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FACULDADE DE ENGENHARIA DE ALIMENTOS
DEPARTAMENTO DE PLANEJAMENTO ALIMENTAR E NUTRIÇÃO

TESE DE DOUTORADO

EFEITO DA RESTRIÇÃO DIETÉTICA NAS ETAPAS INICIAIS DA AÇÃO INSULÍNICA EM RATOS WISTAR MACHOS COM 2 E 14 MESES DE IDADE

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Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas, como parte dos requisitos necessários para a obtenção do título de Doutor em Ciência da Nutrição

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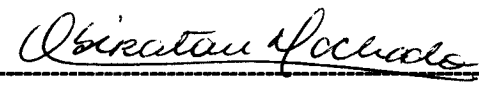

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Dedico este trabalho aos meus pais,
Hiram e Mercedes, pelo exemplo de vida
e, à minha irmã Marta pela amizade
e respeito que sempre nos uniu

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SUMÁRIO

LISTA DE ABREVIATURAS

μCi	microCuri
ATP	adenosina trifosfato
EDTA	ácido etilenodiaminotetracético
GAP	proteína ativadora da proteína G
GLUT-1	transportador de glicose, independente de insulina
GLUT-4	transportador de glicose, depende de insulina
GRB2	proteína ligadora do receptor para fator de crescimento
^{125}I	radioisótopo do iodo
IGF	insulin-like growth factor
IR	receptor de insulina
IRS-1	substrato 1 do receptor de insulina
IRS-2	substrato 2 do receptor de insulina
ITT	teste de tolerância à insulina
Jak2	tirosina quinase da família das proteínas Janus
Kitt	constante de desaparecimento da glicose
ln	logarítmo neperiano
MAPK	proteína quinase ativadora da mitogênese
Nck	proteína que contém domínios SH2 e SH3
PI 3-k	fosfatidilinositol 3-quinase
Raf	serina quinase citoplasmática
Ras	proteína originalmente identificada como um oncogene
SDS-PAGE	gel de poliacrilamida para eletroforese com dodecil-sulfato de sódio

*Os termos estão apresentados de acordo com o uso genérico, o que explica a tradução literal de alguns termos e ausência de tradução em outros.

SH2	segunda homologia ao Src
SH3	terceira homologia ao Src
Shc	molécula adaptadora e substrato do receptor de insulina
SHPTP2 ou Syp	proteína fosfotirosina fosfatase
SOS	fator ativador do Ras
Src	oncogene definido como produto do sarcoma virus Rous
STAT	proteínas ativadoras e tradutoras do sinal de transcrição
Tris	tri(hidroximetil)-aminometano

*Os termos estão apresentados de acordo com o uso genérico, o que explica a tradução literal de alguns termos e ausência de tradução em outros.

1. Introdução

1.1.Sinalização da Insulina

A insulina é um potente hormônio com efeito metabólico e promotor do crescimento, que atua no metabolismo de carboidratos, proteínas e lipídeos, bem como na síntese de RNA e DNA, em praticamente todas as células, através da modificação da expressão ou atividade de uma série de enzimas e sistemas de transporte (FELIG & BERGMAN, 1990). Em mamíferos, os efeitos fisiológicos da insulina incluem estimulação do transporte de hexose, ions e aminoácidos; modificação da atividade enzimática da glicogênio sintetase, da lipase hormônio sensível e da piruvato-desidrogenase; regulação da expressão gênica de algumas enzimas; redistribuição de proteínas de membrana como os transportadores de glicose, os fatores de crescimentos ligados à insulina (IGF) e os receptores de transferrina, e ainda, promoção do crescimento celular (ROSEN, 1987).

A insulina secretada liga-se a receptores específicos da superfície celular, presentes em todos os tecidos dos mamíferos, embora o número oscile de apenas 40 nos eritrócitos a mais de 200.000 unidades receptoras nos adipócitos (SAAD, 1994; CHEATHAN & KAHN, 1995). O receptor de insulina é uma glicoproteína tetramérica composta por duas subunidade α de 135 kDa e por duas subunidades β de 95 kDa unidas através de pontes de sulfeto formando a estrutura α-β-β-α, a subunidade β liga-se externamente à membrana celular e à subunidade α e estende-se para dentro da célula. (SAAD, 1994; DEMEYTS et al., 1990).

Modificações químicas e estudos de mutações sugerem que domínios específicos como as regiões codificadas pelos exons 2 e 3 sejam críticos para o "binding" (DEMEYTS et al., 1990). Aminoácidos cistina na subunidade α e na parte extracelular da subunidade β participam da ligação de pontes dissulfetos entre as subunidades α e β. O componente transmembrana da subunidade β é responsável pela transmissão do sinal insulínico para sua região citoplasmática.

Em 1982, deu-se grande avanço na compreensão da transmissão do sinal insulínico, com a descoberta de que a subunidade β do receptor de insulina é uma proteína com atividade quinase, estimulada pela insulina, capaz de se autofosforilar e fosforilar outros substratos em aminoácidos tirosina. De fato, o receptor de insulina é

uma enzima, da família das tirosina-quinases. Este receptor comporta-se, funcionalmente, como uma enzima alostérica com uma subunidade α regulatória e uma subunidade β catalítica (KASUGA et al., 1982). Quando a insulina se liga à subunidade α ocorre uma alteração na conformação do receptor e a atividade quinase na subunidade β é estimulada (HERRERA & ROSEN 1986; WHITE et al.,1988). Este fato determina a transferência de grupos fosfato do ATP para vários aminoácidos tirosina do receptor e promove a fosforilação de substratos protéicos intracelulares.

A subunidade β pode ser dividida em quatro partes relacionadas às suas ações: porção justamembrana, relacionada à ligação dos substratos e à internalização do receptor (CARPENTIER et al.,1993); região catalítica que contém o sítio de ligação do ATP (Lisina 1030) sendo a região com maior semelhança entre as várias proteínas tirosina-quinases; a região que promove a regulação da atividade quinase e a porção COOH-terminal, onde estão as maiores diferenças entre os receptores de insulina e do "insulin like growth factor-1" (IGF-1) e, por isso, considerada como responsável pela diferenciação entre os efeitos metabólicos e de crescimento da insulina (TAKATA et al.,1988). Há, pelo menos, seis locais de fosforilação das tirosinas: área justamembrana com uma tirosina na posição 972; porção média com tirosinas nas posições 1158, 1162 e 1163; e, COOH-terminal com tirosinas nas posições 1328 e 1334 (WHITE et al.,1988).

Consideráveis evidências foram acumuladas indicando que a atividade tirosina-quinase é essencial para a sinalização insulínica (EBINA et al., 1985; EBINA et al., 1987; WILDEN et al., 1992). A evidência mais convincente deriva de estudos de mutações *in vitro* por substituição do aminoácido lisina da posição 1030 do receptor de insulina por vários outros aminoácidos (metionina, arginina ou alanina). Com base na analogia a outras proteínas da mesma família quinase, este aminoácido é o sítio de ligação do ATP nas quinases (EBINA et al., 1985). Quando tais mutações são expressas em células a ligação da insulina ocorre normalmente porém, estes receptores são totalmente inativos como proteínas quinases e são totalmente ineficientes em mediar a ação metabólica da insulina nestas células (EBINA et al., 1987). Isto é verdadeiro para todos os efeitos da insulina, incluindo efeitos metabólicos e promotores de crescimento. Mutações em um dos sítios de autofosforilação (tirosinas 1158, 1161, 1162) produz efeitos semelhantes porém menos intensos (WILDEN et al., 1992).

Embora estudos ocasionais tenham descrito que receptores com baixa atividade quinase podem exercer efeitos biológicos em algumas enzimas intracelulares (SUNG et al.,1989), para a maioria das ações insulínicas há uma estreita relação entre perda da atividade quinase do receptor e redução da resposta biológica. Além do mais, o receptor de insulina possui sítios de fosforilação de serina e treonina, os quais, quando fosforilados, parecem determinar uma diminuição ou inibição da atividade quinase do receptor (BOLLAG et al.,1986). Esta fosforilação em treonina ou serina pode ser estimulada pelo tratamento prolongado com insulina, por esteres de forbol e análogos de AMP cíclico, e provavelmente é o resultado da ação das proteínas quinase C ou A sobre o receptor (TAKAYAMA et al., 1984; TAKAYAMA et al., 1988). Esta regulação da atividade quinase do receptor por fosforilações em aminoácidos distintos tem um potencial importante como mecanismo de regulação do receptor de insulina em situações fisiológicas e patológicas.

O mecanismo pelo qual o receptor quinase transmite seu sinal não é completamente compreendido. O modelo aceito atualmente demonstra que há uma cascata de fosforilações onde a insulina induz a autofosforilação do seu receptor, ativando a capacidade tirosina-quinase do mesmo, o qual fosforila um ou mais dos seus substratos intracelulares (WHITE et al., 1985; BERNIER et al., 1987; KARASIK et al., 1988; SUN et al., 1991).

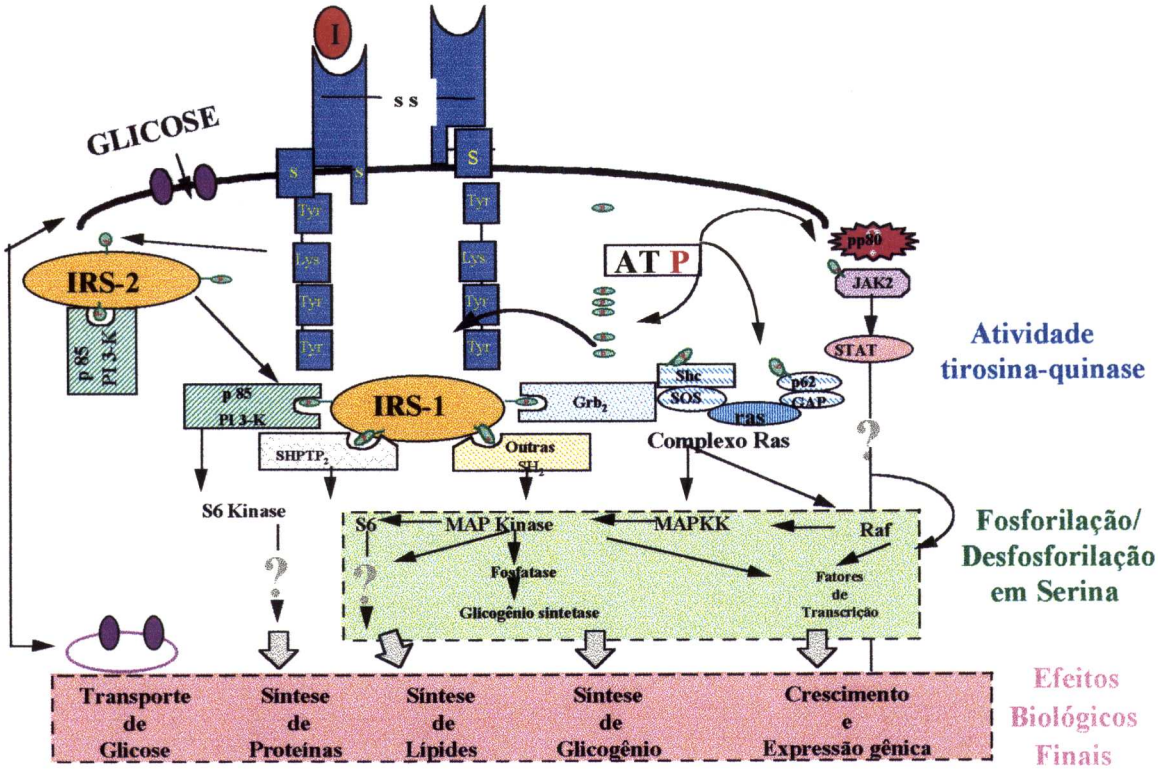
O primeiro e mais estudado substrato do receptor de insulina é uma proteína de aproximadamente 185 kDa denominada pp185 pela sua mobilidade eletroforética (WHITE et al., 1985). Em células transfectadas com o receptor de insulina humano há um marcante aumento da fosforilação da pp185 coincidente com aumento da ação insulínica (SUN et al., 1991). A fosforilação dessa proteína está reduzida em células expressando receptores com mutações nos sítios de ligação do ATP e ou de autofosforilação, proporcional a redução da ativação da capacidade quinase do receptor (WILDEN et al., 1992). Evidências adicionais da função da pp185, vieram através de estudos de mutagênese *in vitro* efetuados no aminoácido tirosina de posição 972, situada na região justamembrana do receptor de insulina. Nesta situação o receptor mutante apresenta ligação normal à insulina com total ativação da capacidade tirosina-quinase *in vitro*, porém quando transfectado em células, este receptor mutante não é capaz de fosforilar a pp185 e é incapaz de transmitir o sinal insulínico (WHITE et al., 1988).

A pp185 foi clonada e denominada substrato 1 do receptor de insulina ou IRS-1 (SUN et al., 1991), e nos últimos dois anos, demonstrou-se que outra proteína também migra nesta banda, que foi então denominada de IRS-2 (TAMEMOTO et al., 1994; ARAKI et al., 1994; SUN et al., 1995). O DNA complementar (cDNA) do IRS-1 prevê uma proteína de 1235 aminoácidos com diversas características interessantes. Primeiro, não há porção transmembrana, é uma proteína de localização citoplamática. Há uma sequência de aminoácidos compatível com as de ligação para nucleotídeos (ATP ou GTP), embora não haja nenhuma homologia com proteínas quinases conhecidas. Ainda em relação a homologia de sequências de aminoácidos, há múltiplos sítios de fosforilação em serina, treonina e tirosina. São 22 sítios potenciais de fosforilação em tirosina, desses, dez mantêm a seqüência YMXM onde Y é o aminoácido tirosina, M é o aminoácido metionina e X corresponde a qualquer aminoácido, três apresentam a seqüência YXXM, e um tem a seqüência EYYE, onde E é o aminoácido glutamina. A sequência de aminoácidos YMXM não existe no receptor de insulina porém tem sido observado em várias outras proteínas tirosina-quinases. Nestas proteínas a sequência YMXM está envolvida em interações não covalentes com moléculas sinalizadoras. Estas moléculas sinalizadoras são caracterizadas pela presença de sequências de aminoácidos denominada SH2 (homologia 2 ao src) que se ligam às sequências YMXM quando o aminoácido tirosina está fosforilado (SHOELSON et al.; 1992).

O IRS-1 apresenta também cerca de 30 outros sítios de fosforilação em serina/treonina que são reconhecidos por várias kinases. Pelo menos 8 resíduos tirosina do IRS-1 são fosforiladas pelo receptor de insulina ativado, e são os seguintes: 608, 628, 939 e 987 na seqüência YMXM e os resíduos 460, 895,1172 e o 1222 nas seqüências YICM, YVNI, YIDL e YASI, respectivamente (WHITE & KAHN, 1994). Os autores também reportaram que a fosforilação dos múltiplos resíduos de tirosina em IRS-1, pelo receptor de insulina, gera sítios de reconhecimentos específicos para o domínio SH2 de várias proteínas incluindo: fosfatidil-inositol 3-quinase - PI 3-kinase (Y460, Y608, Y939 e Y987), o pequeno adaptador de proteína que se liga ao receptor do fator de crescimento - GRB2 (Y895) e a tirosina fosfatase - SHPTP2 (Y1172, Y1222). Segundo WATERS & PESSIN (1996) uma única molécula de IRS-1 pode complexar-se simultaneamente com diferentes proteínas com domínio SH2, sugerindo que um

complexo de sinalização foi formado. É possível que seja necessário a presença de várias proteínas com domínio SH2 ligadas à várias moléculas de IRS-1 para que a especificidade do sinal insulínico sejam efetivados em sua plenitude. A figura 1, abaixo, é um esquema representativo das etapas iniciais da ação insulínica.

Figura 1-*Etapas iniciais da ação insulínica*



Através de estudos *in vivo* em cultura de células e em sistemas reconstituídos *in vitro*, o IRS-1 fosforilado associa-se a enzima fosfatidilinositol 3-quinase (PI 3-quinase) ativando-a (BACKER et al., 1992; FOLLI et al., 1992). Foi demonstrado que esta associação e ativação também ocorre em dois importantes tecidos insulino-sensíveis de ratos, fígado e músculo, em concentrações fisiológicas de insulina (FOLLI et al., 1992; SAAD et al., 1993). A enzima PI 3-quinase possui duas subunidades, uma catalítica de 110 kDa e outra regulatória de 85 kDa que contém duas porções SH2 e uma SH3 (CARPENTER & CANTLEY, 1990). A ligação e ativação descritas envolvem as tirosinas fosforiladas do IRS-1 próximas a metioninas, YMXM, e a porção SH2 da subunidade regulatória da enzima PI 3-quinase. A insulina aumenta a

concentração intracelular de fosfatidilinositol 3-fosfato, sugerindo que a PI 3-quinase é realmente ativada durante a estimulação com este hormônio. Embora a subunidade de 85 kDa da PI 3-quinase seja fosforilada após estímulo com outros fatores de crescimento, isto não parece ocorrer após estímulo com insulina. Ao invés disso, a ativação parece resultar como consequência da ligação do IRS-1 à PI 3-quinase.

Estudos recentes demonstram que a associação do IRS-1 com a PI 3-quinase é essencial para o transporte de glicose em tecido muscular (TSAKIRIDIS et al., 1995) e em tecido adiposo (CLARKE et al., 1994), para a síntese de glicogênio e controle do crescimento celular na maioria dos tecidos (TSAKIRIDIS et al., 1995).

Além da PI 3-quinase, pelo menos três outras proteínas com porção SH2 associam-se ao IRS-1: SHP2, GRB-2 e Nck. Essas associações potencialmente, podem ter um importante papel no crescimento celular. A SHP2 (Syp) é outra proteína de interesse nos estudos de sinalização da insulina; esta proteína se liga ao domínio SH2 do IRS-1 e aumenta sua atividade tirosina fosfatase (LAVAN et al., 1992). HAUSDORFF et al. (1995) demonstraram que a Syp não interfere na translocação de GLUT-4 mas aumenta a expressão de GLUT-1 e como conseqüência o transporte de glicose independente de insulina. A SHP2 ainda tem a capacidade de bloquear a mitogênese induzida pela insulina (HAUSDORFF et al., 1995).

1.2. Resistência à Insulina

A resistência à insulina está associada à diminuição da utilização da glicose, pode surgir em função da obesidade ou inatividade física e, normalmente manifesta-se durante o envelhecimento (NARIMIYA et al., 1984; NISHIMURA et al., 1988). Foi demonstrado por JACKSON et al. (1988) que a intolerância à glicose relacionada à idade é caracterizada por níveis elevados de insulina, aumento na produção hepática da glicose e redução na utilização periférica de glicose.

O envelhecimento e a obesidade em humanos e animais podem ser acompanhados por diminuição da secreção de insulina pelas células β após o estímulo da glicose, diminuição da ligação da insulina ao seu receptor, e ainda, diminuição do GLUT-4 nos músculos (REAVEN et al., 1979; SCHEEN et al., 1996; OLEFSKY & REAVEN, 1975; MARITA et al., 1997; KERN et al., 1992; CARTEE et al., 1997).

Também foi demonstrado por NADIV et al. (1994) que a atividade da proteína fosfatase tirosina sobre o receptor de insulina é elevada no fígado de ratos Wistar velhos, promovendo inibição da atividade quinase tirosina do receptor de insulina.

DEFRONZO & FERRANNINI (1991) e BECK-NIELSEN et al. (1996) relataram que a resistência à insulina é freqüente na população em geral, ocorrendo em qualquer faixa etária e que no grupo jovem, os indivíduos com sensibilidade normal à insulina usam 4 vezes mais glicose do que a maioria daqueles indivíduos resistentes à insulina. Nas condições de resistência à insulina, a intolerância à glicose assim como a diabetes mellitus poderão estabelecer-se devido ao aumento da secreção de insulina, mantendo um estado de hiperinsulinemia crônica (REAVEN, 1988; DEFRONZO & FERRANNINI, 1991).

Estudos em ratos demonstraram que a redução da glicose estimulada pela insulina começa a decair à partir de 2 a 4 meses de idade e decairá progressivamente até os 20 meses de idade (NISHIMURA et al., 1988). A equipe de trabalho que deu apoio a esta tese, demonstrou que ocorre diminuição do IRS-1 em ratos Wistar com idade entre 2 e 5 meses (CARVALHO et al., 1996). Anteriormente, GULVE et al. (1993) já haviam iniciado estudos sobre GLUT-4 em ratos com 1 a 10 meses de idade, quando encontraram que durante este período decresceu o número daquele transportador.

1.3.Relação da Resistência à Insulina com Dietas de Restrição

Estudos em humanos e animais são conduzidos com o objetivo de determinar o início e a extensão de diversas patologias que surgem em decorrência do envelhecimento. Ratos e outras espécies de roedores são mamíferos de vida curta e portanto utilizados em estudos de envelhecimento. BUREK & HOLLANDER (1980), encontraram que a vida média de várias linhagens de ratos situa-se entre 27 e 30 meses. Para os estudos de envelhecimento, 50% da idade de sobrevivência dos roedores foi usada como marco da senescência. Os autores determinaram que ratos machos com 24 meses de idade eram adequados para o estudo de envelhecimento.

A restrição dietética é reconhecida como conduta nutricional capaz de prolongar o tempo de vida de animais e humanos, capaz de retardar o aparecimento de doenças relacionadas à idade e à queda da eficiência fisiológica e imunológica, bem

como poderá proteger contra a ação dos radicais livres (YU, 1994; ROJAS et al., 1993; PIERI et al., 1996; WEINDRUCH, 1996). Outros estudos mostraram que a restrição dietética e a perda de peso reduzem os níveis de glicose e insulina plasmáticas, melhoram a secreção e ação da insulina e consequentemente a tolerância à glicose (KEMNITZ et al., 1994; REAVEN et al., 1993; BERGAMINI et al., 1991; COLMAN et al., 1995).

1.4. Objetivos

Embora esteja claro que a restrição dietética induza melhora na tolerância à glicose e sensibilidade à insulina, o mecanismo molecular que envolve a sinalização da insulina neste processo não está estabelecido. O presente trabalho teve como objetivo estudar o efeito da restrição dietética nas etapas iniciais da ação da insulina utilizando ratos Wistar de 14 meses de idade, com resistência à insulina estabelecida, e em animais de 2 meses de idade considerados em muitos estudos como parâmetro de normalidade.

2. Resumo dos Artigos

A insulina é um hormônio com efeito metabólico e promotor do crescimento, que atua no metabolismo de carboidratos, proteínas e lipídeos (FELIG & BERGMAN, 1990). A resistência à insulina está associada à diminuição da utilização de glicose pelas células (NARIMIYA et al., 1984; NISHIMURA et al., 1988), aos níveis séricos de insulina elevados e à diminuição da produção de glicose hepática (JACKSON et al.,1988).

Estudos têm demonstrado que a restrição dietética é uma conduta nutricional capaz de prolongar o tempo de vida de animais e humanos, retardar o aparecimento de doenças relacionadas à idade, proteger contra a ação dos radicais livres (YU, 1994; ROJAS et al., 1993; PIERI et al., 1996; WEINDRUCH, 1996), reduzir os níveis de glicose e insulina plasmáticas, regular a secreção e a ação insulínica e, finalmente melhorar a tolerância à glicose (KEMNITZ et al., 1994; REAVEN et al., 1983; BERGAMINI et al., 1991; COLMAN et al., 1995).

O objetivo do presente trabalho foi estudar o efeito da restrição dietética de 40%, durante 28 dias, nas etapas iniciais da ação da insulina utilizando ratos Wistar de 14 meses de idade, com resistência à insulina estabelecida, e em animais de 2 meses de idade considerados em muitos estudos como parâmetro de normalidade.

Os animais de 2 meses de idade, sob restrição dietética, tiveram perda de peso de aproximadamente 14 %, durante a primeira semana, ganharam posteriormente mas não conseguiram recuperar o peso inicial, computando assim no final do experimento uma perda de 6% em relação ao peso no início do experimento. Os ratos restritos com 14 meses de idade perderam 18% do peso corporal enquanto que os controle mantiveram o mesmo peso durante os 28 dias de experimento.

A análise bioquímica do sangue dos animais com 2 meses de idade com restrição de dieta mostrou aumento dos níveis séricos de glicose e, não houve aumento dos níveis de insulina, triacilgliceróis, colesterol e albumina quando foram comparados aos animais que consumiram ração *ad libitum*. Os animais restritos de 14 meses de idade apresentaram redução dos níveis séricos de insulina e triacilgliceróis diferindo dos resultados encontrados nos animais mais jovens e, esta diferença pode ser resultado da hiperinsulinemia e hipertriacilgliceridemia presente nos animais de 14 meses de idade quando comparados aos de 2 meses de idade.

O teste de tolerância à insulina (ITT) mostrou que a restrição dietética aumentou a taxa de redução de glicose indicando que os animais restritos de 2 e 14 meses apresentaram maior sensibilidade à insulina e portanto maior captação de glicose pelas células em relação aos controle.

A quantidade da proteína IRS-1 não foi diferente no músculo, fígado e tecido adiposo dos ratos restritos de 2 meses de idade quando comparados ao controle, mas aumentou 159±14% ($p<0,05$) no fígado dos animais de 14 meses que consumiram a dieta restrita, podendo estar relacionada com a redução de 42% ($p<0,05$) nos níveis de insulina destes animais.

A fosforilação em tirosina do IRS-1, aumentou 127±18% ($p<0,05$) no músculo dos animais de 2 meses de idade sob restrição dietética e aumentou 210±40% ($p<0,05$) no músculo e 234±58% ($p<0,05$) no tecido adiposo dos ratos de 14 meses sob restrição, quando comparados aos seus controle. O aumento na fosforilação em tirosina do IRS-1 no músculo dos animais restritos de 14 meses foi acompanhado por aumento de 273±71% ($p<0,05$) na associação IRS-1/PI 3-quinase; entretanto, no tecido adiposo, apesar do aumento da fosforilação em tirosina doIRS-1, a associação IRS-1/PI 3-quinase não aumentou. Nos animais restritos de 2 meses de idade, o aumento da fosforilação do IRS-1, no músculo, não foi acompanhado por aumento da associação IRS-1/PI 3-quinase. Entretanto após estímulo insulínico houve aumento de 157±7% ($p<0,05$) na associação IRS-1/PI 3-quinase no tecido adiposo.

A associação de IRS-1/SHP2 nos tecidos dos ratos restritos de 2 meses de idade aumentou em 138±9% ($p<0,05$) no fígado, 143±17% ($p<0,05$) no músculo e 139±14% ($p<0,05$) no tecido adiposo. Estes resultados sugerem que o aumento da associação IRS-1/SHP2 poderia ter contribuído para aumentar a expressão de GLUT1 e consequentemente o aumentar o transporte de glicose (HAUDSDORFF et al., 1995), nestes tecidos. Os resultados também sugerem que nos animais restritos de 2 meses de idade, a melhora na sensibilidade à insulina foi induzida por aumento na fosforilação em tirosina do IRS-1 no músculo, aumento na associação IRS-1/PI 3-quinase no tecido adiposo e aumento na associação IRS-1/SHP2 no fígado, músculo e tecido adiposo. Nos animais restritos de 14 meses, a melhora na sensibilidade insulínica pode ter ocorrido pelo aumento da fosforilação do IRS-1 do músculo, acompanhado do aumento da associação IRS-1/PI 3-quinase do mesmo. Concluindo, foi demonstrado que a restrição

dietética para ratos Wistar jovens (2 meses de idade) ou adultos (14 meses de idade) melhora a captação tecidual de glicose, estimulada pela insulina, através de mecanismos distintos que envolvem alterações nas vias iniciais de sinalização da insulina.

3. Artigo 1

EFFECT OF DIETARY RESTRICTION ON THE EARLY STEPS OF INSULIN ACTION IN AN ANIMAL MODEL OF INSULIN RESISTANCE

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Key words: Dietary restriction, obesity, IRS-1, insulin resistance, tyrosine phosphorylation, IR

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SUMMARY

The present study investigated whether food ingestion restricted of 40% could revert the decreased sensitvity to insulin seen in 14 month-old male Wistar rats. We have examined the levels and phosphorylation state of the insulin receptor and of insulin receptor substrate 1 (IRS-1), as well as the association between IRS-1 and phosphatidylinositol 3-kinase in liver, muscle and adipose tissue of 14-month-old rats by immunoprecipitation and immunoblotting with anti-insulin receptor, anti-IRS-1, anti-PI 3-kinase and antiphosphotyrosine antibodies. Also we have examined the association of the IRS-1 with SHP2 (protein tyrosine phosphatase) in adipose tissue in these animals.

Rats on a restricted diet had decreased serum insulin and triacylglycerols levels in serum after 28 days. During this time there was an improvement in the sensitivity to insulin. The levels of insulin receptor protein and insulin-induced tyrosyl phosphorylation in the liver, muscle and adipose tissue were unchanged. The IRS-1 protein level increased to 159±14% ($p<0.05$) in the liver of the restricted rats but not in muscle and adipose tissue. In contrast, there was no change in the insulin-induced phosphorylation of hepatic IRS-1, whereas IRS-1 phosphorylation was increased in adipose tissue and muscle. Increased IRS-1/PI 3-kinase association occurred only in muscle.

There were no changes in IRS-1/SHP2 association, suggestting that the insulin-induced IRS-1 phosphorylation in adipose of diet-restricted rats occurs at tyrosine recognition sites specific for other proteins such as Grb or Nck

The improved sensitivity to insulin in rats on a restricted diet may be related to increased IRS-1 phosphorylation accompanied by increased IRS-1/PI 3-kinase association in muscle.

INTRODUCTION

Dietary restriction retards aging (Yu, 1994) and the decline in immunological functions (Utsuyama et al., 1996), decreases experimental gastric mucosal injury (Lee and Devi, 1996), increases expression of the cytoprotective hsp70 gene in the gut (Ehrenfried et al., 1996) and lowers body temperature (Lane et al., 1996). Dietary restriction and weight loss also reduce plasma glucose and insulin levels (Kemnitz et al., 1994), and improve insulin secretion and insulin resistance in aging (Reaven and Reaven, 1981; Reaven et al., 1983; Bergamini et al., 1991; Colman et al., 1995). However, the mechanism for the improvement of insulin action in aging is not known.

Many of the cytosolic proteins involved in intracellular response following insulin binding have been defined during the past decade (White and Kahn, 1994). The insulin receptor is a protein tyrosine kinase which, when activated, undergoes rapid autophosphorylation and phosphorylates intracellular protein substrates, including insulin receptor substrate-1 (IRS-1) (White and Kahn, 1994; White et al., 1985; Momomura et al., 1987; Sun et. al., 1991). Tyrosine phosphorylated IRS-1 acts as a docking protein for several Src homology 2 (SH2) domain-containing molecules, including phosphatidylinositol 3-kinase (PI 3-kinase) and protein tyrosine phosphatase (SHP2) (Lamphere et al., 1994; Backer et al., 1992; Folli et al., 1993; Lavan et al., 1992; Yamauchi et al., 1995). The interaction between the IRS-1 and PI 3-kinase occurs through the p85 regulatory subunit of PI 3-kinase and increases the catalytic activity of its p110 subunit. PI 3-kinase is essential for many insulin-sensitive metabolic processes, including the stimulation of glucose transport and glycogen and protein synthesis (Hara et al., 1994, Cheatham et al., 1994; Clarke et al., 1994).

In order to determine whether the improved sensitivity to insulin induced by dietary restriction is related to modulation of the early steps of insulin action, we examined the phosphorylation status of the insulin receptor and IRS-1 as well as the association of the latter with PI 3-kinase in the liver, muscle and adipose tissue of aging rats fed a restricted diet.

METHODS

Materials

The reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiothreitol, Triton X-100, Tween 20, and glycerol were from Sigma Chemical Co. (St. Louis, MO). Protein A Sepharose 6 MB was from Pharmacia (Uppsala, Sweden), [^{125}I]protein A was from Amersham (Aylesbury, UK) and nitrocellulose paper (BA85, 0.2 μm) was from Schleicher & Schuell (Keene, NH). Sodium thiopental and human recombinant insulin (Humulin R) were from Elli Lilly Co. (Indianapolis, IN). Male Wistar rats were from the UNICAMP'S Central Animal Breeding Laboratory. Monoclonal antiphosphotyrosine, polyclonal anti-insulin receptor, anti-IRS-1, and anti-SHP2 antibodies were from Santa Cruz Technology (Santa Cruz, CA). Polyclonal anti-PI 3-kinase (p85) antibody was from Upstate Biotechnology Incorporated (Lake Placid, NY).

Animals and food restriction plan

Male Wistar rats 14 months old were weighed, and randomly allocated to one of two groups. One group had unlimited access to water and standard rodent chow (Nuvilab CR-1, Nuvital, Curitiba, SP; composition: 22% protein, 3% fat, 9% fiber, 8% minerals, 1.4% calcium and 0.6% phosphorus). In the second group, the food intake was restricted to 60% of the daily consumption of the first group (controls). The amount of food ingested was measured every two days and the rats were weighed once a week interval. The animals were maintained on a 12 h light-dark cycle with lights at 7a.m. All rats were fasted for 14 h before the experiments

Methods

Rats were anesthetized with sodium thiopental (24 mg/kg b.w., ip) and opened 10-15 min later, *i.e.* as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein exposed, and 0.5 ml of normal saline (0.9% NaCl) with or without 6 μg of insulin was injected. Thirty seconds later, a liver sample was removed, minced coarsely and homogenized immediately in approximately ten volumes of solubilization buffer (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 vanadate, 2 mM PMSF and 0.1 mg of aprotinin/ml) at 4 °C using a Polytron PTA 20S generator (model PT 10/35, Brinkmann Instruments, Westbury, NY) operated at maximum speed for 20 s.

Approximately 90 sec and 120 sec after the injection as described above, samples of hindlimb muscle and epididimal adipose tissue, respectively, were quickly excised and homogenized as described for liver. Both extracts were centrifuged at 15,000 rpm at 4°C for 45 min to remove insoluble material and the supernatants were used in the assays described below. Protein determination was performed by the biuret method using the Labtest reagent (Labtest Diagnóstica S.A., M.G., Brazil.) and bovine serum albumin (BSA; Sigma Chemical Co.) as standard. Supernatants of the three tissues were used for immunoprecipitation with anti-insulin receptor or anti-IRS-1 antibody and followed by adsorption with Protein A Sepharose 6 MB.

Immunoblotting

Immunoprecipitated proteins were treated with Laemmli sample buffer (Laemmli, 1970) containing 100 mM dithiothreitol and heated in a boiled in a water bath for 4 min, after which they were separeted by SDS-PAGE (6% bis-acrylamide) in a Bio-Rad miniature gel apparatus (Mini-Protean, Bio-Rad Laboratories, Richmond, CA).

Electrotransfer of the proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin *et al.* (1979), except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high M_r proteins. Non-specific protein binding to the nitrocellulose was reduced by pre-incubating the filter in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20) overnight at 4° C. The nitrocellulose blot was then incubated overnight at 4°C C with antiphosphotyrosine (αPy) (1 μg/ml), anti-insulin receptor (1:100), anti-IRS-1 (1:100), anti-SHP2 (1:100), or anti PI 3-kinase (1:500) antibodies diluted in blocking buffer (0.3% BSA instead of nonfat dry milk) and then washed for 30 min with blocking buffer without milk. The blots were subsequently incubated with 2 μCi of [^{125}I]protein A (30 μCi/μg) in 10 ml of blocking buffer for 2 h at room temperature and then washed again for 30 min as described above. [^{125}I] protein A bound to the antiphosphotyrosine and antipeptide antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak, Rochester, NY) with

Cronex Lightning Plus intensifying screens (DuPont, Wilmington, DE) at -80°C for 12-48 h. Band intensities of the developed autoradiographs were quantitated by densitometry (model GS 300, Hoefer Scientific Instruments, San Francisco, CA).

Estimation of insulin action in vivo using the 16-minute insulin tolerance test (ITT)

Twelve animals from both groups received insulin (6 μg in 0.5 ml.) i.v. and blood samples for glucose determination were collected at 0 (basal), 4, 8, 12 and 16 min post-injection. Tail blood from rats anesthetized, 20μl was collected in TCA, centrifuged at 6000 rpm at 4°C for 5 min and the blood glucose level measured by the glucose oxidase method using a commercial kit. The rate constant for plasma glucose disappearance (Kitt) was calculated from the formula $0.693/t_{1/2}$. The plasma glucose half-life ($t_{1/2}$) was calculated from the slope of the least square analysis of the plasma glucose concentration during the linear phase of decline (Bonora et al, 1989).

Serum analysis

Blood obtained by cardiac puncture of 8 rats of both groups was centrifuged at 6000 rpm, at 20°C for 15 min and the serum was used to insulin, glucose, triacylglycerols, cholesterol and albumin. All analyses were performed using commercial kits.

Statistical analysis

The results are means ± SD or means ± SEM of values obtained from independent experiments. Statistical evaluation was performed by the nonpaired Student's *t* test was used. The level of significance was set at $p<0.05$.

RESULTS

Body weight and serum analyses

The body weights and serum analyses for the two groups of rats after 28 days are shown in Table 1. Dietary restriction produced an 18% lag in body-weight gain. The cholesterol, albumim and glucose were unaffected by a restricted diet whereas insulin and triacylglycerols levels were lower significantly ($p<0.05$) in diet restricted rats. The glucose disappearance rate in diet restricted rats was 2.46±0.9%/min compared to 1.8± 0.6%/min for the control group ($p<0.05$; n=16 each).

Insulin receptor phosphorylation and insulin receptor protein levels in liver

There was no significant difference in the insulin-stimulated tyrosine phosphorylation of the 95 kDa ß subunit of the insulin receptor on in the insulin receptor protein levels in the liver of the two groups of rats (Fig 1).

IRS-1 phosphorylation and protein levels and association IRS-1/PI3-k in liver

The levels of IRS-1 protein in liver of rats diet restricted increased to 159±14% ($p<0.05$) but there was no change in IRS-1 phosphorylation or IRS-1/PI 3-kinase association (Fig. 3).

Insulin receptor phosphorylation and protein levels in the muscle

There was no change in the insulin receptor levels or in the insulin-stimulated phosphorylation of the 95-kDa β-subunit of this receptor in muscle from diet restricted rats (Fig 4).

IRS-1 phosphorylation and protein levels and IRS-1/PI3-k association in muscle

Figure 5 shows that the levels of IRS-1 protein in muscle were similar for diet restricted and control and that after stimulation with insulin the IRS-1 phosphorylation increased to 210±40% ($p<0.05$). Previous studies (Backer et al, 1992; Folli et al, 1993) have shown that there is a relatively stable, high affinity interaction between IRS-1 and the 85-kDa subunit of PI 3-kinase so that both proteins are coprecipitated by antibodies to either protein. When blots of muscle IRS-1 were incubated with antibodies against the 85-kDa subunit of PI 3-kinase, the levels of this protein were found to be increased to 273±71% ($p<0.05$) in diet restricted rats (Fig 5).

Insulin receptor phosphorylation and protein levels in the adipose tissue

The effect of dietary restriction on the insulin receptor protein levels and phosphorylation in adipose tissue were similar to these seen in liver and skeletal muscle, in that there were no significant changes in these parameters in diet restricted rats (Fig.6).

IRS-1 phosphorylation and protein levels and the association between IRS-1/PI3-k and IRS-1/SHP2 in adipose tissue

The amount of IRS-1 in adipose tissue was similar in both groups of rats. After stimulation with insulin, the IRS-1 phosphorylation increased to 234±58% ($p<0.05$) in diet restricted rats but these was no change in the association of PI 3-kinase with IRS-1(Fig 7). Similarly, there was no change in the association between IRS-1/SHP2 in diet restricted and fed rats (Fig7).

DISCUSSION

The physiological effects of dietary restriction are diverse. Kemnitz et al. (1994) found that dietary restriction decreased the amount of glycosylated hemoglobin, as well as decreased levels of serum insulin and fasting plasma glucose in monkeys. A reduction in serum triacylglycerols was reported by Liepa et al. (1980) and was suggested to modulate age-related changes in serum lipid levels. Yamashita et al. (1996) and Björntorp (1997) showed that visceral fat has a high lipogenic activity and that elevated levels of portal free fatty acids may enhance hepatic triacylglycerols synthesis and induce insulin resistance. This could lead to glucose intolerance, hypertension and atherosclerosis.

As shown in Table 1, diet-restriction reduced the serum levels of insulin and triacylglycerols. Our data are in accordance with the results of Reaven and Reaven (1981) that reported a reduction in the serum insulin levels of 12-month-old rats on a restricted diet. A reduction in serum lipids levels as well as in visceral and total body fat may delay the onset of insulin resistance, diabetes mellitus and cardiovascular disease (Semenkovich and Heinecke, 1997; Koyama et al, 1997; Cefalu et al.,1988).

The 16-min ITT showed that after 28 days on a restricted diet there was an improvement in the sensitivity to insulin. The mechanism underlying this improvement is not yet fully understood. Colman et al., 1995 reported an increased sensitivity to insulin in 45-year old men on a restricted diet.

To characterize the role of the early steps of insulin action in improving resistance to insulin, we evaluated the protein levels and insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1 *in vivo*, as well as the association of IRS-1 with PI 3-kinase in liver, muscle and adipose tissue. After stimulation with insulin, a band with expected molecular weight of the regulatory subunit of PI 3-kinase (85 kDa) was present in anti-IRS-1 antibody immunoprecipitates of three tissues from both groups of animals. This observation is consistent with the existence of a stable complex between IRS-1 and PI 3-kinase. In the muscle of diet restricted rats there was an increase in IRS-1/PI 3-kinase association, suggesting enhanced affinity between these two proteins, which may have a role in improving the sensitivity to insulin.

Despite increased insulin-induced IRS-1 phosphorylation in adipose tissue of diet restricted rats, there was no change in the IRS-1/PI 3-k association compared to control rats.

IRS-1 contains 22 potential tyrosine phosphorylation sites which act as specific recognition sites for cellular substrates bearing SH2 domains, including PI 3-kinase (sites Tyr460, Tyr608, Tyr939, Tyr 987), the small adaptor Grb2 (site Tyr 895), and the protein tyrosine phosphatase SHPTP2 (sites Tyr1172, Tyr1222) and other proteins (Sun et al., 1991; Keller et al, 1993; Sugimoto et al, 1994).

The Syp (SHP2) binds to IRS-1 through SH2 domains, leading to increased tyrosine phosphatase activity (Lavan et al., 1992). Noguchi et al. (1994) demonstrated that Syp is an upstream regulator of the Ras/MAP kinase pathway but not of PI 3-kinase, which suggests that Syp is not involved in insulin-stimulated glucose transport. Hausdorff et al. (1995) showed that Syp blocked insulin-induced mitogenesis, but not insulin-stimulated GLUT4 translocation, and increased expression of GLUT1 leading to increased glucose transport activity.

Our results demonstrated that the increase in IRS-1 tyrosine phosphorylation was not accompained by an increase in IRS-1/Syp association in adipose tissue. This suggests that the increase in IRS-1 tyrosine phosphorylation was not responsible for the interaction with PI 3-kinase, and that Syp or other factors can impair these associations.

These results also demonstrate that the improved sensitivity to insulin in food-restricted rats may be due to increased IRS-1 phosphorylation accompanied by an increase in IRS-1/PI 3-kinase association in muscle; the tissue that seems to be more responsive to dietary restriction. These findings are consistent with the fact that a 40% reduction in food intake resulted in an 18% lag of weight gain. This in itself suggests the biochemical system as a whole operates at a higher efficiency rate than in the animal with free access to food.

FIGURE LEGENDS

Table 1: Metabolic characteristics of rats after 28 days on normal (control) and restricted diets.

Figure 1: Insulin receptor protein levels (**a**) and phosphorylation (**b**) in liver of *ad libitum* or control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Thirty seconds later, the liver was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with insulin receptor antibody and immunoblotted with insulin receptor (**a**) or antiphosphotyrosine (**b**) antibody. The insulin receptor protein level, the basal (-) and insulin-stimulated (+) insulin receptor phosphorylations are the mean ± S.E.M. of the scanning densitometries of 13 control and 15 restricted animals to insulin receptor protein level and 8 control and 9 restricted animals to insulin-induced insulin receptor phosphorylation.

Figure 2: IRS-1 protein level (**a**), insulin receptor phosphorylation (**b**) and IRS-1/PI 3-kinase association (**c**) in liver of control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Thirty seconds later, the liver was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with IRS-1 antibody and immunoblotted with anti-IRS-1 (**a**) or antiphosphotyrosine (**b**) or anti-PI 3-kinase (**c**) antibodies. The IRS-1 protein level, the basal (-)and insulin-stimulated (+) responses are mean ± S.E.M. of the scanning densitometries of 12, 10, 6 control animals and 13, 10, 6 restricted animals, respectively. * $p<0.05$.

Figure 3: Insulin receptor protein level (**a**) and phosphorylation (**b**) in muscle of control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Ninety seconds later, the muscle was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with insulin receptor antibody and immunoblotted with anti-insulin receptor (**a**) or antiphosphotyrosine (**b**) antibody. The insulin receptor protein level, the basal (-) and insulin-stimulated (+) insulin receptor phosphorylations are the mean ± S.E.M. of the scanning densitometries of 7 control and 8 restricted animals to insulin receptor protein level and 6 control and 7 restricted animals to insulin-induced insulin receptor phosphorylation.

Figure 4: IRS-1 protein level (**a**), insulin receptor phosphorylation (**b**) and IRS-1/PI 3-kinase association (**c**), in muscle control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Ninety seconds later, the muscle was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with IRS-1 antibody and immunoblotted with anti-IRS-1 (**a**) or antiphosphotyrosine (**b**) or anti-PI 3-kinase (**c**) antibodies. The IRS-1 protein level, the basal (-) and insulin-stimulated (+) IRS-1 responses are the mean ± S.E.M. of the scanning densitometries of 9, 11, 6 control animals and 10, 12, 7 restricted animals, respectively. * $p<0.05$.

Figure 5: Insulin receptor protein level (**a**) and phosphorylation (6) in adipose tissue of control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Two minutes later, the adipose tissue was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with insulin receptor antibody and immunoblotted with anti-insulin receptor (**a**) or antiphosphotyrosine (**b**) antibodies. The insulin receptor protein level, the basal (-) and insulin-stimulated insulin receptor phosphorylation (+) are the mean ± S.E.M. of the scanning densitometries of 7 control and 7 restricted animals to insulin receptor protein level and 5 control and 6 restricted animals to insulin-induced insulin receptor phosphorilation.

Figure 6: IRS-1 protein level (**a**), phosphorylation (**b**), IRS-1/PI 3-kinase association (**c**) and IRS-1/SHPTP2 association (**d**) in adipose tissue of control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Two minutes later, the adipose tissue was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with IRS-1 antibody and immunoblotted with anti-IRS-1 (**a**) or antiphosphotyrosine (**b**) or anti-PI 3-kinase (**c**) or anti-SHPTP2 (**d**) antibodies. The IRS-1 protein level, the basal (-) and insulin-stimulated (+) responses are the mean ± S.E.M. of the scanning densitometries of 13, 11, 8, 6 control animals and 12, 10, 6, 5 restricted animals, respectively. * $p<0.05$.

Table 1: Metabolic characteristics of rats after 28 days on a restricted diet

Diet (*n*)	*Weight* (*g*) (*23*)	*Glucose* (*mg/dl*) (*8*)	*Insulin* (*μUI/dl*) (*8*)	*Triacylglycerols* (*mg/dl*) (*8*)	*Cholesterol* (*mg/dl*) (*8*)	*Albumin* (*g/dl*) (*8*)
Control	524±45	90±13	33±7	108±31	91±15	2.75±0.21
Restricted	413±41*	92±12	19±6*	50±12*	96±18	2.72±0.17

Values are means ± SD of the number (n) of animals used indicated. * $p<0.05$ when compared to controls.

Fig1 - liver

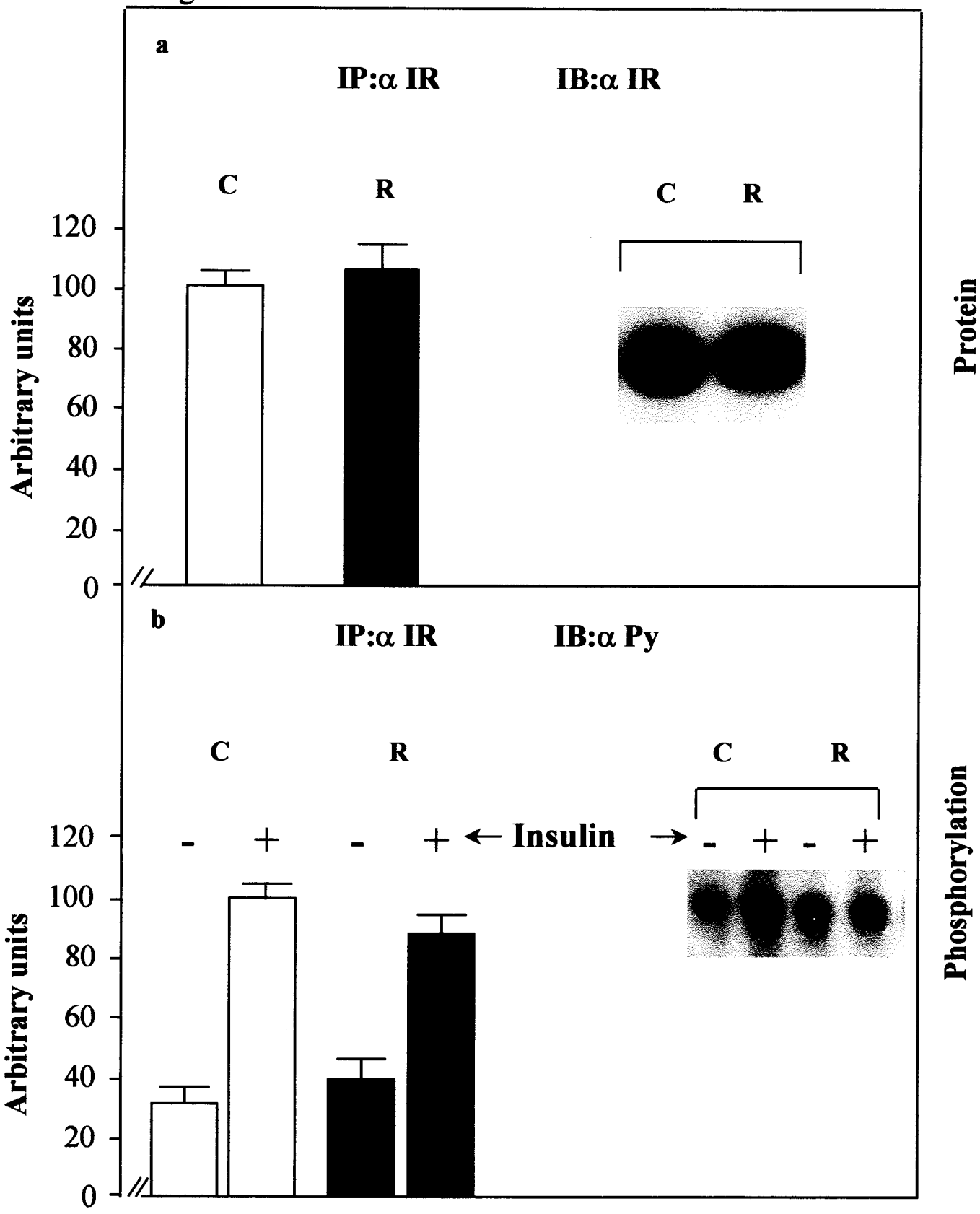

Fig2 - liver

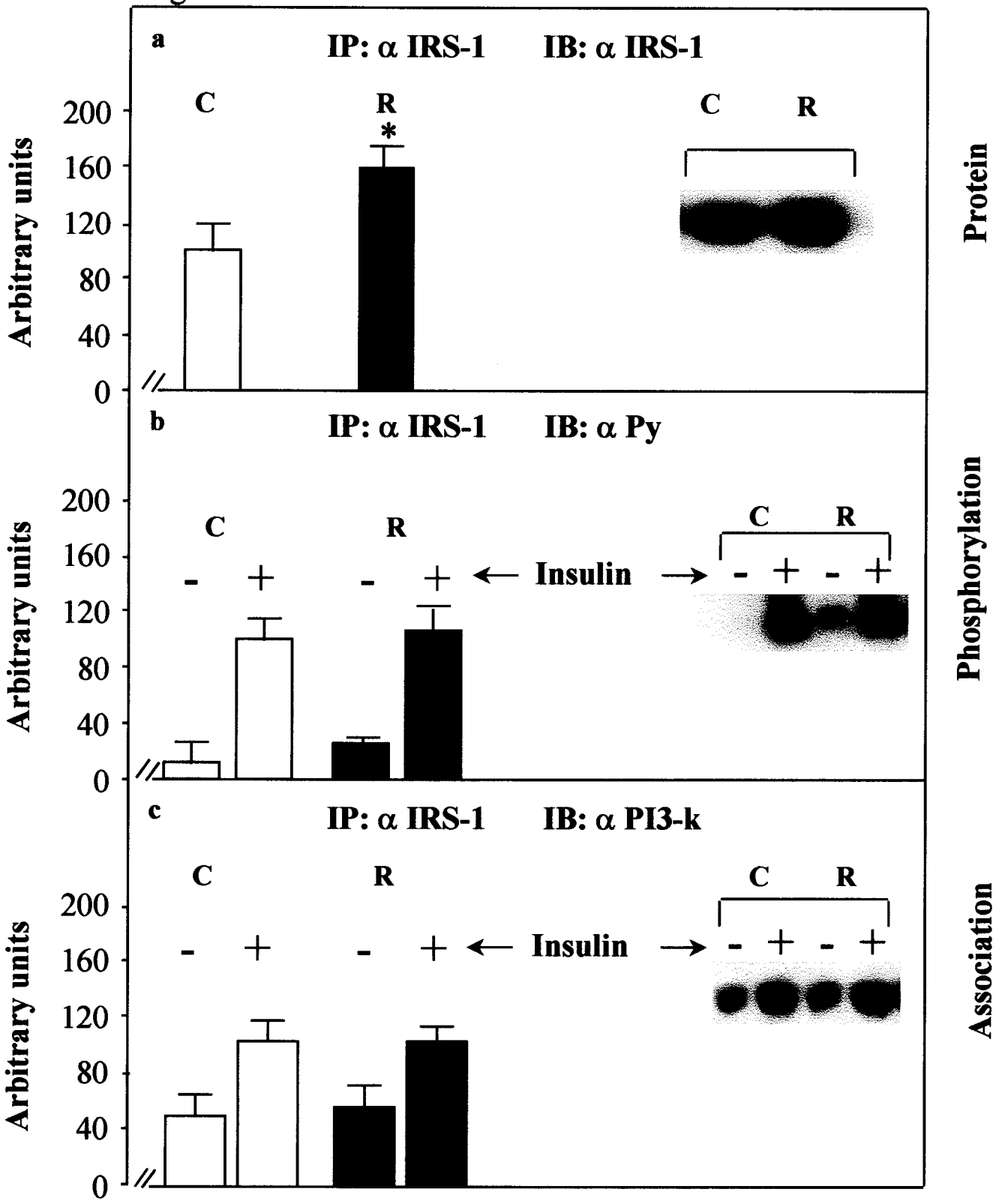
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Fig3 - muscle

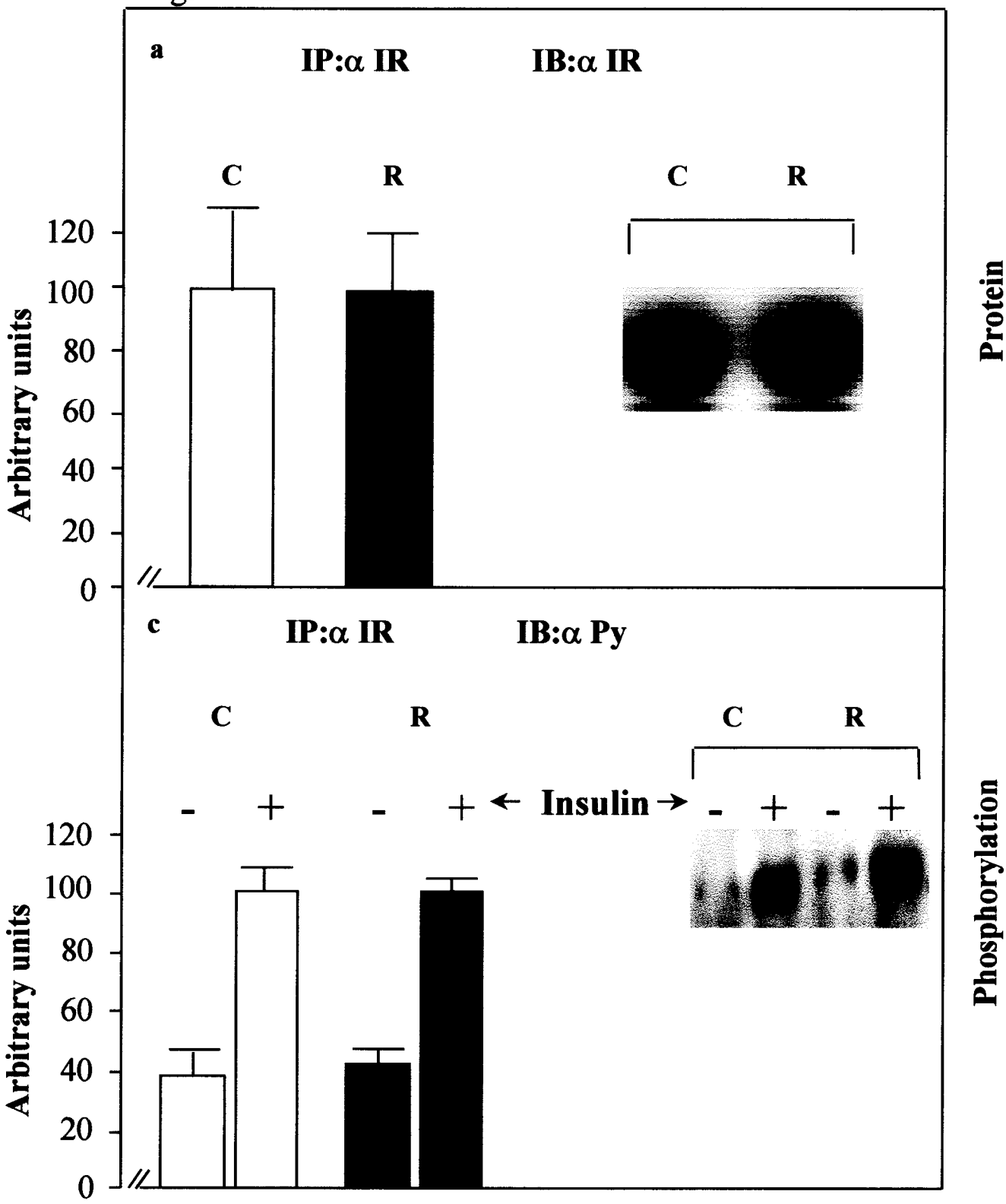
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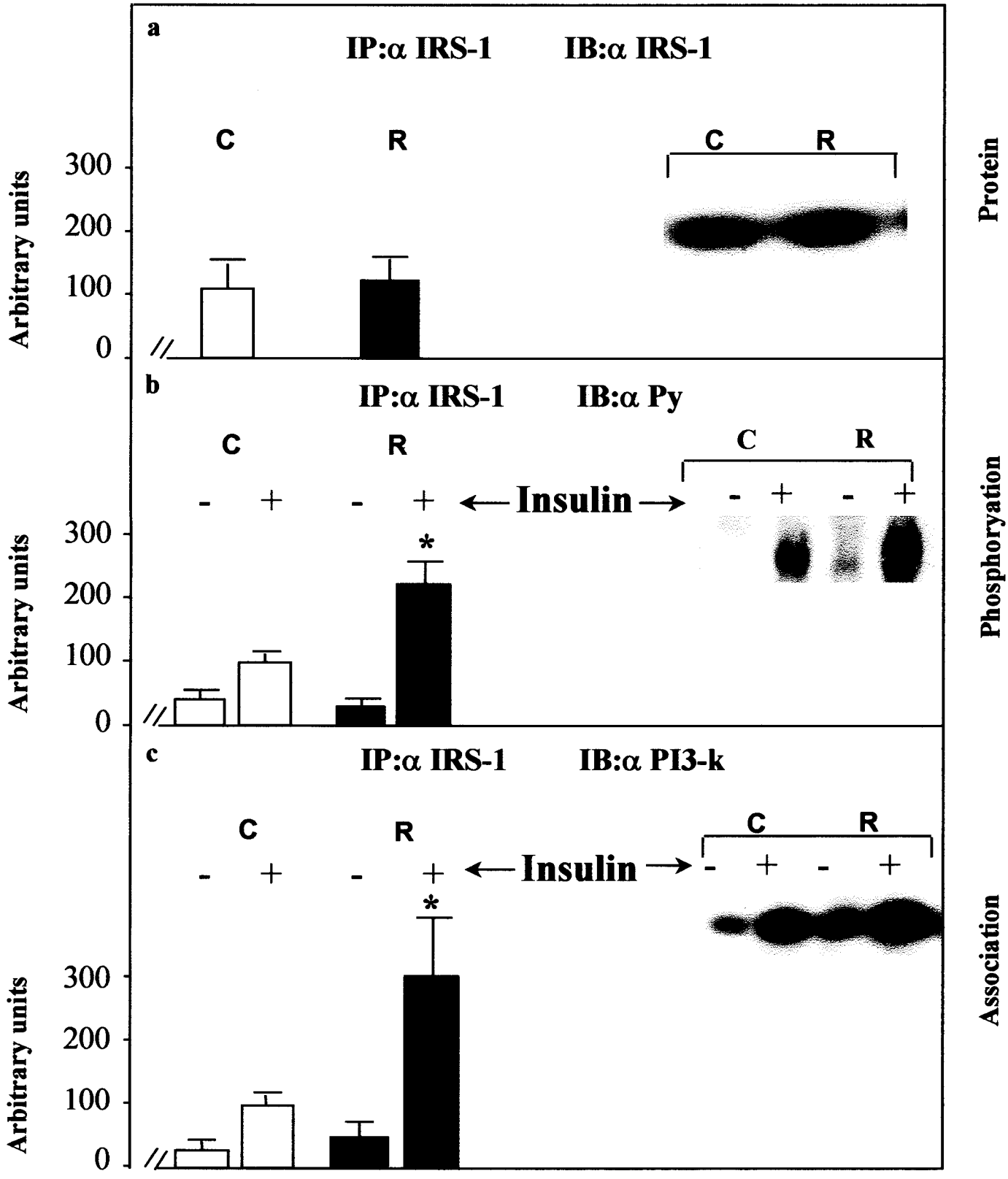
Fig4 - muscle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- + - +
*
300
200
100
0
Arbitrary units
Phosphoryation
c
IP:α IRS-1
IB:α PI3-k
C
R
C
R
- + - + ← Insulin →
- + - +
*
300
200
100
0
Arbitrary units
Association

Fig5 - adipose tissue

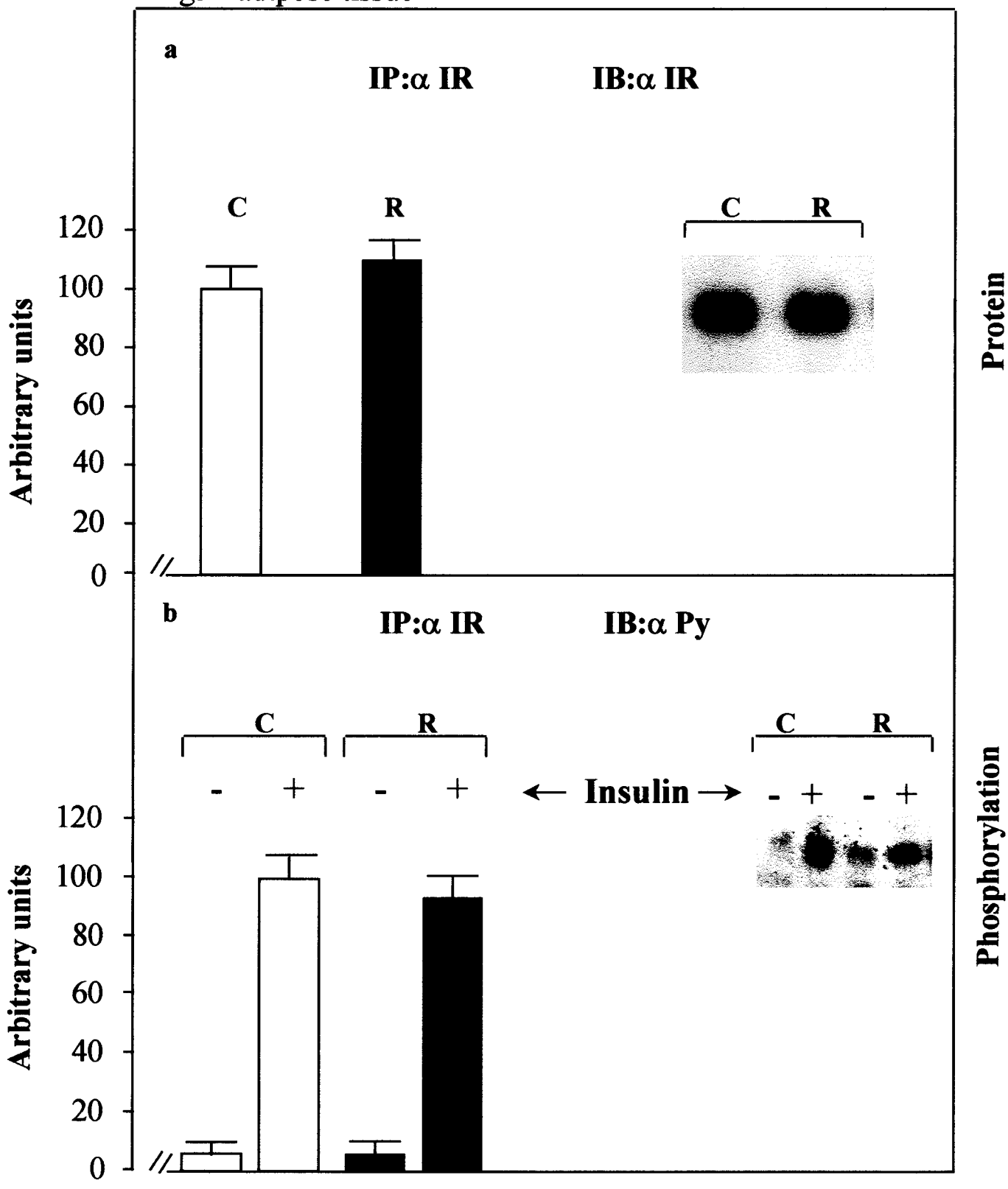
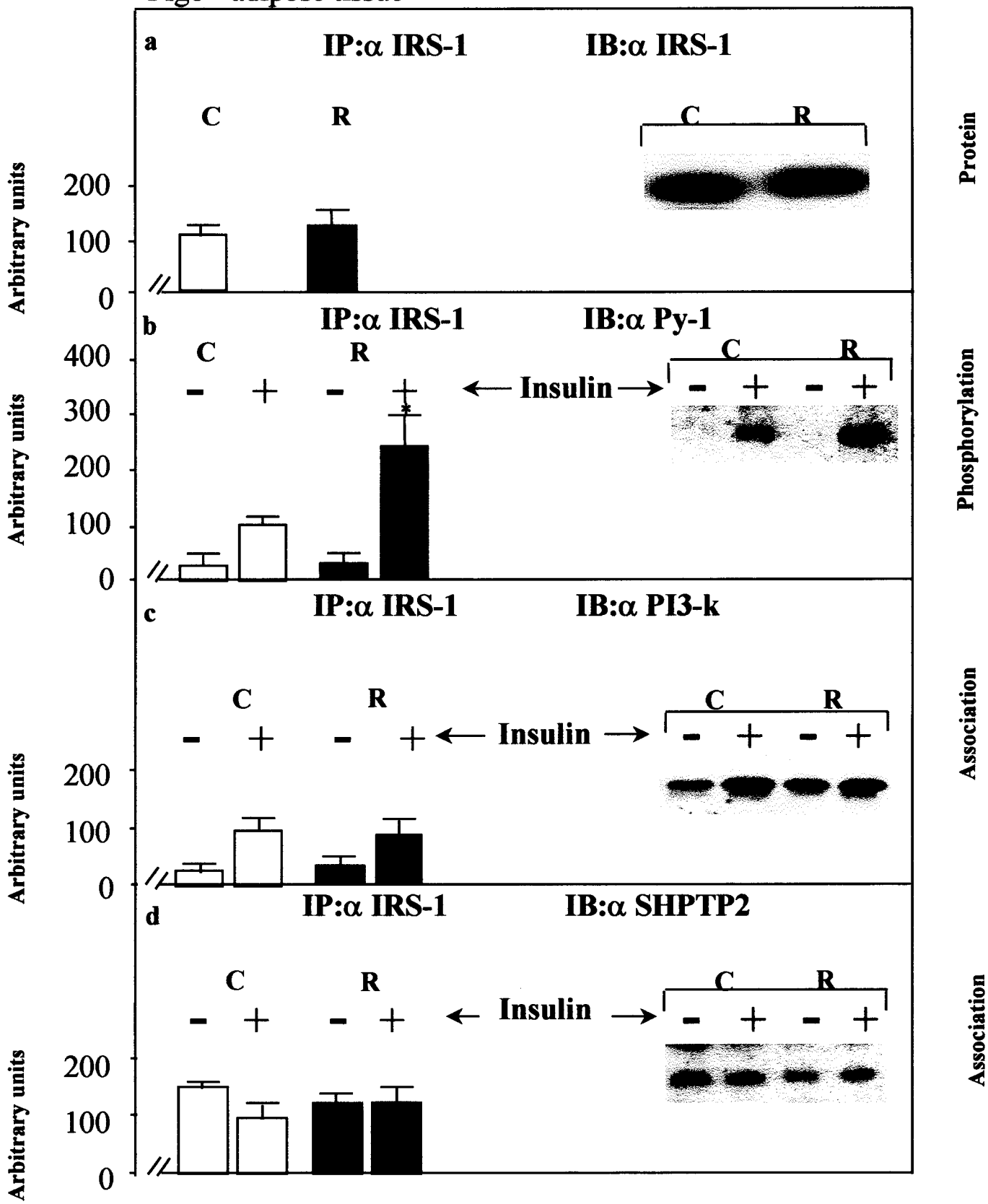


Fig6 - adipose tissue



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4. Artigo 2

DIETARY RESTRICTION IMPROVES INSULIN SENSITIVITY IN TWO-MONTH-OLD RATS

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Key words: Dietary restriction, IRS-1, insulin resistance, tyrosine phosphorylation, insulin receptor

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SUMMARY

The present study investigated whether the dietary restriction could alter the sensitivity to insulin in 2-month old male Wistar rats. We have examined the levels and phosphorylation state of the insulin receptor and insulin receptor substrate 1 (IRS-1), as well as the association between IRS-1 and phosphatidylinositol 3-kinase (PI 3-kinase) in liver, muscle and adipose tissue of 2-month-old rats by immunoprecipitation and immunoblotting with anti-insulin receptor, anti-IRS-1, anti-PI 3-kinase, anti- SHP2 and antiphosphotyrosine antibodies.

The body weight of diet restricted animals declined during the first week and then increased up to the 28th day the study. The fasting glucose levels were elevated in diet restricted rats but there was no alteration in the serum levels of insulin, triacylglycerols, cholesterol and albumin. The sensitivity to insulin was increased in the rats as shown by elevated glucose disappearance rate during the 16-min ITT.

Dietary restriction did not change the levels of insulin receptor protein, insulin-induced insulin receptor phosphorylation, or the IRS-1 protein levels in liver, muscle and adipose tissue. However, muscle IRS-1 phosphorylation was increased but was not accompanied by a rise in IRS-1/PI 3-kinase association. IRS-1 phosphorylation was unaltered in liver and adipose tissue of diet restricted rats but there was an increase in IRS-1/PI 3-k association in adipose tissue and in IRS-1/SHP2 association all three tissue.

In conclusion, dietary restriction improves the sensitivity to insulin in young animals. This improvement is accompanied by increases in IRS-1 tyrosine phosphorylation in muscle, IRS-1/PI 3-kinase association in adipose tissue and IRS-1/Syp association in liver, muscle and adipose tissue.

INTRODUCTION

Insulin initiates its metabolic and growth-promoting effects by binding to the α subunit of its tetrameric receptor, thereby activating the kinase in the β (White and Kahn, 1994). This interaction catalyses the intramolecular autophosphorylation of specific tyrosines residues of the β subunit which further enhances the tyrosine kinase activity of the receptor toward other protein substrates (White and Kahn, 1994). In most cells, this primary event leads to the subsequent tyrosyl phosphorylation of a cytoplasmic protein with an apparent molecular weight of 160-185 kDa, called insulin receptor substrate 1 (IRS-1) is a protein tyrosine kinase which, when activated, undergoes rapid autophosphorylation and phosphorylates intracellular protein substrates, including insulin receptor substrate-1 (IRS-1) (White et al., 1985; Momomura et al., 1987; Sun et. al., 1991). Tyrosine phosphorylated IRS-1 can associate with several Src homology 2 (SH2) domain-containing molecules, including phosphatidylinositol 3-kinase (PI 3-kinase) and protein tyrosine phosphatase (SHP2) (Lamphere et al., 1994; Backer et al., 1992; Folli et al., 1993; Lavan et al., 1992; Yamauchi et al., 1995). The interaction between the IRS-1 and PI 3-kinase is essential for many insulin-sensitive metabolic processes, including the stimulation of glucose transport and glycogen and protein synthesis (Hara et al., 1994, Cheatham et al., 1994; Clarke et al., 1994).

The early steps of insulin action may have a role in the molecular mechanism of insulin resistance. The decrease in IRS-1 tyrosine phosphorylation and in association with PI 3-kinase in the liver and muscle of aging rats is paralleled by the appearance of the insulin resistance in these animals (. Carvalho et al, 1996).

Captropil, an angiotensin-converting enzyme (ACE) inhibitor increases IRS-1 tyrosine phosphorylation and improves insulin action in aging rats. Such an effect is not observed in young rats and suggests that this drug reduce insulin resistance but not insulin action in normal rats. Recently, dietary restriction has been shown to increase the sensitivity to insulin and to modulates the early steps in insulin action in aging rats (data not shown).

This study assessed the effect of dietary restriction on insulin action by examining the phosphorylation status of the insulin receptor and IRS-1 as well as the association of the latter with PI 3-kinase and Syp in the liver, muscle and adipose tissue of young rats maintained on restricted diet.

METHODS

Materials

The reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiothreitol, Triton X-100, Tween 20, and glycerol were from Sigma Chemical Co. (St. Louis, MO). Protein A Sepharose 6 MB was from Pharmacia (Uppsala, Sweden), [^{125}I]protein A was from Amersham (Aylesbury, UK) and nitrocellulose paper (BA85, 0.2 μm) was from Schleicher & Schuell (Keene, NH). Sodium thiopental and human recombinant insulin (Humulin R) were from Elli Lilly Co. (Indianapolis, IN). Male Wistar rats were from the UNICMP'S Central Animal Breeding Laboratory. Monoclonal antiphosphotyrosine, polyclonal anti-insulin receptor, anti-IRS-1, and anti-SHP2 antibodies were from Santa Cruz Technology (Santa Cruz, CA). Polyclonal anti-PI 3-kinase (p85) antibody was from Upstate Biotechnology Incorporated (Lake Placid, NY).

Animals and food restriction plan

Male *Wistar* rats two months old was divided randomly into two groups and each subjected for four weeks to a different dietary regimens. The group without food restriction was fed standard rodent chow (Nuvilab CR-1, Nuvital, Curitiba, SP; composition: 22% protein, 3% fat, 9% fiber, 8% minerals, 1.4% calcium and 0.6% phosphorus) and received water *ad libitum*. Food-restricted rats were provided with water *ad libitum* and 60% of the normal amount of standard rodent chow, calculated from the daily food intake of the controls rats. The amount of food ingested was measured every two days and the weight of each rat determined once a week. The rats were maintained on a 12 h light-dark cycle with light on at 7 a.m.. All rats were fasted for 14 h before the experiments.

Methods

Rats were anesthetized with sodium thiopental (24 mg/kg b.w., ip) and opened 10-15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein exposed, and 0.5 ml of normal saline (0.9%

NaCl) with or without 6 μg of insulin was injected. Thirty seconds later, a liver sample was removed, minced coarsely and homogenized immediately in approximately ten volumes of solubilization buffer (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 vanadate, 2 mM PMSF and 0.1 mg of aprotinin/ml) at 4 °C using a Polytron PTA 20S generator (model PT 10/35, Brinkmann Instruments, Westbury, NY) operated at maximum speed for 20 s.

Approximately 90 sec and 120 sec after the injection as described above, samples of hindlimb muscle and epididimal adipose tissue, respectively, were quickly excised and homogenized as described for liver. Both extracts were centrifuged at 15,000 rpm at 4°C for 45 min to remove insoluble material and the supernatants were used in the assays described below. Protein determination was performed by the biuret method using the Labtest reagent (Labtest Diagnóstica S.A., M.G., Brazil.) and bovine serum albumin (BSA; Sigma Chemical Co.) as standard. Supernatants of the three tissues were used for immunoprecipitation with anti-insulin receptor or anti-IRS-1 antibody and followed by adsorption with Protein A Sepharose 6 MB.

Immunoblotting

Immunoprecipitated proteins were treated with Laemmli sample buffer (Laemmli, 1970) containing 100 mM dithiothreitol and heated in a boiled in a water bath for 4 min, after which they were separeted by SDS-PAGE (6% bis-acrylamide) in a Bio-Rad miniature gel apparatus (Mini-Protean, Bio-Rad Laboratories, Richmond, CA).

Electrotransfer of the proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin et al. (1979), except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high M_r proteins. Non-specific protein binding to the nitrocellulose was reduced by pre-incubating the filter in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20) overnight at 4° C. The nitrocellulose blot was then incubated overnight at 4°C with antiphosphotyrosine (αPy) (1 μg/ml), anti-insulin receptor (1:100), anti-IRS-1 (1:100), anti-SHPTP2 (1:100), or anti PI 3-kinase (1:500) antibodies diluted in blocking buffer (0.3% BSA instead of nonfat dry milk) and then washed for 30 min with blocking buffer without milk.

The blots were subsequently incubated with 2 μCi of [^{125}I]protein A (30 μCi/μg) in 10 ml of blocking buffer for 2 h at room temperature and then washed again for 30 min as described above. [^{125}I]Protein A bound to the antiphosphotyrosine and antipeptide antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak, Rochester, NY) with Cronex Lightning Plus intensifying screens (DuPont, Wilmington, DE) at -80°C for 12-48 h. Band intensities of the developed autoradiographs were quantitated by densitometry (model GS 300, Hoefer Scientific Instruments, San Francisco, CA).

Estimation of insulin action in vivo using the 16 minutes insulin tolerance test (ITT)

Twelve food restricted rats and 13 rats with unrestricted access to food received insulin (6 μg in 0.5 ml.) i.v. and blood samples for glucose determination were collected at 0 (basal), 4, 8, 12 and 16 min post-injection. Tail blood from rats anesthetized, 20μl was collected in TCA, centrifuged at 6000 rpm at 4°C for 5 min and the blood glucose level measured by the glucose oxidase method using a commercial kit. The rate constant for plasma glucose disappearance (Kitt) was calculated from the formula $0.693/t_{1/2}$. The plasma glucose half-life ($t_{1/2}$) was calculated from the slope of the least square analysis of the plasma glucose concentration during the linear phase of decline (Bonora et al, 1989).

Serum analysis

Blood obtained by cardiac puncture of 8 rats of both groups was centrifuged at 6000 rpm, at 20°C for 15 min and the serum was used to insulin, glucose, triacylglycerols, cholesterol and albumin. All analyses were performed using commercial kits.

Statistical analysis

The results are means ± SD or means ± SEM of values obtained from independent experiments. Statistical evaluation was performed by the nonpaired Student's *t* test was used. The level of significance was set at $p<0.05$.

RESULTS

Body weight and serum analysis

The body weights and serum analyses for the two groups of rats after 28 days are shown in Table 1. The restricted diet for 28 days resulted in a 6% lag of weight gain while the control animals showed a 20% gain in weight. The insulin, triacylglycerols, cholesterol and albumin levels were similar in both groups of rats but glucose levels were higher in the animals on restricted diet. The glucose disappearance rate in diet restricted rats (n=13) was 3.79 ± 0.32%%/min compared to 2.57 ± 0.26%/min ($p<0.05$) for the control group (n=14).

Effect of dietary restriction on insulin receptor phosphorylation and protein level, IRS-1 phosphorylation and protein level, IRS-1/PI3-k and IRS-1/SHP2 associations in the liver

There were no significant differences in the insulin-stimulated tyrosine phosphorylation of the 95 kDa ß subunit of the insulin receptor or in the insulin receptor protein levels in the liver (Fig.1) of diet-restricted and non restricted rats. The IRS-1 phosphorylation and protein levels were not different between the two groups of rats (Fig.2). The association of the IRS-1 with PI 3-kinase no change but the association of the IRS-1 with SHP2 (Fig.2) increased to 138±9% *($p<0.05$)* in the liver of diet restricted rats

Effect of dietary restriction on insulin receptor phosphorylation and protein level, IRS-1 phosphorylation and protein level, IRS-1/PI3-k and IRS-1/SHP2 associations in the muscle

The insulin receptor phosphorylation and protein levels were not different between the two groups of rats (Fig.3). The dietary restriction no change the IRS-1 protein levels and increased the IRS-1 phosphorylation to 127±5% *($p<0.05$)* in muscle (Fig.4).Although there was not difference in the IRS-1/PI 3-kinase association between the two groups rats, the IRS-1/SHP2 association increased to 143±17% *($p<0.05$)* in rats on dietary restriction (Fig.4).

Effect of dietary restriction on insulin receptor phosphorylation and protein level, IRS-1 phosphorylation and protein level, IRS-1/PI3-k and IRS-1/SHP2 associations in the adipose tissue

There were no significant differences in the insulin-stimulated tyrosine phosphorylation of the insulin receptor or in the insulin receptor protein levels in adipose tissue (Fig.5) of diet-restricted and controls rats. Also there were not difference in the IRS-1 phosphorylation and protein levels between diet restricted and non-restricted rats. After stimulation with insulin, the association of IRS-1 with PI 3-kinase increased to 157±7% *($p<0.05$)* and the association of IRS-1 with SHP2 increased to 139±9% *($p<0.05$)* in the diet restricted rats.

DISCUSSION

Dietary restriction increase the sensitivity to insulin (data not shown). In obese 14-mo-old which is a model of insulin resistance, dietary restriction improves the action of insulin in parallel with an increase in IRS-1 tyrosine phosphorylation and association with PI 3-kinase. In this study, we investigated the effect of dietary restriction on insulin sensitivity and on the early steps of insulin signal transduction in liver, muscle and adipose tissue of young rats.

The body weight of diet restricted animals declined during the first week, but quickly increased thereafter up to the 28th day. The body weights of these rats were similar to those reported by Koyama et al (1997) for 6-7-week-old Wistar rats with a 42% reduction in food intake for 14 days.

Dietary restriction increased the fasting plasma glucose levels but did not alter the serum insulin, triacylglycerols, and cholesterol and albumin levels (Table 1). In contrast, previous studies (Koyama et al., 1997; Rao, 1995) have shown that food restriction in young rats lowered glucose and insulin levels. This discrepancy may be explained by methodological differences such as the extent of dietary restriction, the age of the rats at the start of the experiments and the strains of the animals used.

Restricted access to food improved the insulin sensitivity in 2-month-old rats after 28 days, as shown by the increase in the glucose disappearance rate during the 16-min ITT. The mechanism underlying this improved sensitivity to insulin not fully understood. Dietary restriction did not change the levels of insulin receptor protein, insulin-induced insulin receptor phosphorylation, and IRS-1 protein in the liver, muscle and adipose tissue of 2-month-old rats.

IRS-1 protein levels in the liver increased during dietary restriction in 14-mo-old rats (data not shown). In the present study, the insulin levels in diet restricted rats were unchanged and contrasted with the decreased levels this hormonal in 14-month-old diet restricted rats. IRS-1 regulation is tissue specific and IRS-1 phosphorylation depends more on insulin receptor kinase activity than on IRS-1 protein levels (Saad et al., 1992). The level of IRS-1 protein in liver was inversely related to the levels of insulin, suggesting that insulin played a role in regulating IRS-1 expression.

IRS-1 phosphorylation increased in the muscle of diet-restricted rats but this was not accompanied by an increased IRS-1/PI 3-kinase association. The elevated IRS-1 phosphorylation in muscle may be of biological significance since the decreased insulin receptor and IRS-1 phosphorylation, seen in aging animals, is correlated with insulin resistance (Carvalho et al., 1996).

IRS-1 phosphorylation was unaltered in the liver and adipose tissue of diet restricted young rats whereas there were an increase in IRS-1/PI 3-k association in the adipose tissue of these animals.

PI 3-kinase is essential for many insulin-sensitive metabolic processes, including the stimulation of glucose transport and glycogen and protein synthesis (Hara et al., 1994, Cheatham et al., 1994; Clarke et al., 1994), and the translocation of GLUT4 and GLUT1 (Hara et al., 1994; Cheatham et al., 1994; Clarke t al., 1994; Yeh et al., 1995; Sanches-Margalet et al., 1994; Stephens et al., 1995; Quon et al., 1995; Haruta et al., 1995; Lam et al., 1994). The increase IRS-1/PI 3-kinase association in adipose tissue of diet restricted rats noted above suggests an enhanced in glucose transport and glucose utilization in this tissue. Since adipose tissue does not contribute significantly to insulin-induced glucose utilization, it is unlikely that the increase in IRS-1/PI 3-kinase interaction in this tissue could account for the improved sensitivity to insulin. Sumida and Machado (1998) reported that dietary restriction increased GLUT-4 expression and improved the sensitivity to insulin in adipose tissue of 2-month-old rats.

IRS-1 contains 22 potential tyrosine phosphorylation sites that are part of specific recognition sites for cellular substrates bearing SH2 domains, including PI 3-kinase (Tyr460, Tyr608, Tyr939, Tyr987), the small adaptor Grb2 (Tyr895), the protein tyrosine phosphatase SHP2 (Tyr1172, Tyr1222) and other proteins (Sun et al., 1991; Keller et al, 1993, Sugimoto et al, 1994). SHP2 (Syp) binds to IRS-1 through SH2 domains and leads to increased tyrosine phosphatase activity (Lavan et al., 1992). Noguchi et al. (1994) demonstrated that Syp is an upstream regulator of the Ras/MAP kinase pathway but not of PI 3-kinase wich suggests that Syp is not involved in insulin-stimulated glucose transport. Hausdorff et al. (1995) showed that Syp blocked insulin-induced mitogenesis, but not insulin-stimulated GLUT4 translocation, and increased expression of GLUT1 leading to increased glucose transport activity.

There was an increase in IRS-1/SHP2 association in the three tissue of diet restricted rats, this was accompanied by increased IRS-1 phosphorylation only in muscle. This finding suggests that the increased IRS-1/SHP2 association in muscle and adipose tissue of diet restricted rats may have eleveted the expression of GLUT1 thereby enhancing glucose transport activity (Hausdorff et al. 1995).

In summary, dietary restriction improves the sensitivity to insulin in young animals. This improvement is accompanied by increases in IRS-1 tyrosine phosphorylation in muscle, IRS-1/PI 3-kinase association in adipose tissue and IRS-1/Syp association in liver, muscle and adipose tissue.

FIGURE LEGENDS

Table 1: Metabolic characteristics of rats after 28 days on a normal (control) and restricted diet.

Figure 1: Insulin receptor protein levels (**a**) and phosphorylation (**b**) in liver of *ad libitum* or control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Thirty seconds later, the liver was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with insulin receptor antibody and immunoblotted with insulin receptor (**a**) or antiphosphotyrosine (**b**) antibody. The insulin receptor protein level, the basal (-) and insulin-stimulated (+) insulin receptor phosphorylations are the mean ± S.E.M. of the scanning densitometries of 10 control and 10 restricted animals to insulin receptor protein level and 8 control and 10 restricted animals to insulin-induced insulin receptor phosphorylation.

Figure 2: IRS-1 protein level (**a**), insulin receptor phosphorylation (**b**) and IRS-1/PI 3-kinase association (**c**) in liver of control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Thirty seconds later, the liver was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with IRS-1 antibody and immunoblotted with anti-IRS-1 (**a**) or antiphosphotyrosine (**b**) or anti-PI 3-kinase (**c**) antibodies. The IRS-1 protein level, the basal (-)and insulin-stimulated (+) responses are mean ± S.E.M. of the scanning densitometries of 9, 9, 8, 6 control animals and 10, 10, 10, 6 restricted animals, respectively. * Significant differences at least at $p<0.05$.

Figure 3: Insulin receptor protein level (**a**) and phosphorylation (**b**) in muscle of control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Ninety seconds later, the muscle was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with insulin receptor antibody and immunoblotted with anti-insulin receptor (**a**) or antiphosphotyrosine (**b**) antibody. The insulin receptor protein level, the basal (-) and insulin-stimulated (+) insulin receptor phosphorylations are the mean ± S.E.M. of the scanning densitometries of 10 control and 10 restricted animals to insulin receptor protein level and 8 control and 8 restricted animals to insulin-induced insulin receptor phosphorylation.

Figure 4: IRS-1 protein level (**a**), insulin receptor phosphorylation (**b**) and IRS-1/PI 3-kinase association (**c**), in muscle control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Ninety seconds later, the muscle was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with IRS-1 antibody and immunoblotted with anti-IRS-1 (**a**) or antiphosphotyrosine (**b**) or anti-PI 3-kinase (**c**) antibodies. The IRS-1 protein level, the basal (-) and insulin-stimulated (+) IRS-1 responses are the mean ± S.E.M. of the scanning densitometries of 11, 9, 10, 8 control animals and 13, 9, 10, 8 restricted animals, respectively. * $p<0.05$.

Figure 5: Insulin receptor protein level (**a**) and phosphorylation (6) in adipose tissue of control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Two minutes later, the adipose tissue was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with insulin receptor antibody and immunoblotted with anti-insulin receptor (**a**) or antiphosphotyrosine (**b**) antibodies. The insulin receptor protein level, the basal (-) and insulin-stimulated insulin receptor phosphorylation (+) are the mean ± S.E.M. of the scanning densitometries of 17 control and 15 restricted animals to insulin receptor protein level and 7 control and 6 restricted animals to insulin-induced insulin receptor phosphorilation.

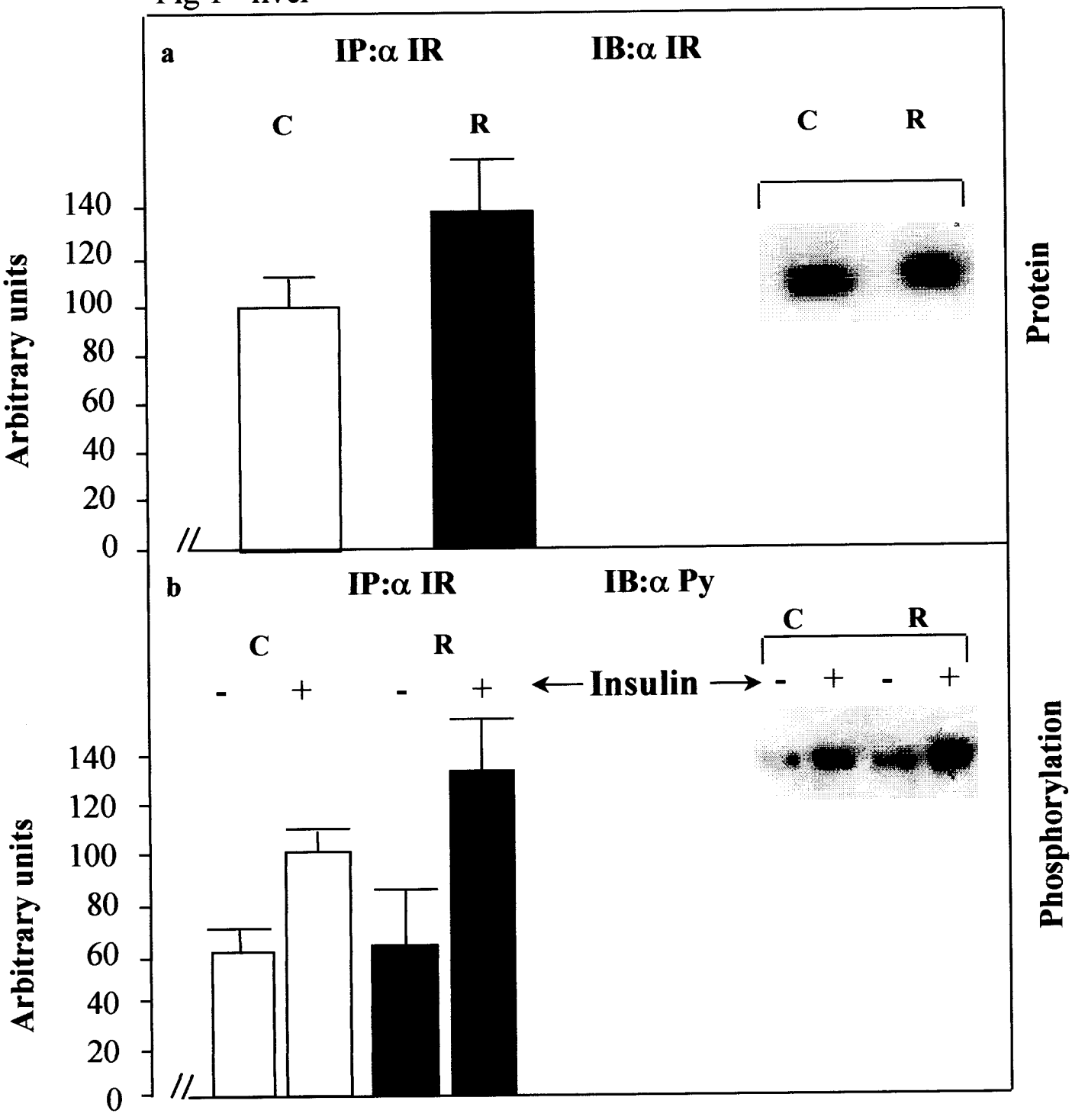
Figure 6: IRS-1 protein level (**a**), phosphorylation (**b**), IRS-1/PI 3-kinase association (**c**) and IRS-1/SHPTP2 association (**d**) in adipose tissue of control (**C**) and diet restricted (**R**) rats. Insulin (6 μg) was administered into the portal vein as a bolus injection. Two minutes later, the adipose tissue was excised and homogenized. Aliquots of the homogenates containing the same amount of protein were immunoprecipitated with IRS-1 antibody and immunoblotted with anti-IRS-1 (**a**) or antiphosphotyrosine (**b**) or anti-PI 3-kinase (**c**) or anti-SHPTP2 (**d**) antibodies. The IRS-1 protein level, the basal (-) and insulin-stimulated (+) responses are the mean ± S.E.M. of the scanning densitometries of 9, 7, 5, 7 control animals and 9, 9, 6, 8 restricted animals, respectively. * $p<0.05$.

Table 1: Metabolic characteristics of rats after 28 days on a restricted diet

Diet	*Weight (g)*		*Glucose (mg/dl)*	*Insulin (μUI/dl)*	*Triacylglycerols (mg/dl)*	*Cholesterol (mg/dl)*	*Albumin (g/dl)*
	Initial	*Final*					
Control	216±18	315±37	78±8	25±8	49±11	32±7	0.8±0.1
n	(32)	(32)	(9)	(8)	(6)	(7)	(7)
Restricted	253±22*	238±18*	93±13*	23±10	56±11	34±5	0.8±0.2
n	(22)	(22)	(13)	(16)	(8)	(9)	(9)

Values are means ± SD of the number (n) of animals indicated. *$p<0.05$ when compared to controls.

Fig 1 - liver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C
R
C
R
140
120
100
80
60
40
20
0
Arbitrary units
Protein
b
IP:α IR
IB:α Py
C
R
C
R
- + - +
Insulin
- + - +
140
120
100
80
60
40
20
0
Arbitrary units
Phosphorylation

Fig2 - liver

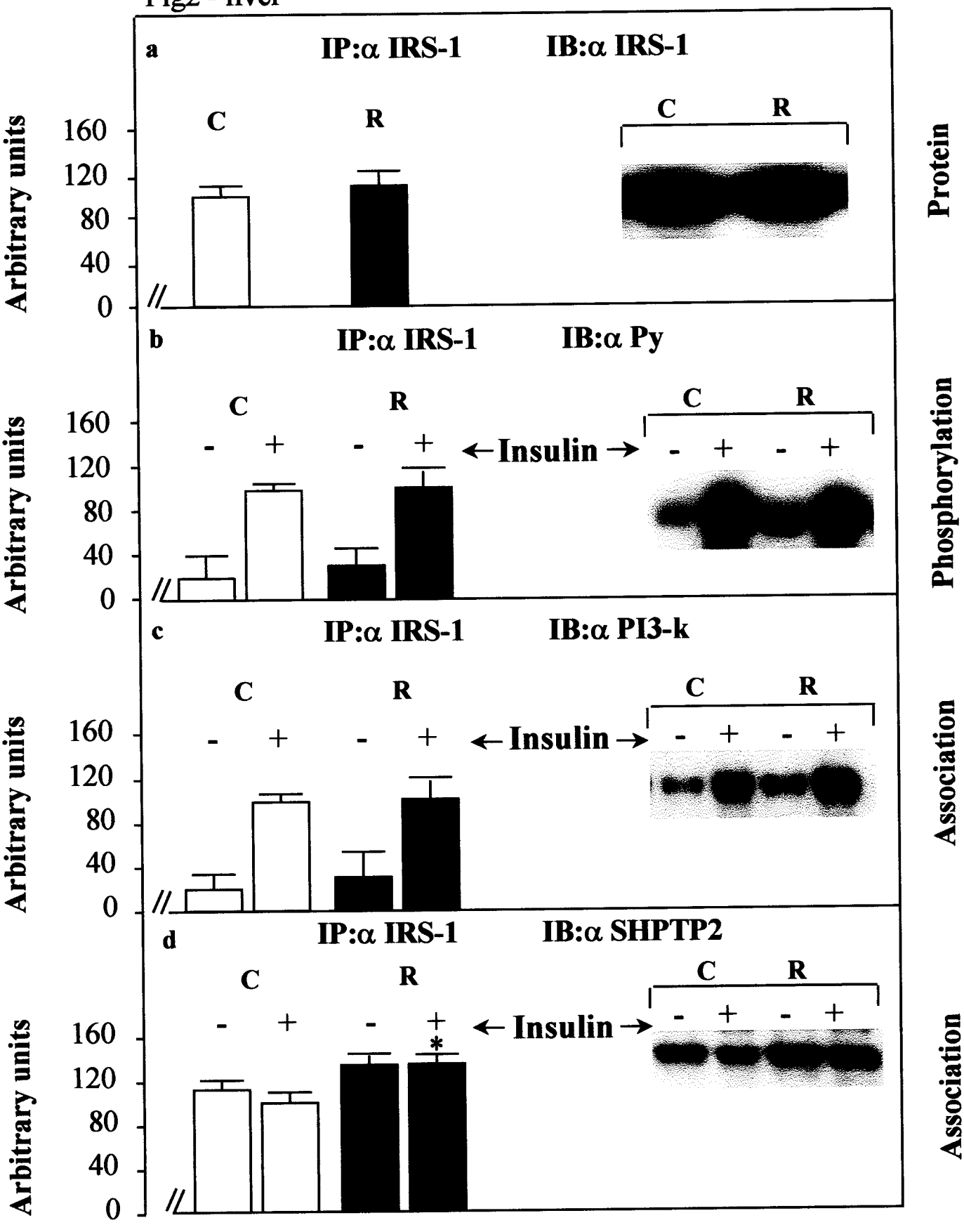
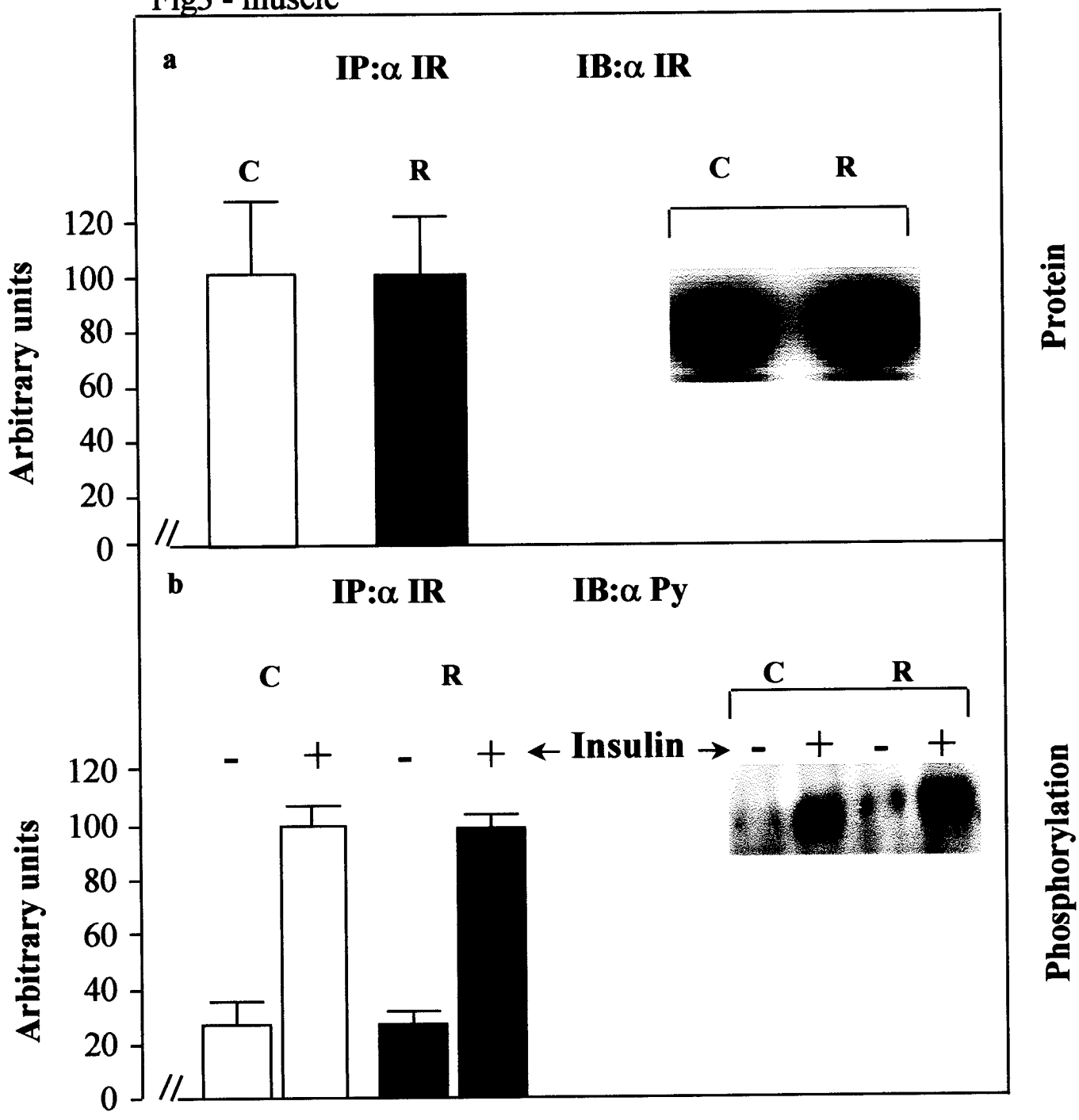

a
IP:α IRS-1
IB:α IRS-1
C
R
160
120
80
40
0
Arbitrary units
Protein
b
IP:α IRS-1
IB:α Py
Insulin
Phosphorylation
c
IP:α IRS-1
IB:α PI3-k
Insulin
Association
d
IP:α IRS-1
IB:α SHPTP2
Insulin
*
Association

Fig3 - muscle


a
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C
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120
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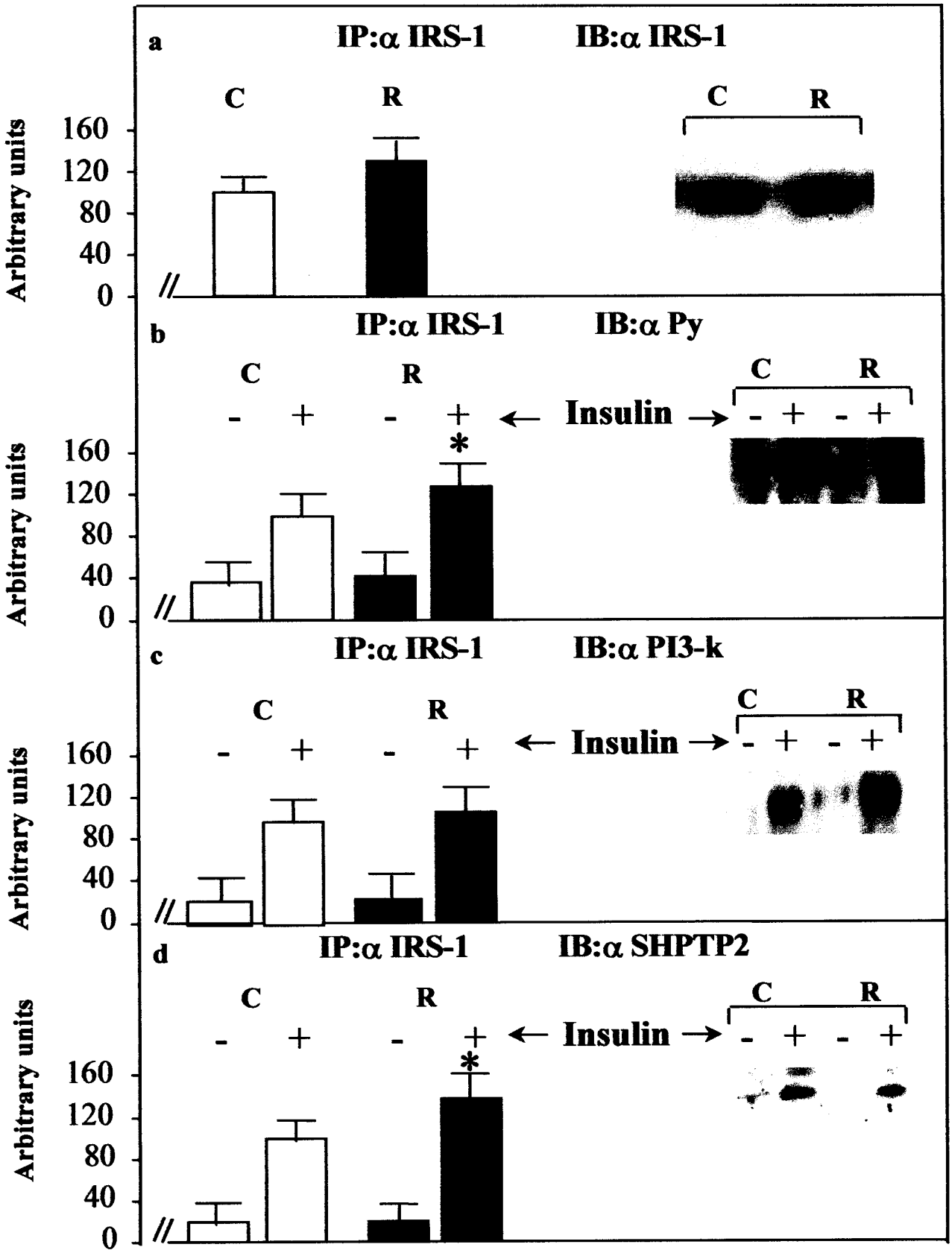
Arbitrary units
Phosphorylation

Fig4 - muscle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160
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Protein
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Arbitrary units
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Phosphorylation
c
IP:α IRS-1
IB:α PI3-k
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R
- + - +
Insulin
- + - +
Arbitrary units
160
120
80
40
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Association
d
IP:α IRS-1
IB:α SHPTP2
C
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C
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Insulin
- + - +
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Arbitrary units
160
120
80
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0
Association

Fig5 - adipose tissue

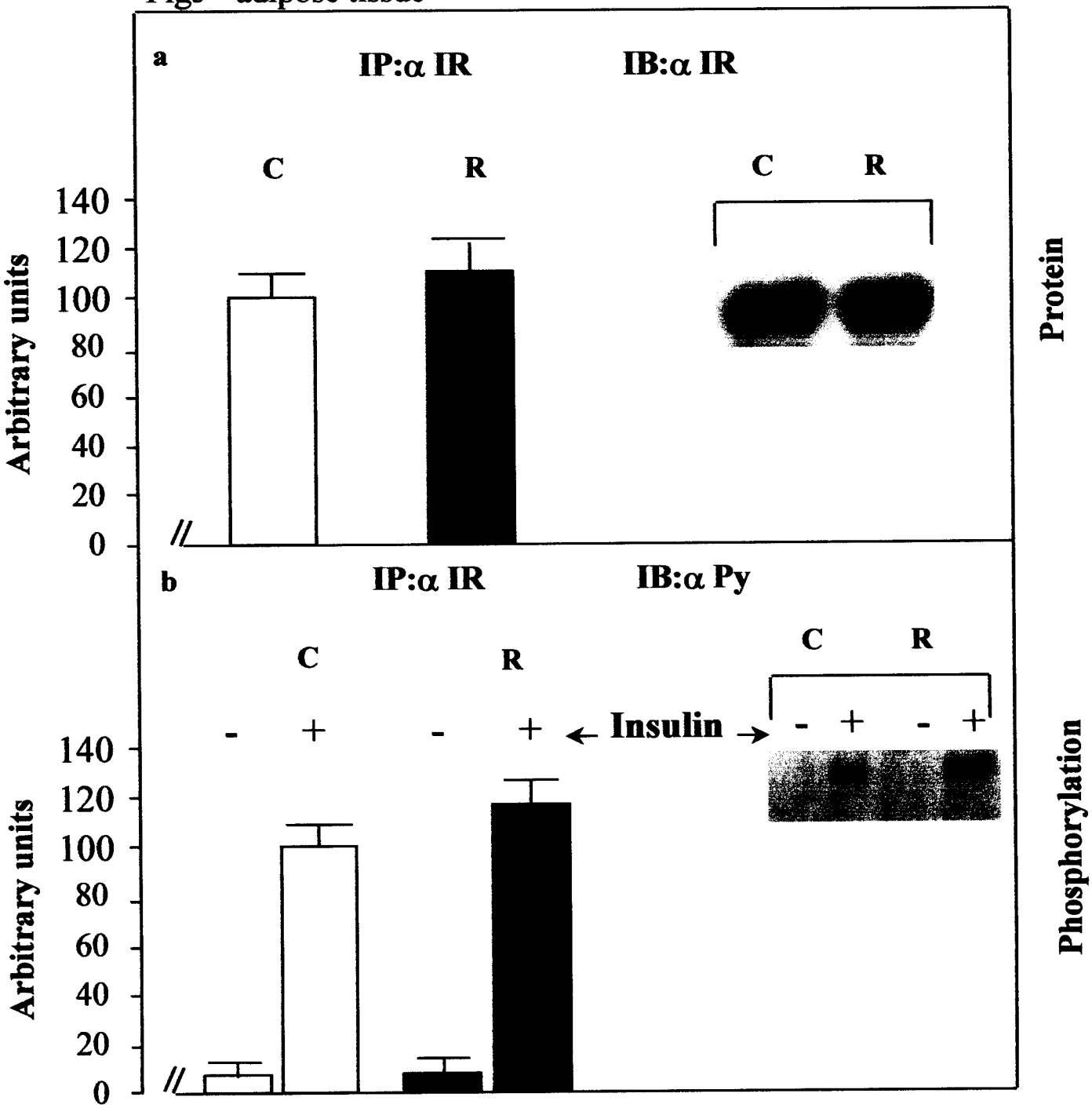
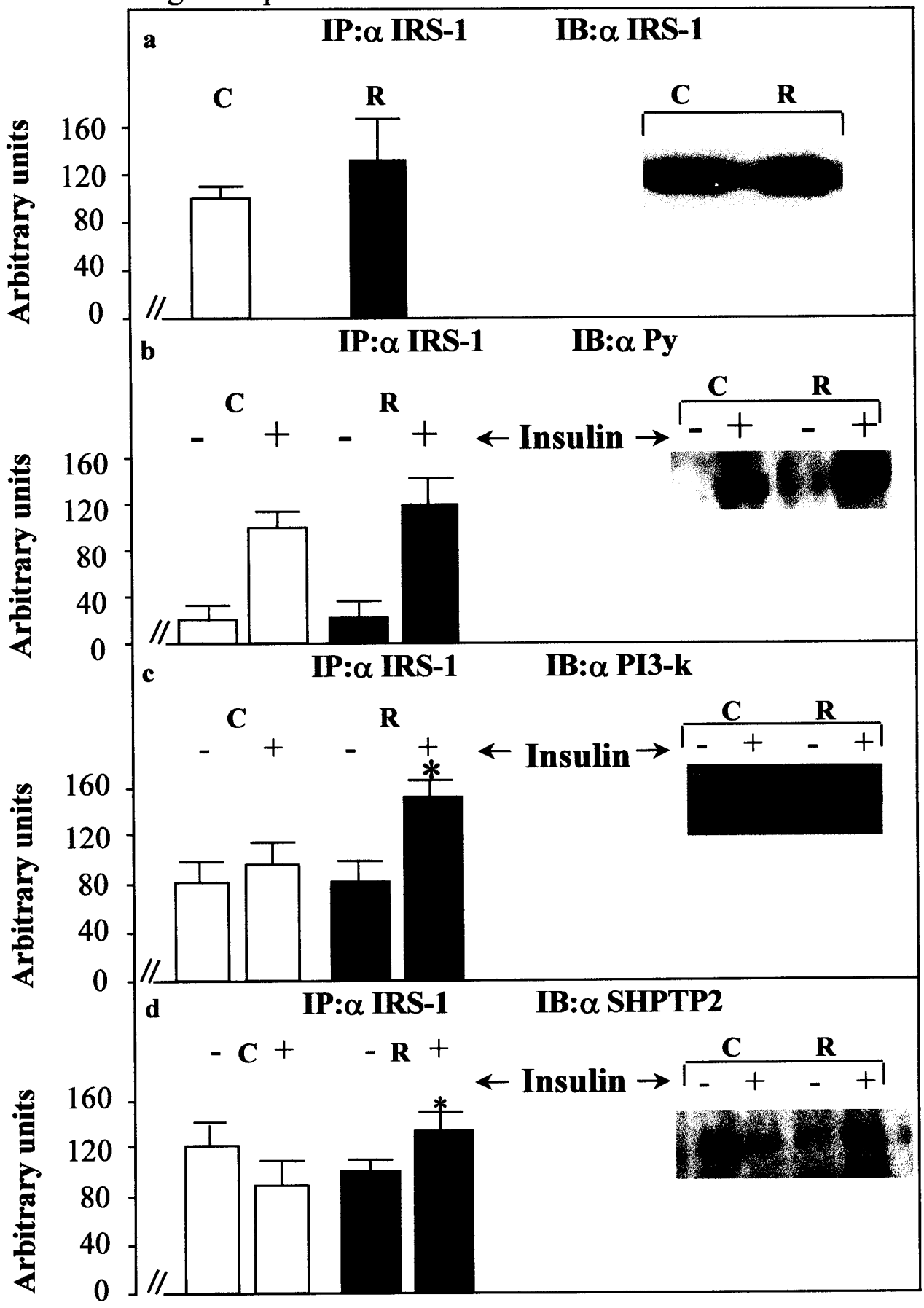


Fig6 - adipose tissue



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5. Discussão

A insulina é um hormônio com efeito metabólico e promotor do crescimento, que atua no metabolismo de carboidratos, proteínas e lipídeos, bem como na síntese de RNA e DNA, em praticamente todas as células, através da modificação da expressão ou atividade de uma série de enzimas e sistemas de transporte (FELIG & BERGMAN, 1990).

Este hormônio liga-se à receptores específicos da superfície celular, presentes em todos os tecidos do mamíferos. A ligação da insulina à subunidade α do seu receptor promove a autofosforilação e implementação da atividade tirosina quinase da subunidade β que então estimulará a fosforilação de resíduos tirosina de outros substratos intracelulares ((WHITE & KHAN, 1985; SAAD, 1994).

O primeiro e mais estudado substrato do receptor de insulina é uma proteína de aproximadamente 185 kDa denominada pp185 pela sua mobilidade eletroforética (WHITE et al., 1985). Segundo revisão de WHITE & KAHN (1994), a fosforilação dos múltiplos resíduos de tirosina em IRS-1, pelo receptor de insulina, gera sítios de reconhecimentos específicos para o domínio SH2 de várias proteínas incluindo: fosfatidil-inositol 3-quinase- PI 3-kinase, o adaptador de proteína que se liga ao receptor do fator de crescimento - GRB2, e a fosfotirosina fosfatase - SHP2.

A PI 3-quinase foi a primeira proteína com domínio SH2 encontrada associada ao IRS-1 (CARPENTER & CANTALEY, 1990). A associação entre PI 3-Kinase e IRS-1 induz a translocação dos transportadores de glicose (GLUT4) da região citoplasmática para a superfície celular seguindo-se a internalização da glicose e síntese de glicogênio através da regulação das proteínas MAPK (proteína kinase ativadora da mitogênese) e da quinase ribossômica S6 (quinases pp70 S6 e pp90 S6) (SAAD, 1994).

A Syp (SHP2), outra proteína de interesse nos estudos de sinalização da insulina, liga-se ao domínio SH2 do IRS-1 aumentando assim sua atividade fosfatase tirosina (LAVAN et al., 1992). HAUSDORFF et al. (1995) demonstraram que a Syp aumenta a expressão de GLUT-1 e como conseqüência o transporte de glicose independente de insulina, e bloqueia a mitogênese mas não a translocação de GLUT-4 induzidos pela insulina (HAUSDORFF et al., 1995).

A resistência à insulina está associada com a diminuição da utilização da glicose, podendo manifestar-se durante o envelhecimento, ou surgir em função da obesidade ou inatividade física (NARIMIYA et al., 1984; NISHIMURA et al., 1988). JACKSON et al.

(1988) demonstraram que a intolerância à glicose relacionada à idade é caracterizada por níveis elevados de insulina, aumento na produção hepática e redução na utilização periférica de glicose.

A restrição dietética é uma conduta nutricional capaz de, prolongar o tempo de vida de animais e humanos, retardar o aparecimento de doenças relacionadas à idade, proteger contra a ação dos radicais livres (Y U, 1994; ROJAS et al., 1993; PIERI et al., 1996; WEINDRUCH, 1996), reduzir os níveis de glicose e insulina plasmática, regular a secreção e a ação insulínica e melhorar a tolerância à glicose (KEMNITZ et al., 1994; REAVEN et al., 1993; BERGAMINI et al., 1991; COLMAN et al., 1995).

O presente trabalho foi conduzido submetendo ratos Wistar jovens com 2 meses e adultos com 14 meses de idade a uma restrição dietética de 40% durante 28 dias. Como controle foram colocados outros 2 grupos de 2 e 14 meses de idade sob a mesma dieta com ingesta *ad libitum*. A dieta consistiu em ração comercial peletizada para roedores (Nuvilab-CR). Os animais com 2 meses de idade, sob restrição dietética, tiveram cerca de 14% de perda de peso, durante a primeira semana. À seguir, nos 21 dias subsequentes, os ratos ganharam peso mas não recuperaram o peso inicial. Ao final do experimento verificou-se uma queda de 6% em relação ao peso corpóreo do início do experimento. Os ratos restritos com 14 meses de idade perderam 18% do peso corporal enquanto que os seus controle mantiveram o mesmo peso durante os 28 dias de experimento. KOYAMA et al. (1997) encontraram o mesmo comportamento de peso nos ratos Wistar de 6 a 7 semanas, com 12 dias de restrição dietética de 42%.

A análise dos parâmetros metabólicos dos ratos restritos com 2 meses de idade mostrou aumento dos níveis séricos de glicose enquanto que os níveis séricos de insulina, triacilgliceróis, colesterol e albumina foram semelhantes aos encontrados nos animais controle. KOYAMA et al. (1997) e RAO (1995) mostraram que ratos jovens com alimentação restrita apresentaram menores concentrações de glicose e insulina em relação aos seus controle. Em nosso trabalho, os ratos restritos com 14 meses de idade reduziram os níveis séricos de insulina e triacilgliceróis, redução esta que não ocorreu para os animais com 2 meses de idade. Consideramos que a redução poderia ser devido à hiperinsulinemia e à hipertriacilgliceridemia já existente nos ratos com 14 meses de idade. De modo parcialmente diverso, LIEPA et al. (1980) demonstraram que a restrição dietética de ratos na faixa etária de 2 a 28 meses, reduziu os níveis séricos de

triacilgliceróis. A discrepância entre os nossos resultados e os de LIEPA et al. (1980) pode ser explicada por diferenças metodológicas tais como o maior tempo de restrição dietética, idade dos ratos no início do experimento e linhagens dos animais utilizados. Confirmando as alterações metabólicas induzidas pela dieta, KEMNITZ et al. (1994) observaram, em macacos, que a restrição dietética reduziu os níveis de insulina e glicose de jejum.

No estudo de tolerância à insulina (ITT) encontramos que a restrição dietética aumenta a taxa de redução da glicose sérica indicando que os ratos restritos do grupo jovem com 2 e do grupo adulto com 14 meses apresentaram, em relação aos seus respectivos controles, maior sensibilidade à insulina e portanto maior transporte de glicose. A perda de peso, obtida sob a restrição dietética, melhorou a utilização de glicose em homens acima de 45 anos, indicando aumento da sensibilidade à insulina (COLMAN et al., 1995). Por outro lado, 40% de restrição dietética em ratos de 1 mês não alterou a homeostase da glicose (RAO, 1995).

GULVE et al. (1993) demonstraram, através de estudos *in vitro,* que o tecido muscular de animais de 1 a 28 meses de idade apresentavam decréscimo gradativo da capacidade de internalização de glicose. No envelhecimento, segundo JACKSON et al. (1988), a intolerância à glicose é caracterizada por elevação nos níveis de insulina, aumento da produção de glicose hepática e redução na utilização periférica de glicose. Para REAVEN et al. (1979) e SCHEEN et al. (1996) o envelhecimento e a obesidade estão associados à diminuição de secreção da insulina pelas células β após estímulo pela glicose.

Estudando modelos de resistência à insulina, pesquisadores encontraram diminuição da quantidade de receptor de insulina e de proteína IRS-1, bem como redução da fosforilação em tirosina e da associação do IRS-1 com PI 3-quinase, após estimulação com insulina (SAAD et al., 1992; CARVALHO et al., 1996; VIDAL et al., 1996 e WIERSMA et al., 1997). Os trabalhos desenvolvidos nesta tese avaliaram a influência da restrição dietética sobre a sinalização insulínica. Estudamos *in vivo* a quantidade protéica e grau de fosforilação em tirosina do receptor de insulina e do IRS-1, assim como as associações do IRS-1 com PI3-quinase e com a Syp em tecidos

hepático, muscular e adiposo de ratos com 2 e 14 meses de idade. Os resultados encontrados nos dois trabalhos, ou seja, nos tecidos dos ratos jovens e dos ratos adultos mostraram que a quantidade protéica e a fosforilação em tirosina do receptor de insulina não diferiram nos animais de 2 meses assim como nos animais de 14 meses de idade submetidos à restrição dietética de 40%, em relação aos que consumiram ração *ad libitum*.

A quantidade de IRS-1 não alterou nos três tecidos analisados dos ratos restritos de 2 meses de idade, e por outro lado aumentou em 159±14% ($p<0,05$) no fígado dos animais de 14 meses sob dieta restrita. Quanto a fosforilação em tirosina do IRS-1, ocorreu aumento de 127±5% ($p<0,05$) no músculo dos ratos restritos de 2 meses de idade; quanto aos ratos restritos de 14 meses de idade ocorreu aumento de 210±40% ($p<0,05$) no músculo e de 234±58% ($p<0,05$) no tecido adiposo em relação aos seus respectivos controle.

O nível protéico de IRS-1 foi diferente nos dois grupos estudados, nos ratos restritos com 14 meses apresentou uma regulação tecido-específica, aumentando no fígado e sem alteração nos tecidos muscular e adiposo. Nos animais com 2 meses de idade não houve alteração da quantidade desta proteína, neste tecidos. Segundo SAAD et al. (1992), a quantidade de IRS-1 tem regulação tecido-específica, e quanto à sua fosforilação, esta poderá depender mais da atividade quinase do receptor de insulina do que da quantidade de IRS-1. Quanto aos níveis de IRS-1, no tecido hepático, eles estariam inversamente relacionados aos níveis insulinêmicos, sugerindo que a insulina poderia regular a expressão gênica do IRS-1. Considerando os nossos resultados, inferimos que o aumento da quantidade de IRS-1 no fígado dos ratos de 14 meses de idade sob restrição dietética provavelmente ocorreu em função da redução dos níveis insulinêmicos de 42% ($p<0,05$) comparados ao controle, enquanto que nos ratos restritos de 2 meses de idade os níveis de insulina foram semelhantes aos controles.

Com relação à associação IRS-1/PI 3-quinase induzida pela insulina os trabalhos sugerem também uma regulação tecido-específica, com aumento de 273±71% ($p<0,05$) no tecido muscular e sem alteração no tecido adiposo no grupo de 14 meses. No grupo com 2 meses de idade ocorreu aumento de 157±7% da associação somente no tecido adiposo. De acordo com CARVALHO et al. (1996), o aumento na fosforilação do IRS-1 no músculo pode ter importância biológica uma vez que a diminuição no nível protéico

de receptor de insulina e o grau de fosforilação do IRS-1 observada em animais velhos correlaciona-se com a resistência à insulina.

A ativação da PI 3-kinase estimulada pela insulina está relacionada coma translocação dos transportadores de glicose (GLUT4) do compartimento intracelular para a membrana plasmática deflagrando a internalização da glicose e a síntese de glicogênio (SAAD, 1994; HARA et al., 1994; CHEATHAN et al., 1994; CLARKE et al., 1994). Portanto, o aumento da associação IRS-1/PI 3-quinase no músculo dos animais restritos com 14 meses e o aumento desta mesma associação no tecido adiposo dos animais restritos com 2 meses sugerem aumento do transporte e utilização de glicose nos tecidos indicados.

O aumento da fosforilação do IRS-1 no tecido adiposo dos ratos restritos com 14 meses de idade, sem aumento na associação IRS-1/PI 3-quinase, permite especular se teria ocorrido um aumento da associação IRS-1/Syp, neste tecido, uma vez que já foram identificados resíduos de tirosina específicos (Y1172 Y1122) para esta associação (WHITE & KAHN, 1994). Nossos resultados mostraram ausência de alteração da associação, IRS-1/Syp, induzida pela insulina, em relação aos ratos controle. Estes dados sugerem que o aumento da fosforilação em resíduos tirosina do IRS-1 nos ratos restritos poderia ocorrer em outros sítios específicos de reconhecimento para outras proteínas com domínio SH2 como Grb ou Nck (SUN et al., 1991; KELLER et al., 1993; SUGIMOTO et al., 1994). No grupo de ratos com 2 meses submetidos à restrição calórica, a associação IRS-1/Syp, aumentou 138±9% ($p<0,05$) , 143±17% ($p<0,05$) e 139±14% ($p<0,05$) no fígado, músculo e tecido adiposo, respectivamente. HAUDSDORFF et al. (1995) demonstraram que a Syp aumenta a expressão de GLUT1, e a captação de glicose. Em animais de 2 meses de idade, com restrição alimentar foi descrito aumento na expressão de GLUT-4 no tecido adiposo, com aumento da sensibilidade à insulina e captação de glicose (SUMIDA & MACHADO, 1998). Portanto, o aumento da associação IRS-1/Syp nos ratos com 2 meses de idade poderia contribuir para aumentar a expressão de GLUT1 e captação de glicose.

Em resumo, nossos resultados demonstraram aumento da sensibilidade à insulina nos grupos de ratos de 2 e 14 meses de idade, acompanhada de alterações das etapas iniciais da ação insulínica, distintas para cada grupo. Nos ratos com 2 meses os animais sob restrição dietética apresentaram aumento da fosforilação do IRS-1 no músculo, da

associação IRS-1/PI 3-quinase no tecido adiposo e da associação IRS-1/Syp no fígado, músculo e tecido adiposo. Nos animais com 14 meses ocorreu um aumento da fosforilação do IRS-1 acompanhado do aumento da associação IRS-1/PI 3-quinase no tecido muscular.

6. Conclusões

Os trabalhos desenvolvidos permitem apresentar as conclusões abaixo enumeradas

1. A restrição dietética reduziu os níveis séricos de triglicerídeos e de insulina nos ratos com 14 meses de idade mas não alterou esses parâmetros nos ratos com 2 meses de idade.
2. A restrição dietética aumentou a sensibilidade à insulina nos ratos com 14 meses de idade (modelos de resistência à insulina) e nos ratos com 2 meses de idade.
3. O aumento da sensibilidade à insulina dos ratos restritos com 14 meses poderia ser devido ao aumento na fosforilação do IRS-1 acompanhada por aumento da sua associação com a PI 3-quinase no tecido muscular.
4. A melhora na sensibilidade à insulina em ratos jovens (2 meses de idade) submetidos à restrição dietética poderia ser devida ao aumento da fosforilação do IRS-1 no músculo, ao aumento da associação IRS-1/PI 3-quinase no tecido adiposo e ao aumento da associação IRS-1/SHP2 no fígado, músculo e tecido adiposo.

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