEK et al., 1998; MELNYK e HIMMS-HAGEN, 1998; YAHATA e KUROSHIMA, 1989; RICQUIER e BOUILLAUD, 2000; RICQUIER et al., 2000).

A atividade do BAT é regulada pelo sistema nervoso simpático principalmente via receptores β3-adrenérgico (LOWELL e FLIER, 1997; DENJEAN et al., 1999; KLEIN et al., 2000; LOWELL e BACHMAN, 2003). Durante a exposição de animais ao frio um elevado tônus simpático é responsável pela indução da expressão da UCP-1 em BAT, que por sua vez aumenta a produção de calor (RICQUIER et al., 2000; PEREIRA-DA-SILVA et al., 2003). Sabe-se também que sinais adrenérgicos regulam a transdução do sinal da insulina (HADCOCK et al., 1992; KLEIN et al., 1999; KLEIN et al., 2000). Em roedores, o uso de agonista β3-adrenérgico melhora a homeostase da glicose e inibe parcialmente a obesidade induzida pela dieta (ROCHET et al., 1988; COLLINS et al., 1997).

Neste contexto, acredita-se que uma completa caracterização da inter-relação molecular entre os receptores β3-adrenérgicos e de insulina (KLEIN et al., 1999; PAEZ-

ESPINOSA et al., 2001) possa contribuir para o avanço da compreensão dos mecanismos de homeostase da glicose em animais expostos ao frio e encontrar novos alvos terapêuticos para diabetes mellitus e obesidade. Assim, na segunda parte do estudo, o papel da sinalização β3-adrenérgica sobre a atividade da sinalização insulínica foi avaliada em BAT de ratos expostos ao frio.

O tratamento de ratos durante oito dias com agonista ou antagonista seletivos β3-adrenérgico não modificou a ingestão alimentar, o peso corpóreo e a temperatura deste animais. Os níveis de insulina e glicose basais também não foram modificados pelos tratamentos, entretanto a glicemia durante o GTT foi reduzida pelo agonista β3-adrenérgico em ratos controle e aumentada pelo antagonista em ratos expostos ao frio. De acordo com o estudo de LIU et al. (1998), o tratamento de ratos obesos e diabéticos durante 14 dias com o agonista β3-adrenérgico CL 316243 promoveu uma significativa perda de peso acompanhada de redução na ingestão alimentar. Neste mesmo estudo, nenhuma modificação significativa do peso corpóreo e da ingestão alimentar foi detectada no grupo controle magro. Além disso, não foi observada alteração dos níveis de insulina basal e somente uma discreta redução da glicemia basal foi detectada (LIU et al., 1998). Todos estes achados foram semelhantes aos resultados do presente estudo e demonstram que a maior parte dos efeitos farmacológicos dos agonistas β3-adrenérgico são clinicamente evidenciados somente em animais obesos e diabéticos.

Quando foram avaliados os efeitos dos compostos β3-adrenérgicos sobre a transdução do sinal da insulina no BAT, observou-se que a expressão do IR, Akt e ERK de ratos controle e expostos ao frio não foi modificada, entretanto uma significativa redução na expressão protéica do IRS-1 e 2 ocorreu em animais expostos ao frio. Sabendo-se que proteínas IRS estão envolvidas na transdução do sinal via *cross-talk* com receptores acoplados à proteína G (GPCRs) (THIRONE et al., 1998; VELLOSO et al., 1996; CARVALHO et al., 2003) e que a frequência de síntese e degradação de proteínas é diretamente influenciada pelo uso das mesmas (RUI et al., 2001), e, levando-se em consideração que o elevado tônus simpático gerado durante a exposição de ratos ao frio possa controlar a atividade de IRS-1 e 2, acredita-se que o tratamento de animais com compostos β3-adrenérgicos possa modular o ritmo de síntese e degradação destas proteínas.

Este também parece ser o caso do receptor β3-adrenérgico, visto que em ratos somente expostos ao frio ou em ratos controle tratados com ambos compostos β3-adrenérgicos houve um significativo aumento da expressão deste receptor. Entretanto, em ratos expostos ao frio e tratados com o agonista ou antagonista β3-adrenérgico ocorreu uma tendência de inibição dos efeitos da exposição ao frio sobre a expressão do receptor.

A seguir, foi observado que em BAT de ratos controle tratados com BRL37344 ocorreu aumento da fosforilação em tirosina induzida pela insulina do IR e IRS-1, bem como aumento da associação do IRS-1 com a PI3-quinase, porém não ocorreu alteração da fosforilação em tirosina do IRS-2. O tratamento com SR59230A não exerceu efeito nos eventos moleculares induzidos pela insulina em BAT de ratos controle. Em ratos expostos ao frio, entretanto, ambos BRL37344 e SR59230A promoveram significativa inibição da fosforilação em tirosina induzida pela insulina do IR, IRS-1 e IRS-2 e da associação induzida pela insulina do IRS-1 e IRS-2 com a PI3-quinase. O BRL37344 foi efetivo em aumentar a fosforilação em serina induzida pela insulina da Akt e fosforilação em tirosina da ERK em ratos controle. Entretanto, em ratos expostos ao frio, a fosforilação em serina da Akt induzida pela insulina foi discretamente inibida pelo SR59230A. Nenhum efeito dos compostos β3-adrenérgicos sobre a fosforilação da ERK induzida pela insulina em ratos expostos ao frio foi observada. O fato de o SR59230A reverter a sensibilização da sinalização da insulina induzida pelo frio sugere que este efeito ocorra, pelo menos em parte, mediado por mecanismos dependentes de receptor β3-adrenérgico. Por outro lado, acredita-se que o efeito inibitório observado com BRL37344 nos animais expostos ao frio deva ocorrer como um sinal de desensibilização de receptor.

Ao contrário dos achados de KLEIN et al. (1999) em adipócitos marrons isolados, no presente estudo o tratamento crônico com o agonista β3-adrenérgico BRL37344 sensibilizou toda a sinalização da insulina, com exceção do IRS-2, em BAT de ratos controle. Esta diferença deve ter ocorrido pois adipócitos em cultura podem perder algumas de suas características originais, ou talvez, outros fenômenos neurais presentes em tecidos intactos devam modular o sinal β3-adrenérgico. Considerando-se também que receptores β3-adrenérgicos estão presentes em outros tecidos, preferencialmente em WAT

(KRIEF et al., 1993), não se pode excluir a possibilidade de que mecanismos indiretos tenham uma função no efeito induzido pelo BRL37344.

O estímulo β3-adrenérgico em ratos controle promoveu um significativo efeito sensibilizador sobre a fosforilação em tirosina induzida pela insulina da ERK e em serina da Akt. Com respeito a ERK, um fenômeno similar foi previamente descrito como conseqüência de uma inter-relação entre insulina e receptor β2-adrenérgico (WANG et al., 2000). Entretanto, esta é a primeira vez que tal efeito é descrito para uma inter-relação entre receptor de insulina e β3-adrenérgico. É importante notar que, em ratos expostos ao frio, diferentemente do efeito inibitório dos compostos β3-adrenérgico sobre a transdução do sinal da insulina através dos IRSs e Akt, nenhuma alteração foi observado para ERK. Tal fenômeno pode ser devido à multiplicidade de mecanismos que levam à ativação da ERK, os quais podem ser exemplificados, em BAT, pela diferente regulação da expressão da UCP-1, por estímulos da insulina e β3-adrenérgico (KLEIN et al., 2000).

Com o objetivo de avaliar se os eventos moleculares descritos acima exercem influência sobre os eventos funcionais controlados pela insulina, foi investigada a expressão protéica de GLUT-4 e a captação de glicose induzida pela insulina em BAT. Assim, a exposição ao frio, bem como o tratamento de ratos controle com BRL37344 ou SR59230A promoveram significativo aumento da expressão do GLUT-4. Entretanto, ambos compostos β3-adrenérgicos significativamente inibiram a hiperexpressão do GLUT-4 induzida pelo frio. Em relação a captação de glicose foi observado que a exposição ao frio significativamente aumentou a captação de glicose induzida pela insulina em BAT. Este efeito não foi modulado pelo BRL37344, mas foi parcialmente inibido pelo SR59230A. Em adipócitos marrons isolados a estimulação β3-adrenérgica inibiu a captação de glicose induzida pela insulina (KLEIN et al., 1999). Entretanto, em ratos o estímulo β3-adrenérgico aumentou a captação de glicose (DE SOUZA et al., 1997) e de acordo com dados deste trabalho, fica claro que somente parte do aumento da captação de glicose dependente do frio pelo BAT é devida à estimulação β3-adrenérgica.

Por fim, a segunda parte deste estudo demonstra a ocorrência de um *cross-talk* entre as vias de sinalização da insulina e β3-adrenérgica em BAT de ratos. Tal mecanismo é responsável por parte do efeito do frio sobre a ação da insulina neste tecido.

Em conclusão, integrando os resultados obtidos em ambas as etapas deste estudo, observamos que a exposição de ratos ao frio promove a mobilização de glicose com maior eficiência que em animais controle, mesmo apresentando um claro padrão de resistência molecular à ação da insulina, pelo menos em WAT e músculo esquelético, que são importantes tecidos-alvo para a ação da insulina. Os efeitos da exposição ao frio sobre a sinalização da insulina são tecido-específicos e para cada tecido, parecem ser função-específicos, de modo que algumas das respostas controladas pela insulina são modificadas positivamente e outras negativamente. Além disso, estes estudos evidenciaram uma inter-relação positiva entre a sinalização da insulina e β3-adrenérgica em BAT de ratos. Esta inter-relação deve mediar parte do efeito do frio sobre a sinalização da insulina neste tecido. O restante do efeito do frio pode ocorrer em função de outros receptores adrenérgicos (CHERNOGUBOVA et al., 2004), bem como outros fatores circulantes. Estes achados podem ser úteis no avanço do entendimento da homeostase da glicose em animais expostos ao frio e assim evidenciar novos potenciais alvos para terapêutica do diabetes mellitus.

6. CONCLUSÃO GERAL

Os resultados deste trabalho demonstraram que:

O modelo experimental de exposição de ratos ao frio levou a maior mobilização da glicose, mesmo diante de reduzida secreção de insulina, e promoveu modulação tecido-específica dos mecanismos moleculares de resposta à insulina. Os efeitos da exposição ao frio sobre cada tecido parecem ser função-específica, influenciando positivamente algumas das respostas controladas pela insulina e negativamente outras.

Em tecido adiposo marrom

- A exposição ao frio promoveu aumento da captação de glicose e modulação positiva de etapas fundamentais da transdução do sinal da insulina neste tecido.

Em tecido adiposo branco

- A exposição ao frio promoveu aumento da captação de glicose acompanhada de inibição de etapas fundamentais da via de transdução do sinal da insulina neste tecido.

Em músculo esquelético

- A exposição ao frio promoveu aumento da captação de glicose acompanhada de inibição de etapas fundamentais da via de transdução do sinal da insulina neste tecido.

Em fígado

- A exposição ao frio promoveu uma dicotomia nos efeitos fisiológicos da insulina e sua via de sinalização. A captação de glicose não foi alterada, enquanto que os conteúdos de glicogênio hepático foram significativamente aumentados. Além disso, etapas fundamentais da via de transdução do sinal da insulina foram moduladas de forma diversa, ocorrendo aumento da fosforilação do IR e IRS-2, e diminuição da fosforilação da Akt neste tecido.

Sinalização adrenérgica versus insulínica em tecido adiposo marrom

- O tônus simpático elevado durante a exposição de animais ao frio, atuando em receptores β3-adrenérgicos, exerce, por meio de um *cross-talk* intracelular, um efeito estimulatório sobre a via de sinalização da insulina. Entretanto, nem todos os efeitos da exposição ao frio sobre a ação insulínica neste tecido são decorrentes deste mecanismo.

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8. ANEXO

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

C E R T I F I C A D O

Certificamos que o Protocolo nº **503-2** sobre "**Avaliação do Cross-Talk entre a Via de Sinalização da Insulina e a Via de Sinalização Adrenérgica em Tecidos Periféricos de Ratos**", sob a responsabilidade de **Prof. Dr. Licio Augusto Velloso** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de **21 de Março de 2003**.

C E R T I F I C A T E

We certify that the protocol nº **503-2**, entitled **"Cross-Talk Between the Insulin and Adrenergic Signaling in Peripherical Tissue of Rats"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on **March 21, 2003**.

Campinas, 21 de Março de 2003.

Profa. Dra. Liana Verinaud
Presidente
CEEA/IB/UNICAMP

Fátima Alonso
Secretária Executiva
CEEA/IB/UNICAMP

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