UNIVERSIDADE ESTADUAL DE CAMPINAS

DANIELA FALEIROS BERTELLI

MECANISMOS MOLECULARES ENVOLVIDOS NA RESISTÊNCIA À INSULINA EM MÚSCULO SÓLEO DESNERVADO DE RATO – PARTICIPAÇÃO DA FOSFATASE SHIP2

Tese apresentada ao Instituto de Biologia para a obtenção do Título de Mestre em Biologia Funcional e Molecular, na área de Fisiologia

ORIENTADOR: Prof. Dr. Antonio Carlos Boschero

CO-ORIENTADOR: Prof. Dr. Lício Augusto Velloso

CAMPINAS
2003

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA - UNICAMP

Bertelli, Daniela Faleiros

B461f Mecanismos moleculares envolvidos na resistência à insulina em músculo sóleo desnervado de rato – Participação da fosfatase SHIP2/Daniela Faleiros Bertelli. –
Campinas, SP:[s.n], 2002.

Orientador: Antonio Carlos Boschero
Co-Orientador: Lício Augusto Velloso
Dissertação (mestrado) – Universidade Estadual de Campinas. Instituto de Biologia

1.Insulina. 2.Músculo esquelético. I. Boschero, Antonio Carlos. II. Velloso, Licio Augusto. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

Data da Defesa: 21/02/2003

Membros da Banca Examinadora

Prof. Dr. Antonio Carlos Boschero (Orientador) _____

Prof. Dr. Rui Curi _____

Prof. Dr. José Antônio Rocha Gontijo _____

Prof. Dr. Carlos Alberto da Silva _____

Profa. Dra. Helena Coutinho Franco de Oliveira _____

"O maior pecado do ser humano, é ignorar suas forças interiores,
seus poderes criadores e sua herança.
Estuda-te...
Vê quanta coisa és capaz de fazer..."

(O. S. Marden)

DEDICATÓRIA

Dedico este trabalho:
Aos meus pais, Enderson e Sônia
Por uma vida toda de dedicação.
Agradeço a vocês por tudo que sou
E tenho conquistado...
Eu os amo muito.

AGRADECIMENTOS

Ao meu orientador,
Prof. Boschero

Sua amizade, compreensão e confiança
orientaram-me a esta conquista...
Com o senhor não só aprendi sobre
resistência à insulina, diabetes,
e um milhão de outras coisas, mas também,
a conviver com as dificuldades,
a ter disciplina
e acima de tudo a manter o bom humor...

O senhor me abriu as portas e
eu não as fecharei, pois assim,
o senhor poderá observar melhor
os passos de sua fiel discípula
que tanto o estima e
admira muito...

Saiba que enquanto puder ver o sol
e sentir a brisa...
seguirei seus passos...
pois as suas condutas são o alicerce
para a construção científica
da minha vida...

Minha eterna gratidão...

Ao meu co-orientador,
Dr. Lício

Agradeço pela competência, seriedade e emoção
com que sempre tratou meu trabalho.
Sinceramente, agradeço pela maneira gentil
e solícita com que sempre me recebeu...

Nosso convívio foi fundamental
para formar o perfil da minha vida científica,
pois foi com seu entusiasmo, que aprendi a amar a pesquisa e
a buscar continuamente o conhecimento
de maneira inteligente e prazerosa como você assim faz...

Suas elegantes palavras sempre me ajudavam
nos momentos mais difícieis,
nos quais, mais animada
eu buscava forças para seguir nosso projeto...

Sempre terei você como exemplo de respeito,
dedicação, profissionalismo e
acima de tudo aquele que tem o dom de saber ensinar...

Obrigada por tudo...

Agradeço à Deus
Pela vida abençoada que tenho
e por estar comigo na realização deste sonho...

Aos meus avós,
Francisco, Maria Aparecida e Iná
Pelo exemplo de vida, de força e humildade.
Mesmo distante, me incentivaram, oravam e
torceram pelos meus objetivos...
Eu os Amo muito.

Aos meus tios e tias
Agradeço pelo amor e carinho que sempre me deram
no decorrer da minha vida acadêmica.
Obrigada pelo incentivo e apoio. Vocês foram sempre muito
importantes na minha vida.
Obrigada por tudo.

A minha irmã Priscila
Que os laços que nos unem
aumentem com o decorrer dos anos,
e sejam capazes de nos dar força e esperança a
cada novo caminho a ser conquistado.
A amo muito.

Ao querido Nicolau
Pelo apoio e paciência
nos momentos de maior dificuldade...
Obrigada por tudo, principalmente pelo carinho.

Ao amigo Carlos Silva,
Por mais que penso,
sei que é pouco dizer muito obrigada...
Você também é responsável por esta conquista.

Ao amigo Prof. Dr. Rinaldo Guirro
Pela enorme receptividade e incentivo
que foram fundamentais durante este período...
Muito obrigada.

Aos Profs. Dr. Mário Saad, Dr. José Antonio Rocha Gontigo e
Dr. Everardo Magalhães Carneiro
Pela contribuição ímpar e pela oportunidade de realização
deste trabalho em seus laboratórios.

A Prof. Dra. Carla Roberta O. de Carvalho
Pelo apoio e credibilidade que me foi confiada
e ainda pela maneira simples
que me conduziu no princípio de tudo.

As amigas Mirian e Maria Esméria
Pela paciência e grandiosa ajuda no desenvolvimento deste trabalho,
pelos ensinamentos doados, pela grande amizade,
e sobretudo pelas pessoas maravilhosas que são.
Obrigada.

Aos amigos Vivian, Eliana, Emiko, Fernanda, Rosângela, Alessandra, Rachel,
Henrique, Wilson, Márcio Torsoni, Márcio Pereira, Cláudio,
José Barreto, Brahma, Marcos Toyama e outros colegas do dia à dia
Pela forma atenciosa e prestativa com que atenderam
aos meus inúmeros pedidos de ajuda.
Muito Obrigada.

As amigas Telma, Patrícia, Francélia, Letícia e Daniela
Apesar da distância que nos separa, os pensamentos positivos tiveram
uma participação especial e significativa durante este período.
Obrigada pela presença e pelo apoio constante.

Ao funcionário Sr. Luis
Tenho grande estima pela valiosa ajuda que me deu e
nunca me esquecerei o quanto o senhor fez por mim.
Sei que muito obrigada não é o bastante...

LISTA DE ABREVIATURAS

αPy	antifosfotirosina
μCi	microCi
Akt1/PKB	proteína serina/treonina quinase B
ATP	adenosina trifosfato
cDNA	ácido desoxirribonucléico complementar
DTT	ditiotreitol
EDTA	ácido etilenodiaminotetracético
GLUT1	transportador de glicose 1
GLUT4	transportador de glicose 4
Grb2	proteína ligadora do receptor para o fator de crescimento
GS	glicogênio sintase
GSK-3	glicogênio sintetase 3 quinase
HPLC	cromatografia líquida por alta pressão

^{125}I	isótopo de iodo 125
IR	receptor de insulina
IRS1	substrato 1 do receptor de insulina
IRS2	substrato 2 do receptor de insulina
kDa	quilo Dalton
MAPK	proteína quinase ativadora da mitogênese
PI3-kinase	fosfatidilinositol 3-quinase
PI	fosfatidilinositol
PDK1	quinase 1 dependente de fosfatidilinositol
PMSF	fluoreto de fenilmetil sulfonila
PTEN	fosfatase e homólogo tensina
PY	fosfotirosina
mRNA	ácido ribonucléico mensageiro
SDS-PAGE	eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
SH2	segunda homologia do src

SH3	terceira homologia do src
Shc	molécula adaptadora e substrato do receptor de insulina
SHIP	família da inositol polifosfatase que contém o domínio SH2
SHIP2	inositol 5-fosfatase que contém o domínio SH2
TLC	cromatografia de camada fina

Resumo

A desnervação aguda é um modelo reprodutível de resistência à insulina. Músculos *soleus* de ratos controles e de ratos submetidos à desnervação foram utilizados para investigar as bases moleculares da resistência à insulina. A desnervação aguda induziu uma redução significativa na taxa de captação de glicose (62%) analizada pelo *clamp euglicêmico-hiperinsulinêmico* de membro isolado, a qual foi associada à diminuição significativa da fosforilação em tirosina do IR (73%), IRS1 (69%), IRS2 (82%) e fosforilação em serina da Akt (39%), estimuladas pela insulina. Além disso, a desnervação reduziu a associação entre IRS1/IRS2 e p85/PI3kinase, induzida pela insulina. No entanto, a desnervação causou um aumento da atividade da PI3-kinase associada ao IRS1 (275%) e IRS2 (180%), embora o conteúdo de fosfoinositóis fosforilados, detectados por HPLC, fosse significativamente reduzido em frações lípides. Diante dessa aparente discrepância, avaliamos a expressão e atividade da fosfatase SHIP2 e a fosforilação em serina da p85/PI3-kinase. Não houve diferença na expressão da SHIP2 entre músculos controles e desnervados. Entretanto, a fosforilação em serina da p85/PI3-kinase foi reduzida no músculo desnervado, enquanto que o bloqueio da expressão da SHIP2 pelo tratamento com oligonucleotídeo antisense específico restaurou parcialmente o conteúdo de fosfoinositóis fosforilados e normalizou a captação da glicose. Concluindo, alterações no estado funcional da SHIP2 podem contribuir para a resistência à insulina induzida pela desnervação.

Abstract

Short–term muscle denervation is a reproducible model of tissue specific insulin resistance. To investigate the molecular basis of insulin resistance in denervated muscle the downstream signaling molecules of the insulin-signaling pathway were examined in intact and denervated *soleus* muscle of rats. Short–term denervation induced a significant fall in glucose clearance rates (62% of control, $p<0.05$) as detected by *euglycemic-hyperinsulinemic clamp*, and was associated with significant decrease in insulin-stimulated tyrosine phosphorylation of IR (73% of control, $p<0.05$), IRS1 (69% of control, $p<0.05$) and IRS2 (82% of control, $p<0.05$) and serine phosphorylation of Akt (39% of control, $p<0.05$). Moreover, denervation reduced insulin-induced association between IRS1/IRS2 and p85/PI3-kinase. Notwithstanding, denervation caused an increase in IRS1 (275%, $p<0.05$) and IRS2 (180%, $p<0.05$) associated PI3-kinase activity, but the contents of phosphorylated phosphoinositides detected by HPLC were significantly reduced in lipid fractions. In face of the apparent discrepancy we evaluated the expression and activity of the 5-inositol, lipid phosphatase SHIP2 and the serine phosphorylation of p85/PI3-kinase. No major differences in SHIP2 expression were detected between intact and denervated muscle. However, serine phosphorylation of p85/PI3-kinase was reduced in denervated muscle, while the blockade of SHIP2 expression by antisense oligonucleotide treatment led to partial restoration of phosphorylated phosphoinositide contents and to improved glucose uptake. Thus, modulation of the functional status of SHIP2 may be a major mechanism of insulin resistance induced by denervation.

ÍNDICE

LISTA DE FIGURAS

1. Introdução

Diabetes mellitus é uma das principais causas de morbidade e mortalidade em humanos e, pode ser definida como um distúrbio metabólico multifatorial resultante da falência na produção de insulina bem como da resistência à ação deste hormônio. Uma pequena parcela de pacientes apresenta diabetes mellitus tipo 1a, resultante da destruição autoimune das células beta pancreáticas, e que se traduz por deficiência absoluta de insulina (Nathan, 1993). Diabetes mellitus tipo 2 é a forma mais freqüente da doença, caracterizada por grau variável de insulinopenia associada à resistência periférica à insulina (Taylor, 1999). Em ambas as formas de apresentação, seja diabetes mellitus clinicamente manifesto ou reduzida tolerância à glicose, tecidos muscular, adiposo e hepático tornam-se resistentes à ação da insulina. Tal estado pode ser também observado em outras situações clínicas comuns tais como, obesidade, síndromes de ovários policísticos, hiperlipidemia, hipertensão e aterosclerose (Saltiel & Kahn, 2001).

A insulina estimula a captação e o armazenamento de glicose em músculo e tecido adiposo, ao mesmo tempo que inibe a produção hepática de glicose, servindo assim, como um regulador primário da concentração de glicose sanguínea. Este hormônio estimula também uma série de outros parâmetros fisiológicos tais como, o crescimento e a diferenciação celular. No músculo, a insulina age através da estimulação da síntese de proteínas e de glicogênio, enquanto no tecido adiposo, através da estimulação da lipogênese (Klip & Paquet, 1990). No fígado, a insulina inibe a glicogenólise e a gliconeogênese, e estimula a síntese de glicogênio. Portanto, a resistência à insulina resulta da regulação anormal desses processos, produzindo concentrações elevadas de glicose e lipídeos séricos tanto nos períodos de jejum como no estado pós-alimentado (Saltiel & Kahn, 2001).

Desde sua descoberta em 1921, muito esforço tem sido dedicado ao entendimento dos mecanismos de ação da insulina. Porém, o exato mecanismo molecular de sinalização intracelular que é mediado pelo receptor da insulina e que resulta em diversas respostas biológicas, ainda não está completamente elucidado. Na busca do entendimento dos processos que levam à resistência à insulina, vários modelos foram propostos nas últimas décadas, dentre estes, a desnervação.

Desnervação é um modelo reprodutível de resistência à insulina, caracterizado por uma diminuição da habilidade do hormônio em estimular a captação da glicose, a síntese de glicogênio, e o transporte de aminoácidos através da membrana plasmática (Elmendorf et al., 1997), além da redução da expressão do mRNA dos transportadores de glicose, GLUT1 e GLUT4 (Henriksen et al., 1991; Coderre et al., 1992). Esses efeitos já podem ser observados 3 horas após a secção do nervo (Turinski et al., 1998). Buse e Buse (1959) foram os primeiros a mostrar que a interrupção do nervo que supre os músculos esqueléticos resulta no desenvolvimento da resistência à insulina nos mesmos. Assim, a desnervação passou a ser um modelo tecido-específico de resistência à insulina, uma vez que os sinais da resistência ao hormônio ocorrem somente na musculatura desnervada, sendo o músculo da pata contralateral utilizado como controle.

O músculo desnervado difere ainda do normal, uma vez que a interrupção completa da inervação motora promove a perda imediata da atividade voluntária e reflexa do mesmo, atrofia muscular progressiva, além da perda de substâncias neurais que suprem o nervo como, por exemplo, a acetilcolina (Hofmann, 1987). Em 2001, Day et al. observaram que após oito semanas de desnervação, o músculo gastrocnêmio apresentou diminuição de diâmetro, de peso e, ainda, redução da força de contração. Esta perda de

peso foi de 30%, 50% e 60% a 80% após 30, 60 e 120 dias, respectivamente (Sunderland, 1978). Os efeitos morfofisiológicos da desnervação prolongada não foram corrigidos pela reinervação dos músculos sóleo, gastrocnêmio lateral e tibial anterior, realizada vinte e uma semanas após a secção do nervo ciático (Paassen, et al., 2002).

Dentre os principais substratos utilizados pelo tecido muscular como fonte de energia destaca-se a glicose que, após ser captada, é convertida em glicogênio que é reconvertido em glicose para ser utilizada de acordo com as necessidades energéticas das fibras musculares. Nesse tecido, a insulina estimula a síntese de glicogênio através da desativação da proteína GSK3, em resposta ao aumento da atividade da Akt1, o que resulta na fosforilação da enzima glicogênio sintase (GS) (Markuns et al., 1999). No entanto, no músculo desnervado, a síntese de glicogênio está diminuída em 80% depois de 3 dias. Nessas condições, a insulina perde a capacidade de estimular a fosforilação da glicogênio sintase (GS) contribuindo, a médio e longo prazos para a degeneração muscular (Smith et al., 1988). As observações descritas nos parágrafos anteriores foram confirmadas mais recentemente (Lin et al., 2002). Esses pesquisadores observaram ainda uma redução da atividade da glicogênio sintase (GS) em média de 65%, após 24 horas de desnervação.

Além da síntese de glicogênio, a insulina estimula o transporte da glicose através das membranas das fibras musculares. Este transporte envolve diferentes proteínas que promovem a translocação do GLUT4 dos sítios intracelulares para a membrana plasmática. Interessante notar que o efeito da desnervação reduz o transporte da glicose em 28% no músculo desnervado por 24 horas, quando estimulado pela insulina *in vitro* (Wilkes et al., 2000). Tal redução é também observada no músculo *in vivo* após 24 horas de desnervação, atingindo valores de 80% na queda do transporte da glicose (Turinsky, et al., 1998). Quanto

à desnervação aguda, de 3 a 6 horas, o transporte da glicose mediado pela insulina também é reduzido de 20% a 58% (Turinsk, 1987). Quanto à redução no transporte da glicose citada acima, observa-se que o número do transportador GLUT4 é reduzido, embora a translocação para a membrana plasmática das vesículas contendo GLUT4 está preservada no músculo desnervado. Porém, existe um aumento de duas vezes nos níveis das vesículas contendo GLUT1, que passam à responder à insulina, e estão presentes na superfície celular. A formação dessas vesículas contendo GLUT1 pode ser considerada como um mecanismo compensatório da diminuição do nível do transportador GLUT4 nos músculos desnervados (Zhou et al., 2000). Estudos comparativos, entre músculos de ratos tornados diabéticos por estreptozotocina e músculos de ratos controles desnervados (sete dias de desnervação), mostram que o transporte da glicose, estimulado pela insulina, é duas vezes menor no músculo desnervado (40%-60%) do que no músculo do animal diabético (redução de 18%-24%). Esta diferença é associada à redução mais significativa na expressão do mRNA da proteína GLUT4 no músculo desnervado (75%) comparada ao músculo do animal diabético (30%-40%) (Han et al., 2000).

A essa altura é necessário lembrar que o fluxo sanguíneo no músculo desnervado (Turinsky, 1998) e a ligação da insulina ao seu receptor localizado na membrana plasmática das fibras musculares (Forsayeth, 1992) não se alteram, e portanto não contribuem para o aumento da resistência à insulina. O mesmo se aplica à perda da atividade contrátil do músculo pela desnervação (Etgen, 1993).

A ação da insulina em células alvo inicia-se através da sua união ao seu receptor que está localizado na membrana celular (Freytchet et al., 1971; Cuatrecasas, 1972; Khan, 1985). Este receptor está presente em praticamente todos os tecidos dos mamíferos, sendo

que nas células do músculo esquelético ele exerce uma função importante na mediação da captação de muitos substratos metabólicos, sendo a glicose o substrato mais importante. Além disso, a insulina participa do controle da captação de aminoácidos utilizados no crescimento e reparo das fibras musculares (Holfmann, 1987). O receptor de insulina é uma glicoproteína heterotetramérica constituída por duas subunidades α, cada uma com 135 kDa e duas subunidades ß, cada uma com 95 kDa, unidas por ligações dissulfeto (Khan, 1985). A subunidade α é inteiramente extracelular e contém o sítio de ligação da insulina. A subunidade ß é uma proteína transmembrana e responsável pela transdução do sinal.

Em 1982, Kasuga et al., demonstraram que a subunidade ß do receptor de insulina é uma proteína com atividade tirosina-quinase, capaz de se autofosforilar e de fosforilar outros substratos em resíduos de tirosina após a ligação do hôrmonio à subunidade α. O ATP age como doador de fosfatos e a fosforilação ocorre em aminoácidos tirosina. Camundongos nos quais a expressão do receptor de insulina em músculo foi suprimida por tecnologia recombinante apresentam tolerância normal à glicose (Bruning et al.,1998).

Têm sido descritos vários substratos endógenos para os receptores de insulina (Bornier et al., 1987). Estas proteínas são fosforiladas em tirosina pela ativação do receptor. A primeira a ser descrita foi uma proteína de aproximadamente 185 kDa denominada pp185 ou substrato 1 do receptor de insulina (White et al., 1985). O componente de maior peso molecular (pp185), clonado em 1991 por Sun et al., foi denominado substrato 1 do receptor de insulina (IRS1) o qual é localizado no citoplasma das células-alvos da insulina. Camundongos com *knockout* do IRS1 exibem retardo no crescimento pré e pós-natal, assim como, resistência dos tecidos periféricos à ação da insulina além de tolerância reduzida à

glicose (Tamemoto et al., 1994; Araki et al., 1994). Desnervação por sete dias do músculo sóleo reduz em 34% a fosforilação do IRS1, após exposição à insulina (Hirose et al., 2001).

Insulina promove a associação entre a enzima fosfatidilinositol 3'-kinase que possui dois domínios SH2 (Carpenter & Cantley, 1990), com o IRS1 (Folli et al., 1992). Essa associação, está relacionada com a ativação do transportador de glicose (Sanches-Margalet et al., 1994; Cheataham & Khan, 1995). Contudo, nenhuma alteração na atividade da PI3-kinase foi verificada no músculo sóleo após 24 horas de desnervação (Elmendorf et al.,1997).

Há sete anos, outro constituinte da banda pp185, o IRS2, foi purificado e a seqüência cDNA determinada apresentando, aproximadamente, 45% de homologia com o IRS1. Aparentemente, o IRS2 atua sinergicamente com o IRS1 na ativação da PI3-kinase, tendo, portanto, uma função importante nos eventos que controlam o metabolismo de glicose nos tecidos-alvos da insulina (Sun et. al., 1995). Camundongos com *knockout* do IRS2 exibem resistência à insulina no músculo, tecido adiposo e fígado, e apresentam alterações no crescimento de alguns tecidos, incluindo certas regiões do cérebro, ilhotas e retina (Withers et al., 1998; Kido et al., 2000). Nestes camundongos, a resistencia à insulina combinada com a diminuição significativa da massa das células-beta resulta no desenvolvimento da diabetes mellitus tipo 2 (Withers et al., 1998).

O IRS1 e o IRS2 quando fosforilados associam-se à PI3-kinase ativando-a, tanto *in vivo* como *in vitro* (Folli et al., 1992; Saad et al., 1995). A ativação da PI3-kinase aumenta os níveis teciduais de fosfatidilinositol 3 fosfato, que é um intermediário essencial na ativação da serina-kinase Akt1/PKB (proteína kinase B) (Kohn et al., 1996; Krook et al., 1998).

A Akt1-kinase é essencial para o transporte da glicose e para a síntese de glicogênio em tecidos insulino-sensíveis. Essa enzima é fosforilada por uma kinase ainda não identificada, numa reação que depende da formação de 3,4,5 trifosfato de fosfatidilinositol induzida pela PI3-kinase. A Akt1-kinase medeia a captação da glicose estimulada pela insulina através da translocação do GLUT4 para a membrana plasmática como verificado em adipócitos 3T3-L1 (Turinsky et al., 1998). Após três dias de desnervação do músculo sóleo a atividade da Akt1-kinase foi reduzida em 86% enquanto que o transporte da glicose estimulado pela insulina foi reduzido em 60% (Turinsky et al., 1998).

Como dito acima, a ativação da serina kinase Akt1/PKB depende da sua ligação aos fosfatidilinositóis $PI_{3,4}P_2$ e $PI_{3,4,5}P_3$ gerados pela ação da PI3-kinase. Uma vez ligada aos fosfatidilinositóis, a Akt1 torna-se disponível para a atividade catalítica da PDK1, a qual medeia a fosforilação em treonina, e também de uma serina kinase desconhecida necessária para a ativação completa da Akt1 (Coffer et al., 1998).

A ação da insulina é reduzida por tirosinas fosfatases, que catalizam a desfosforilação do seu receptor e de seus substratos. Mais recentemente, foi identificada a SHIP2, uma inositol 5-fosfatase, envolvida na sinalização da via da PI3-kinase. A SHIP2 tem por função desfosforilar fosfoinositóis ligados à membrana celular pela retirada do fósforo da posição 5'.

A SHIP2 é um membro da família da inositol 5-fosfatase que contém um domínio SH2 na porção NH_2-terminal, um domínio 5-fosfatase na porção central e um domínio rico em prolina na porção COOH-terminal (Dyson et al., 2001) e, atua como um potente regulador negativo da sinalização e da sensibilidade à insulina *in vivo*. A proteína-fosfatase

SHIP2 foi detectada em fibroblastos, miócitos, e em diferentes áreas do cérebro de ratos (Muraille et al., 1999). Em camundongos, no décimo quinto dia de vida intrauterina, a SHIP2 foi marcadamente expressa no fígado, regiões específicas do sistema nervoso central, timo e pulmão. Já no camundongo adulto, o mRNA da SHIP2 está presente apenas no cérebro e no timo (Schurmans et al., 1999).

A superexpressão da SHIP2 em mioblastos L6 inibiu a fosforilação e ativação da Akt induzida pela insulina. A fosforilação e inativação da GSK3, a ativação da glicogênio sintase (GS) e a síntese de glicogênio foram também reduzidas nessas células pela superexpressão da SHIP2. Esses efeitos não ocorreram pela superexpressão de uma variante inativa dessa enzima (Sasaoka et al., 2001). A superexpressão da SHIP2 não alterou a fosforilação em tirosina do IRS1, a sua associação com a subunidade p85 da PI3-kinase e a ativação desta, porém, inibiu a ativação da Akt. A superexpressão da SHIP2 não alterou também a fosforilação em tirosina da subunidade β do receptor de insulina e a fosforilação da Shc, e ainda, inibiu a associação da Shc com a Gbr2, resultando assim, na redução da ativação da MAP-Kinase, proteína responsável pela via da mitogênese induzida pela insulina (Ishihara et al., 1999).

Camundongos com *knockout* da SHIP2 apresentam aumento da sensibilidade à insulina, caracterizada por hipoglicemia grave. Nesses animais foi observado ainda, expressão alterada dos genes envolvidos no processo de gliconeogênese hepática e morte dos animais no período perinatal. Em camundongos adultos, que apresentavam mutação heterozigota para o gene da SHIP2, observou-se aumento da tolerância à glicose e da sensibilidade à insulina associado ao aumento do número do transportador de glicose

GLUT4 na membrana plasmática, e ainda, aumento da síntese de glicogênio no músculo esquelético (Clement et al., 2001).

A função da fosforilação em tirosina da SHIP2 sobre sua atividade fosfatase ainda é controversa. Inicialmente, sugeriu-se que a fosforilação em tirosina dessa enzima poderia reduzir a atividade da fosfatase SHIP1 (Osborne et al., 1996; Sattler et al., 1997). Entretanto, a regulação da fosforilação em tirosina da SHIP2 pela insulina e por outros fatores de crescimento, parece influir no controle positivo da sua atividade fosfatase (Ishihara et al., 1999; Taylor et al., 2000) que em outras palavras, significa torná-la mais eficaz quanto suas ações antagônicas à insulina.

Sabe-se ainda que mutações no gene INPPL1 que codifica a SHIP2 contribuem para o aumento da susceptibilidade ao diabetes mellitus tipo 2 em ratos e humanos, uma vez que tais mutações resultam no aumento da expressão do mRNA repórter e da proteína *in vitro*, e ao aumento da expressão da SHIP2 *in vivo*, o que leva a diminuição da sensibilidade à insulina (Marion et al., 2002).

Assim, distúrbios da expressão e função da SHIP2 podem levar a maior susceptibilidade ao diabetes mellitus tipo 2, uma desordem metabólica freqüente, caracterizada pela presença da resistência à insulina bem como por defeitos na produção e secreção de insulina pelas células beta pancreática.

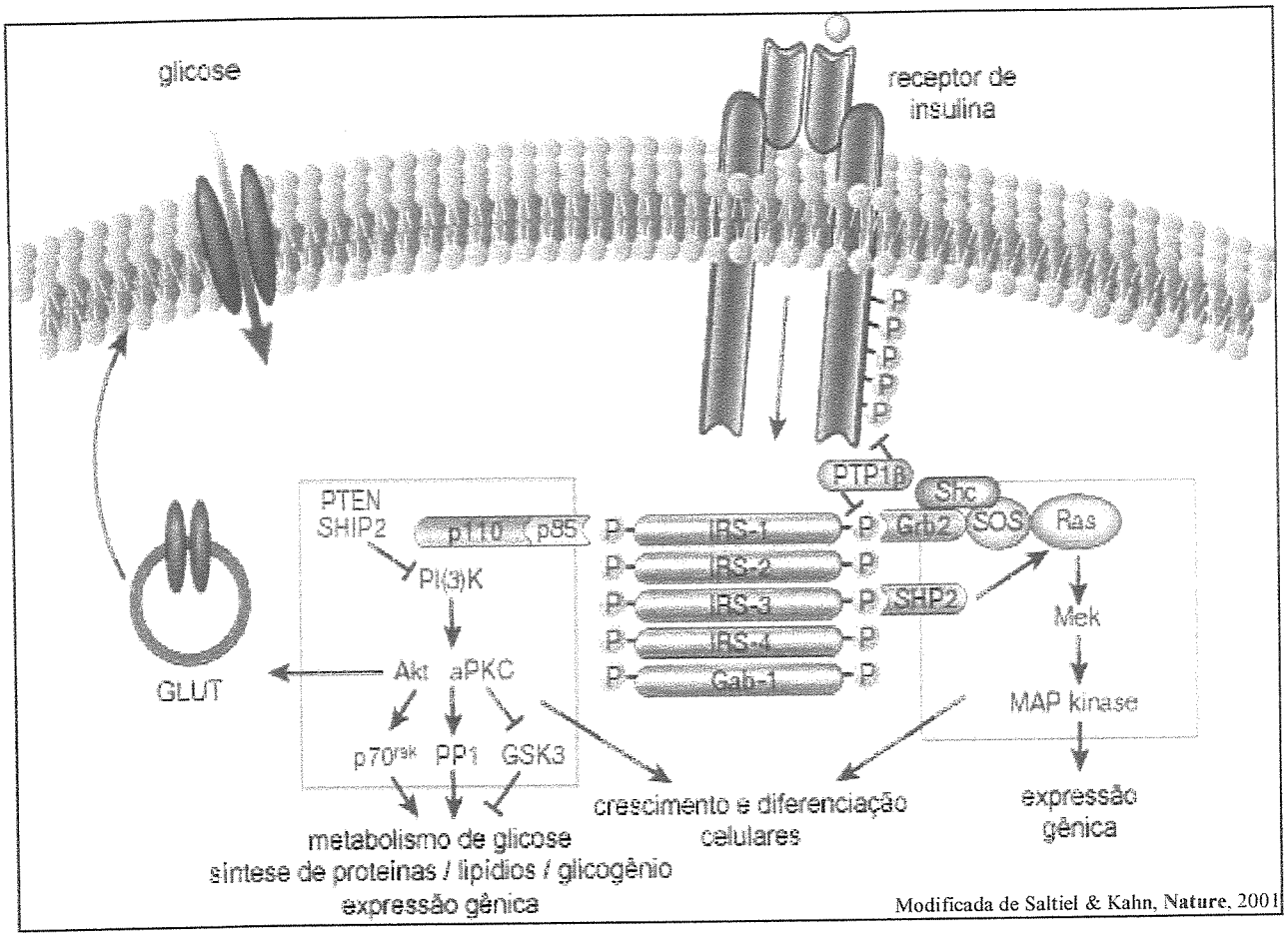

Figura A. Vias intracelulares da transmissão do sinal da insulina

2. Objetivos

Neste trabalho avaliamos a expressão e atividade funcional dos elementos participantes da via de sinalização insulínica em um modelo animal de resistência à insulina induzida pela desnervação aguda.

3. Resultados

Os resultados desta tese originaram o seguinte trabalho:

Reversal of denervation-induced insulin resistance by SHIP2 protein synthesis blockade

Esse trabalho foi apresentado:

- No 38 th Annual Meeting of European Association for the study of Diabetes (EASD) realizado de 01 a 05 de setembro de 2002 em Budapeste-Hungria

- No 1° Internacional Simposium on cellular and molecular mechanisms involved in the physiopathology of diabetes mellitus and therapeutic advances realizado de 15 a 17 de agosto de 2002 em Campinas, SP

- No V Congresso Paulista de Diabetes e Metabolismo realizado de 25 a 27 de abril de 2002 em Águas de São Pedro, SP

- Foi publicado pelo *American Journal Physiology – Endocrinology and Metabolism*

Reversal of denervation-induced insulin resistance by SHIP2 protein synthesis blockade

DANIELA F. BERTELLI[1], MIRIAM UENO[2], MARIA E. C. AMARAL[1], MARCOS HIKARI TOYAMA[1], EVERARDO M. CARNEIRO[1], SERGIO MARANGONI[1], CARLA R. O. CARVALHO[3], MÁRIO J. A. SAAD[2], LÍCIO A. VELLOSO[2], A. CARLOS BOSCHERO[1]

[1]Department of Physiology and Biophysics and [2]Internal Medicine, University of Campinas, and [3]Department of Physiology and Biophysics, University of São Paulo, Brazil
L.A.V. and A.C.B. contributed equally as advisors to the present study.

Address correspondence and reprint requests to Dr. A. Carlos Boschero
Departamento de Fisiologia e Biofisica, IB – UNICAMP, Campinas SP, Brazil.
Email: boschero@unicamp.br
FAX: 55 19 3289 3124

Abbreviations: Akt, protein kinase B; GLUT-1 and GLUT-4, glucose transporters 1 and 4; HPLC, high pressure liquid chromatography; IR, insulin receptor; IRS1 and IRS2, insulin receptor substrates 1 and 2; PI3-kinase, phosphatidylinositol 3-kinase; PI, phosphatidylinositol; PDK1, phosphoinositide-dependent kinase1; PTEN, phosphatase and tensin homolog deleted on chromosome ten; SHIP2, SH2 domain-containing inositol phosphatase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

ABSTRACT

Short–term muscle denervation is a reproducible model of tissue specific insulin resistance. To investigate the molecular basis of insulin resistance in denervated muscle the downstream signaling molecules of the insulin-signaling pathway were examined in intact and denervated *soleus* muscle of rats. Short–term denervation induced a significant fall in glucose clearance rates (62% of control, $p<0.05$) as detected by *euglycemic-hyperinsulinemic clamp*, and was associated with significant decrease in insulin-stimulated tyrosine phosphorylation of IR (73% of control, $p<0.05$), IRS1 (69% of control, $p<0.05$) and IRS2 (82% of control, $p<0.05$) and serine phosphorylation of Akt (39% of control, $p<0.05$). Moreover, denervation reduced insulin-induced association between IRS1/IRS2 and p85/PI3-kinase. Notwithstanding, denervation caused an increase in IRS1 (275%, $p<0.05$) and IRS2 (180%, $p<0.05$) associated PI3-kinase activity, but the contents of phosphorylated phosphoinositides detected by HPLC were significantly reduced in lipid fractions. In face of the apparent discrepancy we evaluated the expression and activity of the 5-inositol, lipid phosphatase SHIP2 and the serine phosphorylation of p85/PI3-kinase. No major differences in SHIP2 expression were detected between intact and denervated muscle. However, serine phosphorylation of p85/PI3-kinase was reduced in denervated muscle, while the blockade of SHIP2 expression by antisense oligonucleotide treatment led to partial restoration of phosphorylated phosphoinositide contents and to improved glucose uptake. Thus, modulation of the functional status of SHIP2 may be a major mechanism of insulin resistance induced by denervation.

Key words: Denervation, SHIP2, PI3 kinase, insulin-resistance.

INTRODUCTION

Muscle denervation is a reproducible model of insulin resistance. It is characterized by a decreased ability of insulin to stimulate glucose uptake, glycogen synthesis, and amino acid transport (9). Several studies have attempted to characterize the major mechanisms involved in the development of impaired insulin action following denervation, and as it stands now, we know that modulation of muscle blood flow (31), reduced binding of insulin to its receptor (13), and loss of mechanical activity (10) are not responsible for the phenomenon. Defects in different steps of the insulin-signaling pathway are currently under scrutiny and some advances have been achieved. No study was able to demonstrate a major loss of IR kinase activity (1, 20), while one study (14) demonstrated a reduced insulin-stimulated IRS1 phosphorylation after seven days denervation. The activity of the lipid metabolizing enzyme PI3-kinase was shown to be unaltered 30 min and 24 h after denervation, and decreased three days after denervation (2, 9). The activity of Akt was shown to be unaltered one day and reduced three days after denervation (30), while the ability of insulin to induce dephosphorylation of glycogen synthase was shown to be reduced three days after denervation (27). Finally, glucose transporters GLUT1 and GLUT4 undergo significant decrease in mRNA and protein expression one day after denervation with no changes if analyzed after shorter periods (4). Thus, a yet unclear picture suggests that defects at several levels and branches of the insulin signaling cascade, occurring at different time points following denervation, participate in the mechanisms that produce the final phenotype of insulin resistance.

The objective of the present study was to evaluate the expression and functional activity of molecular elements participating in insulin signaling from IR to Akt, in a model

of skeletal muscle insulin resistance due to short-term denervation. Besides differences in insulin induced tyrosine or serine phosphorylation in most elements analyzed, an apparent incongruent finding of increased IRS1 and IRS2 associated PI3-kinase activity paralleled by lower insulin-stimulated levels of phosphorylated phosphoinositides in denervated muscle lipid extracts led us to investigate the role of the recently described lipid phosphatase SHIP2. Although no changes in SHIP2 protein expression could be detected, a reversal of insulin resistance was achieved by treating denervated rats with an antisense oligonucleotide capable of blocking SHIP2 protein synthesis.

MATERIALS AND METHODS

Materials. Reagents for SDS-polyacrylamide gel electrophoresis and immunoblotting were from Bio-Rad (Richmond, CA). HEPES, phenylmethylsulfonyl fluoride, antipain, aprotinin, leupeptin, peptstatin, benzamidine hydrochloride, dithiothreitol, ATP, phosphatidylinositol 4-monophosphate, Triton X-100, Tween 20, glycerol, and bovine serum albumin (fraction V) were from Sigma (St. Luis, MO). Nonindet P-40 was from Calbiochem (La Jolla, CA), phosphatidylinositol was from Avanti (Alabaster, AL), silica gel thin-layer chromatography plates were from Merck (Gibbstown, NJ), protein A-Sepharose 6MB was from Pharmacia (Uppsala, Sweden), ^{125}I-protein A was from ICN Biomedicals (Costa Mesa, CA), [γ-^{32} P]ATP was from Du Pont-New England Nuclear (Beverly, MA) 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). Polyclonal anti-phosphotyrosine antibodies were raised in rabbit

and affinity-purified on phosphotyramine columns (19). Anti-IR (rabbit; sc-711), anti-IRS1 (rabbit; sc-559), anti-IRS2 (goat; sc-1555), anti-SHIP2 (goat; sc-14504) and anti-phospho [Ser^{473}]Akt (goat; sc-1618) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rat-p85/PI3-kinase (rabbit; #06-195) antiserum was from UBI (Lake Placid, NY). Anti-phospho-serine (rabbit; AB1603) antiserum was from Chemicon. The catheters were made of Tygon (Norton Performance Plastics, CO), polyvinyl (Silverwater B.C., Australia) and polyethylene (PE 50 – Clay Adams, Parsippany).

Experimental animals. Eight-week-old Wistar male rats (150-180 g) were allowed access to standard rodent chow and water *ad libitum.* Food was withdrawn 8 hours before experiments. For each set of experiments rats were submitted to surgical denervation of only one hind limb and *soleus* muscle specimens were collected from both the denervated and from the intact limb. The experimental protocol was approved by the University of Campinas Ethical Committee.

Denervation. The hind limb was denervated as previously described (29). Rats were briefly anesthetized with sodium thiopental (15 mg/kg body weight, i.p.) and used 10-15 min thereafter, *i.e.* as soon as anesthesia was assured by loss of pedal and corneal reflexes. A small superficial incision was made at the most proximal portion of the hind-limb, the thigh muscles were bluntly separated from the lateral side, the sciatic nerve was exposed and approximately 0.5 cm of the nerve was removed. A sham operation was performed in the contralateral leg. Incisions on both legs were closed with surgical clips, and a topical disinfectant was applied to the skin.

Protein extraction, immunoprecipitation and immunoblotting. Four hours after denervation, the rats were anaesthetized again with sodium thiopental (15 mg/kg body weight, i.p.), the abdominal cavity was opened and 0.5 ml of saline (0.9% NaCl), with or without 10^{-6} mol/l insulin, was injected in the cava vein. Ninety seconds after insulin injection, the *soleus* muscle was excised and homogenized. The extracts were centrifuged at 12,000 rpm at 4°C for 1 h to remove insoluble material, and equal amounts of the supernatant were used in immunoprecipitation experiments with anti-IR, anti-IRS1, or anti-IRS2 antibodies. The samples were processed for SDS-polyacrylamide gel electrophoresis and Western blotting (6).

For protein analysis by immunoblotting the samples were treated with Laemmli sample buffer containing 100 mmol/l DTT and heated in a boiling water bath for 4 min. For total extracts, 200 μg aliquots were subjected to SDS-PAGE (6% Tris-acrylamide) in a Bio-Rad miniature lab gel apparatus (Mini-Protean, Bio-Rad).

The nitrocellulose blots were incubated for 4 h at 22°C with anti-phospho-tyrosine antibody, anti-IR antibody, anti-IRS1 antibody, anti-IRS2 antibody, anti-p85/PI3-kinase antibody, anti-phospho-serine antibody or anti-phospho[Ser^{473}]Akt. The blots were incubated with 2 μCi of [^{125}I] Protein A (30 μCi/μg) in 10 ml of blocking buffer for 1 h at 22°C and washed again as described above for 2 h. [^{125}I] Protein A bound to the antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak, Rochester, NY) with Cronex Lighting Plus intensifying screens (Du Pont, Wilmington, DE) at -70°C for 12-48 h. Band intensities were quantified by densitometry (Scion software, ScionCorp) of the developed autoradiogram.

PI3-kinase activity assay. PI3-kinase activity was measured by *in vitro* phosphorylation of phosphatidylinositol as previously described with minor modifications (17, 21, 32). Shortly, following insulin injection into the cava vein, a portion of the hind limb *soleus* muscle was removed and homogenized. Insoluble material was removed by centrifugation at 15,000 rpm in a Ti-70 rotor (Beckman) for 50 min. IRS1 and IRS2 were immunoprecipitated from aliquots of the supernatants containing 4 mg of total protein utilizing anti-IRS1 or anti-IRS2 antisera followed by protein A-Sepharose 6MB. Alternatively, anti-rat p85/PI3-kinase antibodies (whole serum, 1 μl/ml) were used. The immunoprecipitates were washed successively in appropriate ice-cold phosphate-buffered saline. To each pellet was added 10 μl of 100 mmol/l $MgCl_2$ and 10 μl of phosphatidylinositol (2 μg/μl) sonicated in 10 mmol/l Tris (pH 7.5) with 1 mmol/l EGTA. The PI3-kinase reaction was started by the addition of 10 μl of 440 μmol/l ATP containing 30 μCi of [^{32} P]ATP. After 10 min at room temperature with constant shaking, the reaction was stopped by the addition of 20 μl of 8 N HCl and 160 μl of $CHCl_3$: methanol (1:1). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate (Merck) coated with 1% potassium oxalate. TLC plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2), dried, and visualized by autoradiography. The radioactivity in spots, which co-migrated with a PI-4 standard, was measured by densitometry of the autoradiographic images obtained (Scion software).

Clamp Studies: All procedures for clamp studies followed a previously published description of the method (7). After 6 h fast, a 2 h *hyperinsulinemic euglycemic clamp* study was performed in denervated or sham operated limb. Under sodium thiopental

anesthesia and aseptic conditions, a mono-occlusive polyethylene catheter was inserted into the femoral artery towards the limb for infusion of insulin and glucose. A second polyvinyl catheter was inserted into the femoral vein for blood sampling, and the animal was kept in a heated box (37°C) throughout the study. During the first phase of the study (30 min) a priming dose of insulin was infused followed by a rate of glucose infusion necessary to reach a plateau. After glucose equilibration, insulin infusion (3 $mU.Kg^{-2}.min^{-1}$) was maintained for 2 h with constant rate (0.20 ml/h) and a variable infusion of glucose (5% solution) was adjusted to maintain the plasma glucose concentration at approximately 120 mg/dl. Blood samples were collected from the femoral vein every 5 min for plasma glucose and every 30 min for plasma insulin determinations. Insulin was measured in duplicate by radioimmunoassay and oscillated between 6,0 and 11,0 ng/ml in denervated limb blood samples, and between 4,0 and 9,0 ng/ml in sham operated limb blood samples.

HPLC analysis: The methods used were previously published (16, 26, 34). For identification of phosphoinositols (IPs), *soleus* muscle was excised and homogenized in 1.8 ml of a mixture of methanol:chloroform:$HClO_4$ (8%) (20:10:1). After addition of 500 µl of chloroform and 500 µl of 1% $HClO_4$, the lower organic phase was collected, washed twice with 1% $HClO_4$ and evaporated. Deacylation was made as described previously (16). The product of the deacylation was resolved on an anion-exchange column (Shodex Aschipak, E8502 N7C) with the gradient of 980 µM (A)-3M NaH_2PO_4 (B), pH 3.8. The linear gradient rose to 7% over 30 min, a 1 min step to 15% was followed by a linear gradient to 30% B at 60 min, followed by a linear gradient to 60% B at 80min. Finally, buffer B was increased to 100% over 5 min. Non-phosphorylated IP control was obtained by resolving

highly purified IP from Avanti (which peaked at 25 min) in parallel to sample analysis. Phosphorylated IPs were detected as a peak occurring at 28-29 min.

Sense and antisense oligonucleotide studies : Sense and antisense oligonucleotides were diluted to a final concentration of 20 μmol/l in dilution buffer containing 10 mmol/l Tris-HCl and 1 mmol/l EDTA. The rats were injected (i.p) with 200 μl of dilution buffer containing or not sense or antisense oligonucleotides 2 h before and immediately after denervation. Phosphothioate-modified oligonucleotides were designed according to NM 022944 *Rattus norvegicus* SHIP-2 sequence and were composed of sense (5'-CTG CGG AGG AGC TGC T-3') and antisense (5'-AGC AGC TCC TCC GCA G-3').

Statistical Analysis: The results obtained in the denervated limb were always compared with data obtained in the sham-operated limb, in experiments performed in parallel. Student's *t*-test for paired samples was used for comparison. The level of significance was set at $p < 0.05$.

RESULTS

Protein expression and insulin-induced activation/phosphorylation of elements participating of the insulin-signaling cascade. Binding of circulating insulin to its receptor promotes β subunit autophosphorylation and triggers a series of intracellular events responsible for the final effects of insulin in a given cell. In the present model of muscle insulin resistance no changes in IR protein content was detected (Fig 1A), but a significant fall in insulin-induced IR tyrosine phosphorylation was observed (Fig. 1B, 73% of control, $p<0.05$). The protein contents of the main substrates of the IR (IRS1 and IRS2)

were unaffected by short-term denervation (Figs. 2A and 3A). Nonetheless, insulin induced tyrosine phosphorylation of either IRS1 (69%, $p<0.05$) or IRS2 (82%, $p<0.05$) were reduced (Figs. 2B and 3B). A metabolically active branch of the insulin-signaling cascade depends on activation of the lipid-metabolizing enzyme PI3-kinase. In order to be activated by insulin stimulus, the p85 subunit of the PI3-kinase must be engaged by either IRS1 or IRS2. In shortly denervated *soleus* muscle the insulin-induced association of p85 with IRS1 (73%, $p<0.05$) or IRS2 (73%, $p<0.05$) were significantly reduced (Fig. 2C and 3C). However, the activity of the associated PI3-kinase, as measured by its capacity to incorporate phosphorus in phosphoinositide (PI) was unexpectedly increased (275%, $p<0.05$ for IRS1 associated and 180%, $p<0.05$ for IRS2 associated) (Fig. 2D and 3D). Divergence between IRS1- and IRS2-associated p85/PI3-kinase amounts and the associated PI3-kinase activity have been observed in other situations, and differences in the level of serine phosphorylation of p85/PI3-kinase was pointed as a possible reason for the observed phenomenon (11, 12, 32). Therefore, membranes from IRS1 and IRS2 immunoprecipitates were stripped and reblotted with anti-phospho-serine antibodies. As depicted in Figure 4 a significantly lower level of serine phosphorylation is detected in the associated p85/PI3-kinase (52% and 46% of control for IRS1- and IRS2-associated respectively, $p<0.05$), which possibly explains the increased associated PI3-kinase activity, despite of its lower association with the insulin receptor substrates. The resulting $PI_{3',4'}P_2$ and $PI_{3',4',5'}P_3$ serve as docking sites for the enzymes Akt and PDK1. Apparently, PDK1 binds preferentially to $PI_{3',4'}P_2$ and once in the same subcellular location of Akt (which binds with higher affinity to $PI_{3',4',5'}P_3$) catalyzes its serine phosphorylation and activation. In spite of the higher IRS1 and IRS2 associated PI3-kinase activity, in shortly denervated *soleus* muscle a significantly

lower level of serine phosphorylated Akt was detected (39%, $p<0.05$) (Fig. 5). In face of the incongruent finding of higher IRS1/IRS2 associated PI3-kinase activity in a known model of insulin resistance and occurring in parallel with reduced insulin-induced IR, IRS1 and IRS2 tyrosine phosphorylation and insulin-induced Akt serine phosphorylation, we decided for measuring the amounts of phosphorylated species of PI in lipid extracts of insulin stimulated intact (sham-operated) and denervated *soleus* muscle.

Glucose clearance and accumulation of P IPs. Muscle denervation is known to produce insulin resistance. In order to investigate if the present model was in accordance with the current literature, denervated and intact rats were submitted to an isolated limb *hyperinsulinemic euglycemic clamp*. As expected, denervation produced a significant fall in the rate of glucose uptake under high insulin stimulus (62% of control, $p<0.05$) (Fig. 6). Akt is one of the major participants in the insulin-induced activation of GLUT4 trafficking from an intracellular to a membrane pool. As stated above the presence of $PI_{3',4'}P_2$ and mostly $PI_{3',4',5'}P_3$ are necessary for activation of Akt and thus, for mediating insulin-induced glucose uptake. Therefore, we evaluated the amount of P IPs in lipid extract from *soleus* muscles obtained from intact and denervated hind limbs. By using an anion exchange column the IPs of the membranes were resolved by HPLC and a significant reduction in total amount of phosphorylated membrane inositols were detected (Fig. 7B). Since approximately 95% of membrane IPs are composed by the 5' phosphorylated forms of IPs, we suspected that a dysfunction of the 5'-position phosphoinositide phosphatase SHIP2 could participate in the phenomena observed in shortly denervated muscle. Thus, we measured the protein amounts and phosphorylation status of SHIP2.

Expression and tyrosine phosphorylation of SHIP2. SHIP2 is an SH2 domain-containing 5'inositide phosphatase involved in the control of the insulin signal (3). As a significant reduction in total phosphorylated IPs was detected in *soleus* muscle of hind limb denervated rats (Fig. 7B), we performed experiments to determine the protein amount and tyrosine phosphorylation status of SHIP2. No difference in SHIP2 protein content was detected when comparing total extracts obtained from *soleus* muscle of control and denervated hind limbs (Fig. 8A, first and second lanes). However, when insulin-induced tyrosine phosphorylation of SHIP2 was evaluated, a significant increase was detected in denervated hind limb *soleus* muscle as compared to control (Fig. 8B). Moreover, even in non-insulin stimulated samples a higher-than-control tyrosine phosphorylation of SHIP2 was found, which matched the levels of tyrosine phosphorylation of SHIP2 present in insulin-stimulated control muscle (Fig. 8B).

Rate of glucose clearance and accumulation of P IP in rats treated with SHIP2 antisense oligonucleotide. To test the hypothesis of involvement of SHIP2 in the mechanisms of denervation-induced insulin resistance control and denervated rats were treated with two doses of 6 nmol phosphothioate sense or antisense oligonucleotides. Treatment with antisense oligonucleotide significantly reduced SHIP2 protein expression (Fig. 9A, 5^{th} and 6^{th} lanes). Antisense, but not sense oligonucleotide, partially restored the rate of insulin stimulated glucose clearance as detected by isolated limb *hyperinsulinemic euglycemic clamp* (Fig. 10) and promoted a partial recovery of total phosphorylated IPs amounts as detected by HPLC (Fig 9G).

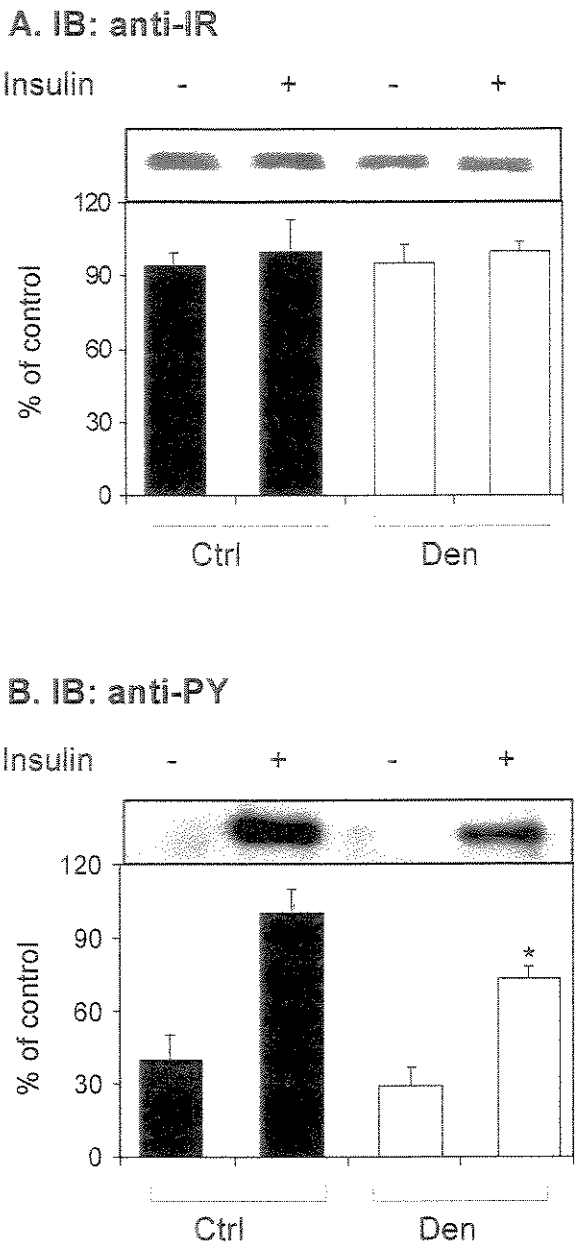

Figure 1. Expression of insulin receptor and levels of tyrosine phosphorylation of insulin receptor in denervated and sham-denervated muscle. Equal amounts of protein (4 mg), extracted from sham-denervated (Ctrl) and denervated muscles (Den), were subjected to immunoprecipitation (IP) with anti-IR antibody and immunoblotting (IB) with anti-IR antibody (**A**) or anti-phosphotyrosine (PY) (**B**). The black bars and white bars represent control and denervated muscle, respectively. Intensities of the bands in the autoradiograms were normalized to the levels of the insulin-stimulated sham-denervated muscles, noted as 100%. The results are expressed as means ± SEM of 10 independent experiments (*$p<0.05$, vs. Ctrl + insulin).

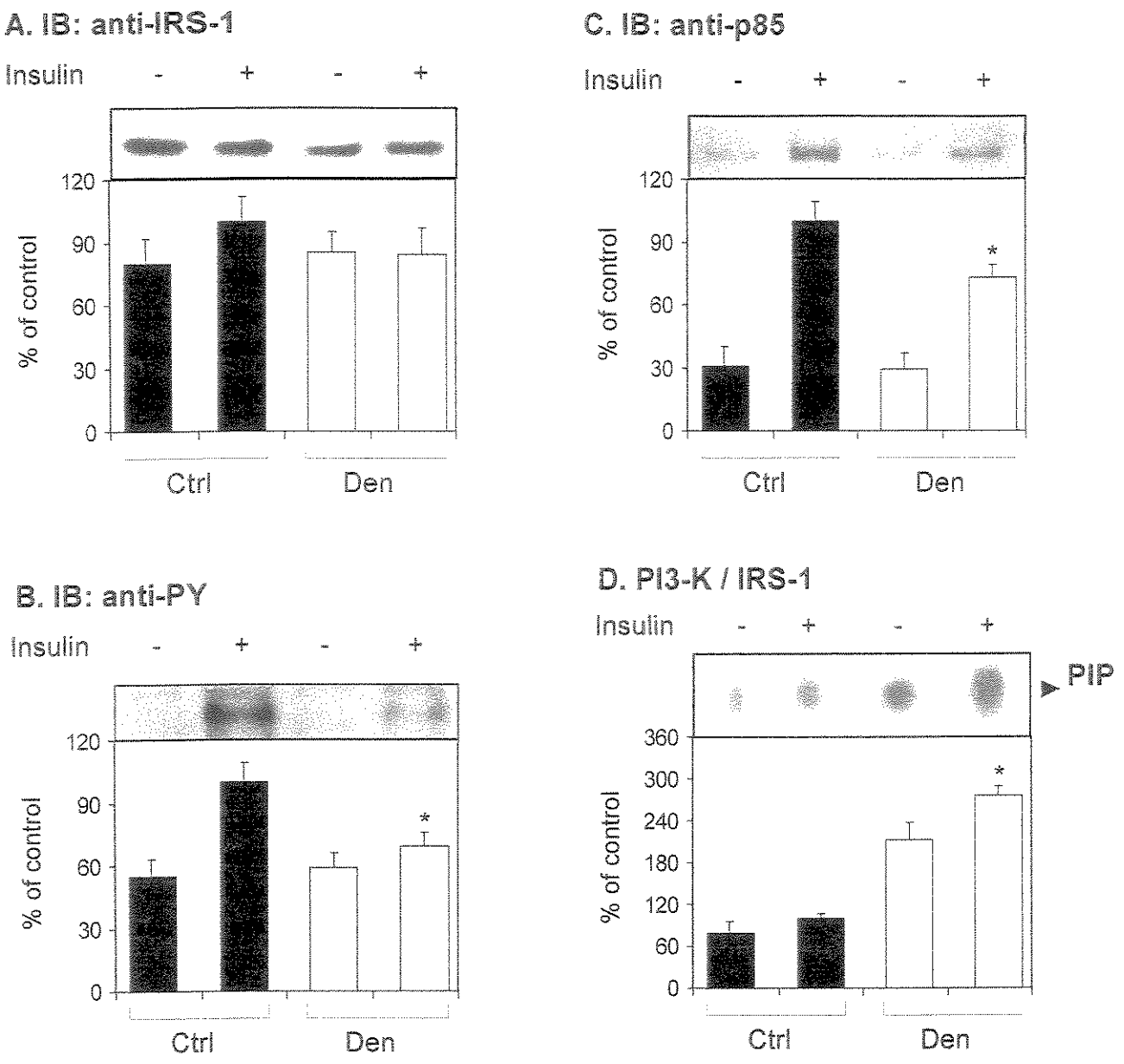

Figure 2. Expression and levels of tyrosine phosphorylation of IRS1, insulin-stimulated association of IRS1 with PI3-kinase, and IRS1 associated PI3-kinase activity in denervated and sham-denervated muscle. Equal amounts of protein (4 mg), extracted from sham-denervated (Ctrl) and denervated muscles (Den), were subjected to immunoprecipitation (IP) with IRS1 antibody (**A-D**) and immunoblotting (IB) with IRS1 antibody (**A**), anti-phosphotyrosine (PY) (**B**) and with anti-p85 (**C**). The IRS1 associated PI3-kinase activity is showed in (**D**). The black bars and white bars represent control and denervated muscle, respectively. Intensities of the bands in the autoradiograms were normalized to the levels of the insulin-stimulated sham-denervated muscles, noted as 100%. The results are expressed as means ± SEM of 4 to 15 independent experiments (*$p<0.05$, vs. Ctrl + insulin).

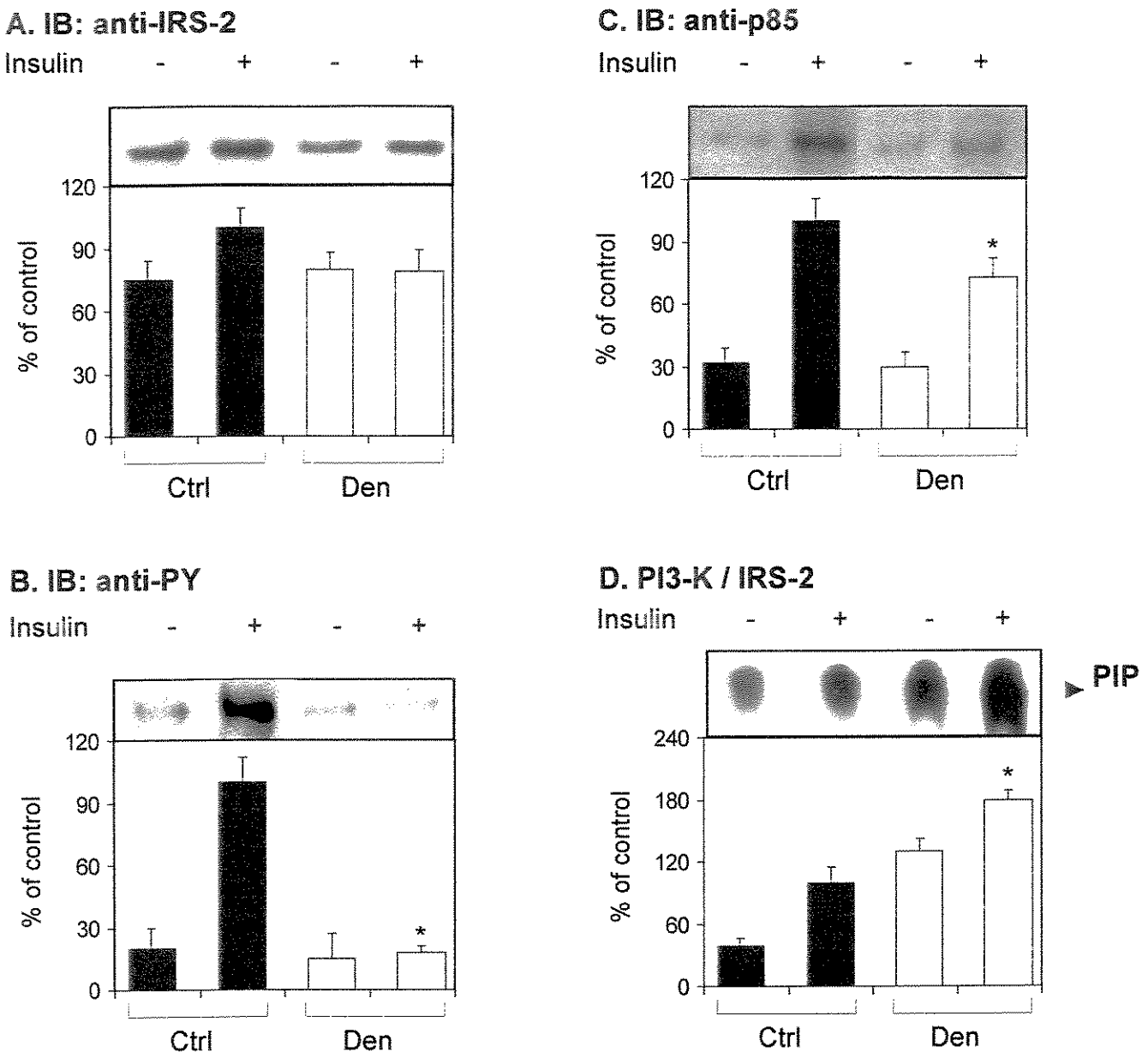

Figure 3. Expression and levels of tyrosine phosphorylation of IRS2, insulin-stimulated association of IRS2 with PI3-kinase, and IRS2 associated PI3-kinase activity in denervated and sham-denervated muscles. Equal amounts of protein (4 mg), extracted from sham-denervated (Ctrl) and denervated muscles (Den), were subjected to immunoprecipitation (IP) with IRS2 antibody (**A** to **D**) and immunoblotting (IB) with IRS2 antibody (**A**), anti-phosphotyrosine (PY) (**B**) and with anti-p85 (**C**). The IRS2 associated PI3-kinase activity is showed in (**D**). The black bars and white bars represent control and denervated muscle, respectively. Intensities of the bands in the autoradiograms were normalized to the levels of the insulin-stimulated sham-denervated muscles, noted as 100%. The results are expressed as means ± SEM of 4 to 12 independent experiments (*$p<0.05$, vs. Ctrl + insulin).

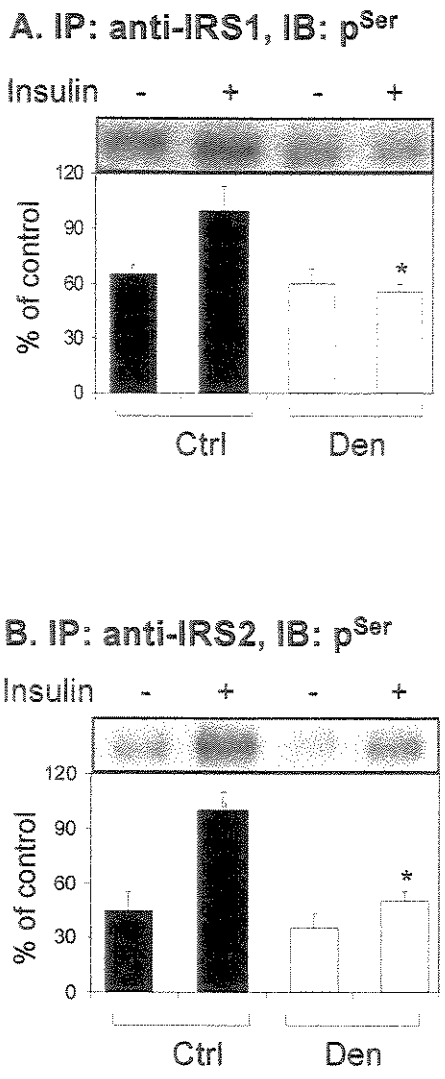

Figure 4. Serine phosphorylation of IRS1- and IRS2-associated p85. Membranes from the experiments presented in Figures 2 and 3 were stripped and reblotted with anti-phosphoserine (pSer) antibody. Bands appearing at 85 kDa, corresponding to IRS1- (A) or IRS2- (B) associated p85-PI3kinase were scanned and densitometricaly measured. The black bars and white bars represent control (Ctrl) and denervated (Den) muscle, respectively. Intensities of the bands in the autoradiograms were normalized to the levels of the insulin-stimulated sham-denervated muscles, noted as 100%. The results are expressed as means SEM of 6 independent experiments (*$p<0.05$, vs. Ctrl + insulin).

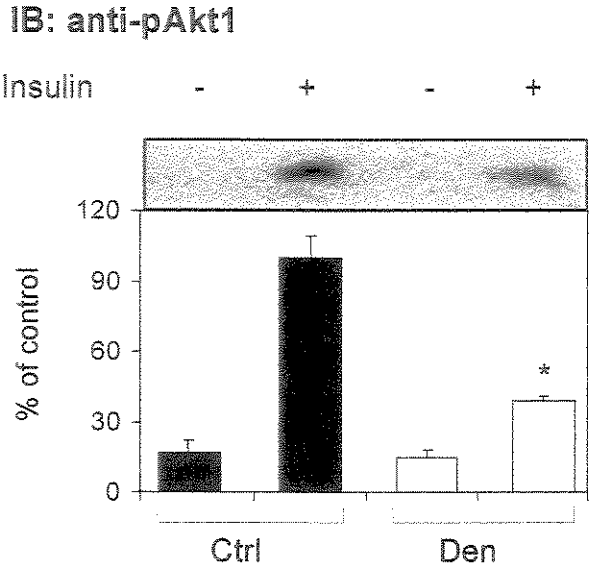

Figure 5. Levels of [ser^{473}]-phosphorylation of Akt1 in denervated and sham-denervated muscles. Equal amounts of protein (200 mg), extracted from sham-denervated (Ctrl) and denervated muscles (Den), were subjected to immunoblotting (IB) with [ser^{473}]-pAkt antibodies. The black bars and white bars represent control and denervated muscle, respectively. Intensities of the bands in the autoradiograms were normalized to the levels of the insulin-stimulated sham-denervated muscles, noted as 100%. The results are expressed as means ± SEM of 3 independent experiments (*$p<0.05$, vs. Ctrl + insulin).

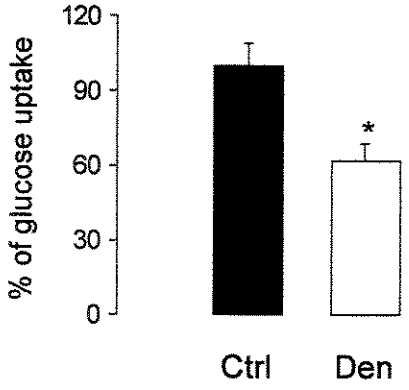

Figure 6. Hind limb glucose uptake as evaluated by hyperinsulinemic clamp study. The two-hour-study of euglycemic-hyperinsulinemic clamp performed in isolated limbs detected a 38% reduction of glucose uptake in denervated (Den) as compared to sham-denervated (Ctrl) muscle (n = 6, *$p<0.05$, vs. Ctrl + insulin).

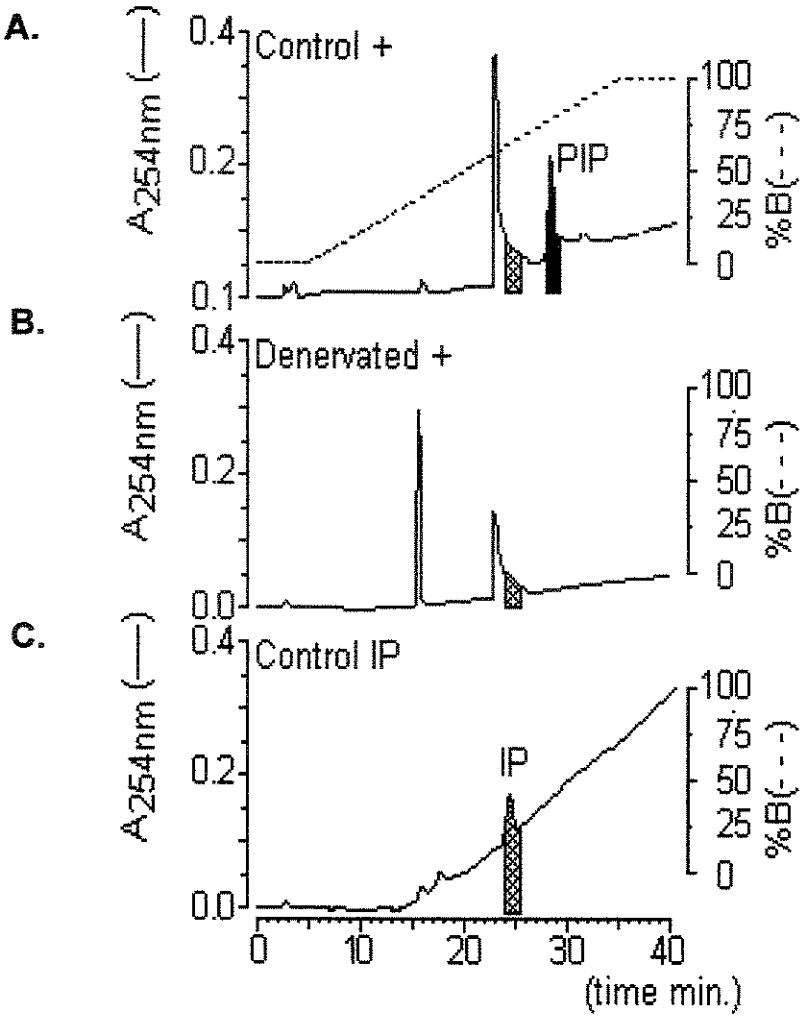

Figure 7. Measurement of phosphorylated phosphoinositols in denervated and sham-denervated muscle by HPLC. After extraction and deacylation the phospholipids were resolved on anion-exchange column as described in Research Design and Methods. The identification of the peaks was determined based on comparison with the mobility of IP. The peak appearing at 25 min corresponds to non-phosphorylated phosphoinositols (IP) as determined by the resolution of purified IPs from Avanti (**C**). Phosphorylated phosphoinositols peaks at 28-29 min and is present in high amounts in sham-denervated muscle (**A**), but is almost absent in denervated muscle samples (**B**) (n = 3).

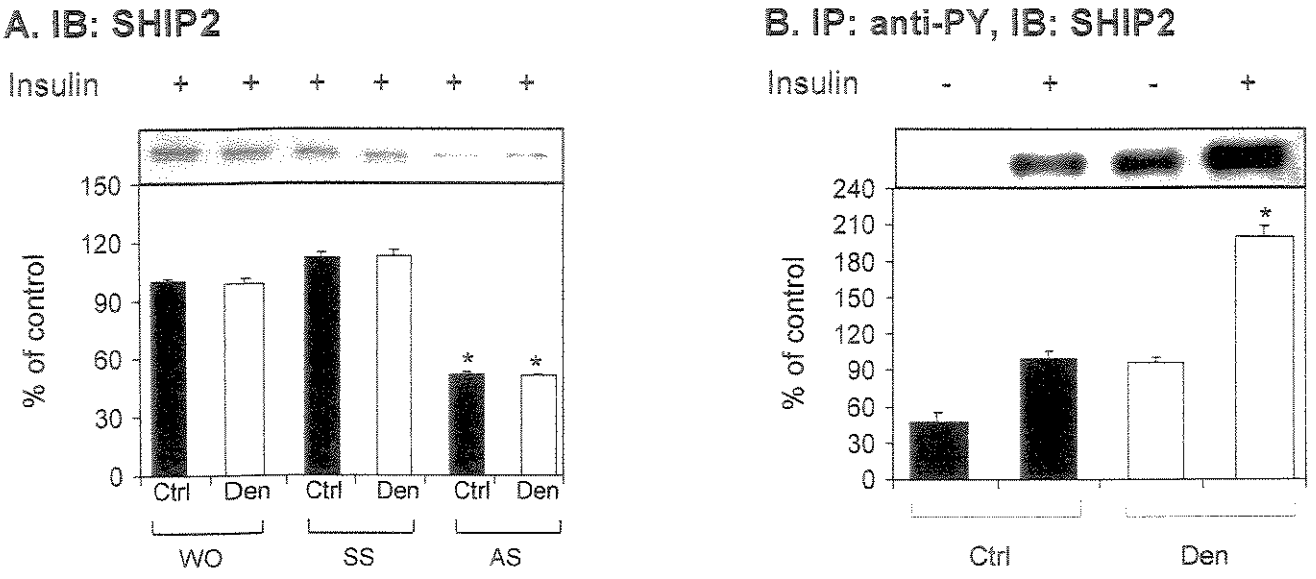

Figure 8. Expression and levels of tyrosine phosphorylation of SHIP2 in denervated and sham-denervated muscle. Equal amounts of protein (200 mg for immunoblot and 4 mg for immunoprecipitation), extracted from sham-denervated (Ctrl) and denervated muscle (Den), were subjected to immunoblotting (IB) with anti-SHIP2 antibody (A) or immunoprecipitation (IP) with anti-phosphotyrosine (PY) and immunoblotting (IB) with anti-SHIP2 antibody (B). The black bars and white bars represent control and denervated muscle, respectively. Intensities of the bands in the autoradiograms were normalized to the levels of the insulin-stimulated sham-denervated muscles, noted as 100%. The results are expressed as means ± SEM of 5 independent experiments (*$p<0.05$, vs. Ctrl + insulin). In A, WO means not exposed to oligonucleotide; SS exposed to sense SHIP2 oligonucleotide; and AS exposed to antisense SHIP2 oligonucleotide.

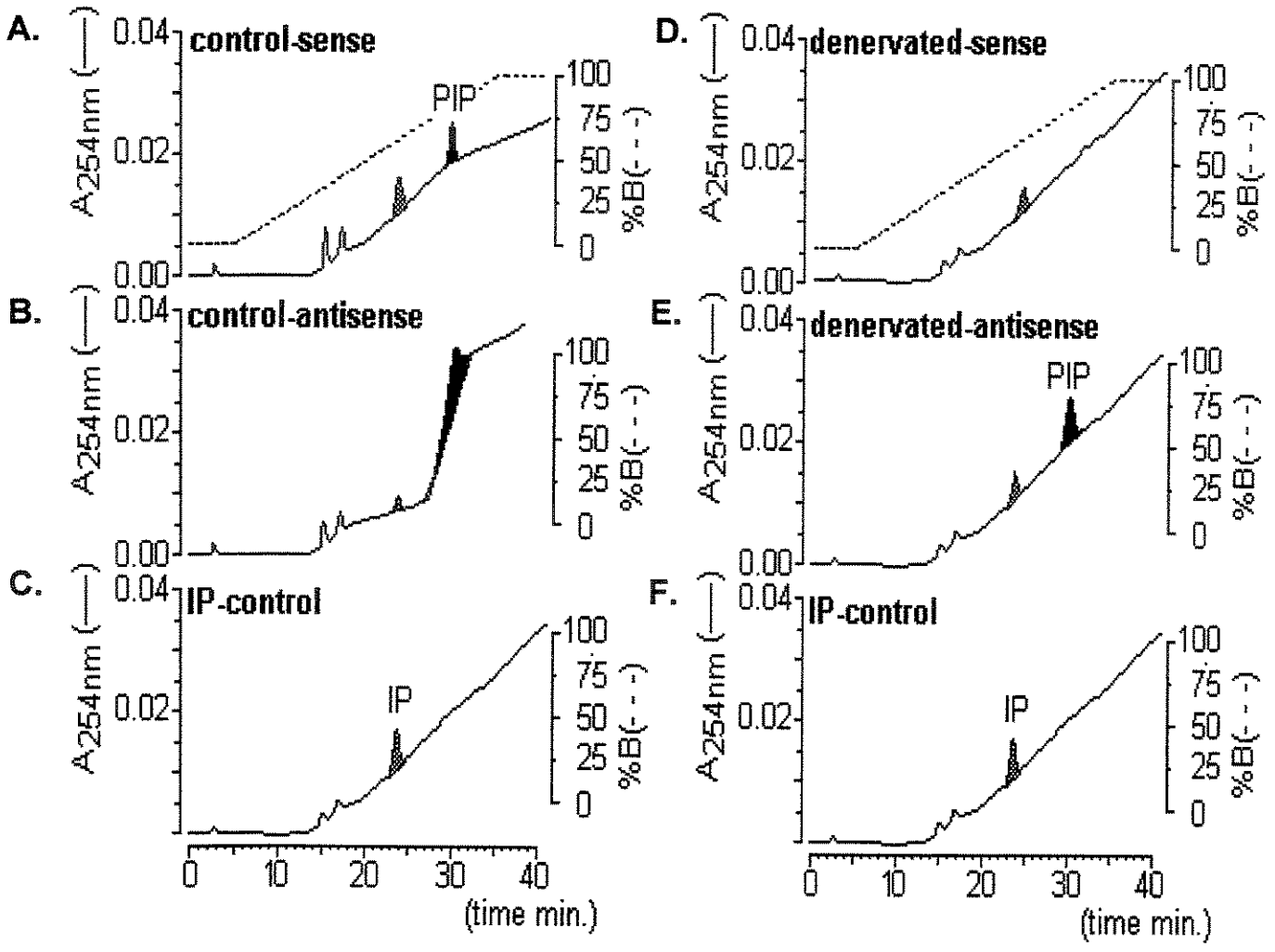

Figure 9. HPLC analysis of PIP derivatives associated with SHIP2 oligonucleotide treatment. For HPLC (**A** to **F**), the tissues were extracted 4 hours after denervation, phospholipids were extracted, deacylated and resolved on anion-exchange column as described in Research Design and Methods. The identity of the peaks was determined based on comparison with the mobility of IP. (**A**) sham-denervated treated with sense SHIP2 oligonucleotide, (**B**) sham-denervated treated with antisense SHIP2 oligonucleotide, (**C**) IP-control, (**D**) denervated treated with sense SHIP2 oligonucleotide, (**E**) denervated treated with antisense SHIP2 oligonucleotide, (**F**) IP-control (n = 3, *p<0.05, vs. Ctrl + insulin).

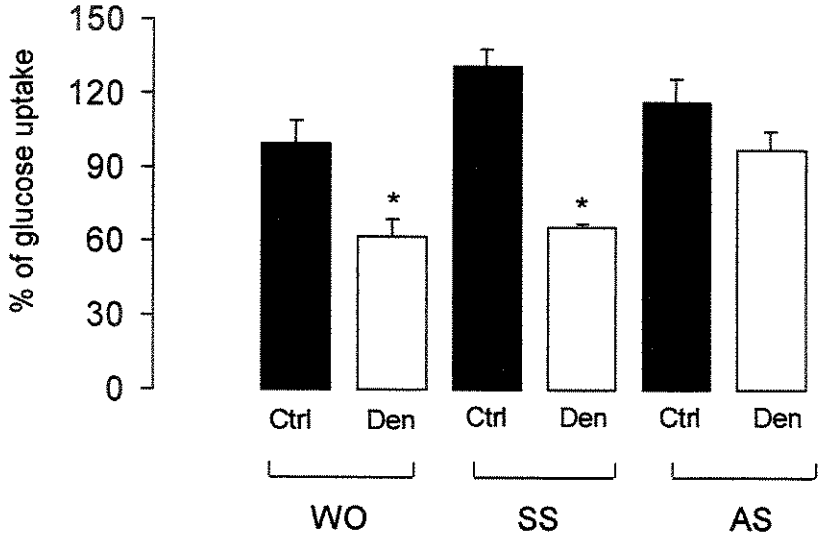

Figure 10. Hind limb glucose uptake as evaluated by hyperinsulinemic clamp study in rats treated antisense SHIP2 oligonucleotide. The two-hour-study of euglycemic hyperinsulinemic clamp performed in isolated limb of rats treated with antisense (AS) SHIP2 oligonucleotides revealed a significant improvement of glucose uptake as compared to animals treated with no oligonucleotide (WO) or treated with sense SHIP2 oligonucleotide (SS) (n = 4, *$p<0.05$, vs. Ctrl + insulin).

DISCUSSION

The negative modulation of different steps of the insulin-signaling pathway has been demonstrated in several clinical and experimental situations where insulin resistance prevails (22). Thus, reduction in protein expression or functional status of the IR, IRS1, IRS2, PI3-kinase and Akt are known to occur at various degrees and displaying specific characteristics during aging, dexamethasone or epinephrine treatment, hypertension, and obesity, among other situations (23). The characterization of the molecular mechanisms of insulin signaling, in such a diversity of situations, known to couple with insulin resistance has contributed to reinforce the concepts of multifactoriality and complexity of type 2 diabetes and insulin resistance.

In the present series of experiments it is demonstrated that short-term denervation leads to impaired glucose uptake strictly in the denervated portion of the body. As in sham-operated limb, muscle from the denervated side presents no changes in the protein amount of IR, as well as no modulation in IRS1 and IRS2 protein expression. However, insulin-induced IR, IRS1 and IRS2 tyrosine phosphorylation and p85-IRS1/2 association are significantly reduced in denervated muscle. Up to these steps of the insulin signaling cascade the data herein presented are somewhat similar to those presented in a few recent studies (14). Notwithstanding, when analyzing the next step of the pathway we were surprised by an apparently incongruent finding. Thus, although a significant reduction in insulin-induced IRS1 and IRS2 tyrosine phosphorylation was accompanied by reduced binding of p85/PI3-kinase in *soleus* muscle from denervated limbs, a significant increase in insulin-induced IRS1 and IRS2 bound PI3-kinase activity was detected. Finally, and in accordance with recent studies (33), a significant reduction of insulin stimulated serine

phosphorylation of Akt was detected in denervated *soleus* muscle. Divergence between catalytic activity and association of p85/PI3-kinase with either IRS1 and IRS2 was previously observed in angiotensin II-induced activation of elements of the insulin signaling pathway (11, 12, 32), which was accounted for by differential serine phosphorylation of p85/PI3-kinase. In the present model of insulin resistance a significant reduction of IRS1- and IRS2-associated p85/PI3-kinase activity was detected in *soleus* muscle of denervated hind limb, which might respond for the increased PI3-kinase activity detected.

PI3-kinase plays a pivotal role in the molecular linkage between early and late cellular events triggered by a number of hormones, growth factors and cytokines (8). Active PI3-kinase mediates the incorporation of phosphate at 3' position in membrane bound phosphoinositides generating phosphatidylinositol 3,4-bisphosphate ($PI_{3,4}P_2$) and phosphatidylinositol 3,4,5-trisphosphate ($PI_{3,4,5}P_3$). These phosphorylated PIs participate in the control of cellular mitogenesis, apoptosis, membrane trafficking, oncogenesis and nutrient uptake and stocking. As a substrate of the insulin-signaling pathway, PI3-kinase is activated downstream to IRS1 and IRS2 and induces the activation of the serine kinase Akt. The activation of Akt/PKB depends on its binding through a pleckstrin homology (PH) domain to $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$ present in cellular membrane. Once bound to membrane PIs Akt becomes available for the catalytic activity of PDK1, which mediates threonine phosphorylation, and for a yet unknown serine kinase necessary for full activation of Akt (5). Since recent studies have emphasized the predominant role of $PI_{3,4,5}P_3$ as target and mediator of Akt activation, and as 5' position phosphorylated PIs correspond to more than 90 % of the membrane's PIs, we measured by HPLC, the total amount of PIs in the cell

membranes of denervated and sham operated limb muscle. As shown in Fig. 7 denervation led to substantial reduction in the amount of phosphorylated PIs in cell membrane. Thus, although an apparent mechanistic explanation for reduced insulin-induced serine phosphorylation of Akt in denervated muscle was found, a question about the outcome of an increased PI3-kinase activity induced by insulin remained. Supposing that enough substrate for PI3-kinase ($PI_{4,5}P_2$ and PI_4P_1) should be available, a rate of PIs dephosphorylation that would surplus the rate of phosphate incorporation might explain the present findings. Two recently identified phosphatases involved in PI3-kinase signaling are known to dephosphorylate membrane bound phospho-PIs, PTEN and SHIP2. PTEN is the protein product of the *PTEN* tumor suppressor gene that is highly associated with development of glioblastomas and endometrial carcinomas when mutated or lost (25). PTEN is a 3' position PI phosphatase, which antagonizes the actions of PI3-kinase by reducing the amount of membrane bound $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$. The loss of PTEN activity leads to increased Akt activation, while hyperexpression of PTEN reduces the amounts of serine-phosphorylated Akt inducing cell cycle arrest and increased rate of apoptosis (28). As stated above, highest levels of membrane bound PIs are accounted by the 5' position phosphorylated PIs. Therefore, as in the present model a drastic reduction in total phosphor-PIs in the membranes was detected, an increased activity of 5' position phosphatases should become the primary candidate to be investigated. SHIP2 is a member of the inositol polyphosphatase 5-phosphatase family and a potent negative regulator of insulin signaling and insulin sensitivity *in vivo*. In response to stimulation by insulin, SHIP2 is closely linked to signaling events mediated by PI3-kinase and Ras/mitogen-activated protein kinase. The loss of SHIP2 leads to increased sensitivity to insulin

associated with an increased recruitment of the GLUT4 glucose transporter and increased glycogen synthesis in skeletal muscle (3). In the present model no difference in SHIP2 protein content was detected between control and denervated muscle when performing immunoblot analysis of total protein extracts. However, when evaluating tyrosine phosphorylation status of the 5' phosphatase, a significant difference was detected between control and denervated *soleus* muscle, both in insulin-treated and non-insulin treated rats. In fact, muscle from non-insulin stimulated animals presented a SHIP2 tyrosine phosphorylation status that was similar to that detected in insulin stimulated control muscle.

The role for tyrosine phosphorylation of SHIP2 upon its phosphatase activity is a matter of controversy. In initial reports it was suggested that tyrosine phosphorylation would hamper SHIP1 phosphatase activity (18, 24). However, in recent studies the regulation of SHIP2 tyrosine phosphorylation by insulin and other growth factors was demonstrated, and suggestively participates in the positive control of its phosphatase activity (15, 28).

To investigate the participation of SHIP2 in denervation-induced insulin resistance we designed phosphothioate-modified antisense oligonucleotides against SHIP2 mRNA, and treated the experimental animals with two doses of the compound which resulted in 50 % reduction of SHIP2 content in both control and denervated *soleus* muscle (Fig. 8A). Restrained SHIP2 expression led to significant increase in membrane bound total phosphorylated IPs and coincided with a reversal of denervation-induced decrease in insulin-induced glucose uptake as evaluated by *hyperinsulinemic euglycemic clamp*.

Site specific insulin resistance generated by acute denervation is accompanied by a complex interplay of functional modulatory events occurring upon several participants of

the insulin signal transduction cascade. In the present study it is demonstrated that significantly reduced amounts of phosphorylated PIs is present in the membrane of denervated muscle, which is accompanied by increased steady-state and insulin-induced tyrosine phosphorylation of SHIP2. The blockade of SHIP2 protein expression by antisense oligonucleotide treatment reverted the most remarking clinical manifestation of muscle insulin resistance i.e., insulin induced glucose uptake. Thus, it is suggested that functional modulation of SHIP2 may participate in the genesis of denervation-induced insulin resistance and approaches that hamper SHIP2 action may serve as therapeutic methods in diabetes mellitus and syndromes of insulin resistance.

Acknowledgements. These studies were supported by grants from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), CNPq/ Pronex (Coordenação Nacional de Pesquisa e Desenvolvimento).

REFERENCES

1. Burant, C. F., M. K. Treutelaar, and M. G. Buse. In vitro and in vivo activation of the insulin receptor kinase in control and denervated skeletal muscle. *J Biol Chem* 261: 8985-93., 1986.

2. Chen, K. S., J. C. Friel, and N. B. Ruderman. Regulation of phosphatidylinositol 3-kinase by insulin in rat skeletal muscle. *Am J Physiol* 265: E736-42., 1993.

3. Clement, S., U. Krause, F. Desmedt, J. F. Tanti, J. Behrends, X. Pesesse, T. Sasaki, J. Penninger, M. Doherty, W. Malaisse, J. E. Dumont, Y. Le Marchand-Brustel, C. Erneux, L. Hue, and S. Schurmans. The lipid phosphatase SHIP2 controls insulin sensitivity. *Nature* 409: 92-7., 2001.

4. Coderre, L., M. M. Monfar, K. S. Chen, S. J. Heydrick, T. G. Kurowski, N. B. Ruderman, and P. F. Pilch. Alteration in the expression of GLUT-1 and GLUT-4 protein and messenger RNA levels in denervated rat muscles. *Endocrinology* 131: 1821-5., 1992.

5. Coffer, P. J., J. Jin, and J. R. Woodgett. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J* 335: 1-13., 1998.

6. de L. A. Fernandes, M. L., M. J. Saad, and L. A. Velloso. Insulin induces tyrosine phosphorylation of the insulin receptor and SHC, and SHC/GRB2 association in cerebellum but not in forebrain cortex of rats. *Brain Res* 826: 74-82., 1999.

7. DeFronzo, R. A., J. D. Tobin, and R. Andres. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 237: E214-23., 1979.

8. Del Aguila, L. F., R. K. Krishnan, J. S. Ulbrecht, P. A. Farrell, P. H. Correll, C. H. Lang, J. R. Zierath, and J. P. Kirwan. Muscle damage impairs insulin stimulation of IRS-1,

PI 3-kinase, and Akt-kinase in human skeletal muscle. *Am J Physiol Endocrinol Metab* 279: E206-12., 2000.

9. Elmendorf, J. S., A. Damrau-Abney, T. R. Smith, T. S. David, and J. Turinsky. Phosphatidylinositol 3-kinase and dynamics of insulin resistance in denervated slow and fast muscles in vivo. *Am J Physiol* 272: E661-70., 1997.

10. Etgen, G. J., Jr., R. P. Farrar, and J. L. Ivy. Effect of chronic electrical stimulation on GLUT-4 protein content in fast-twitch muscle. *Am J Physiol* 264: R816-9., 1993.

11. Folli, F., C. R. Kahn, H. Hansen, J. L. Bouchie, and E. P. Feener. Angiotensin II inhibits insulin signaling in aortic smooth muscle cells at multiple levels. A potential role for serine phosphorylation in insulin/angiotensin II crosstalk. *J Clin Invest* 100: 2158-69., 1997.

12. Folli, F., M. J. Saad, L. Velloso, H. Hansen, O. Carandente, E. P. Feener, and C. R. Kahn. Crosstalk between insulin and angiotensin II signalling systems. *Exp Clin Endocrinol Diabetes* 107: 133-9, 1999.

13. Forsayeth, J. R., and M. K. Gould. Inhibition of insulin-stimulated xylose uptake in denervated rat soleus muscle: a post-receptor effect. *Diabetologia* 23: 511-6., 1982.

14. Hirose, M., M. Kaneki, H. Sugita, S. Yasuhara, C. Ibebunjo, J. A. Martyn, and C. Iebunjo. Long-term denervation impairs insulin receptor substrate-1-mediated insulin signaling in skeletal muscle. *Metabolism* 50: 216-22., 2001.

15. Ishihara, H., T. Sasaoka, H. Hori, T. Wada, H. Hirai, T. Haruta, W. J. Langlois, and M. Kobayashi. Molecular cloning of rat SH2-containing inositol phosphatase 2 (SHIP2) and its role in the regulation of insulin signaling. *Biochem Biophys Res Commun* 260: 265-72., 1999.

16. Lips, D. L., and P. W. Majerus. The discovery of a 3-phosphomonoesterase that hydrolyzes phosphatidylinositol 3-phosphate in NIH 3T3 cells. *J Biol Chem* 264: 19911-5., 1989.

17. Luciano, E., E. M. Carneiro, C. R. Carvalho, J. B. Carvalheira, S. B. Peres, M. A. Reis, M. J. Saad, A. C. Boschero, and L. A. Velloso. Endurance training improves responsiveness to insulin and modulates insulin signal transduction through the phosphatidylinositol 3- kinase/Akt-1 pathway. *Eur J Endocrinol* 147: 149-57., 2002.

18. Osborne, M. A., G. Zenner, M. Lubinus, X. Zhang, Z. Songyang, L. C. Cantley, P. Majerus, P. Burn, and J. P. Kochan. The inositol 5'-phosphatase SHIP binds to immunoreceptor signaling motifs and responds to high affinity IgE receptor aggregation. *J Biol Chem* 271: 29271-8., 1996.

19. Pang, D. T., B. R. Sharma, and J. A. Shafer. Purification of the catalytically active phosphorylated form of insulin receptor kinase by affinity chromatography with O-phosphotyrosyl- binding antibodies. *Arch Biochem Biophys* 242: 176-86., 1985.

20. Robinson, K. A., K. P. Boggs, and M. G. Buse. Okadaic acid, insulin, and denervation effects on glucose and amino acid transport and glycogen synthesis in muscle. *Am J Physiol* 265: E36-43., 1993.

21. Ruderman, N. B., R. Kapeller, M. F. White, and L. C. Cantley. Activation of phosphatidylinositol 3-kinase by insulin. *Proc Natl Acad Sci U S A* 87: 1411-5., 1990.

22. Saad, M. J. Molecular mechanisms of insulin resistance. *Braz J Med Biol Res* 27: 941-57., 1994.

23. Saad, M. J., F. Folli, and C. R. Kahn. Insulin and dexamethasone regulate insulin receptors, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in Fao hepatoma cells. *Endocrinology* 136: 1579-88., 1995.

24. Sattler, M., R. Salgia, G. Shrikhande, S. Verma, J. L. Choi, L. R. Rohrschneider, and J. D. Griffin. The phosphatidylinositol polyphosphate 5-phosphatase SHIP and the protein tyrosine phosphatase SHP-2 form a complex in hematopoietic cells which can be regulated by BCR/ABL and growth factors. *Oncogene* 15: 2379-84., 1997.

25. Simpson, L., J. Li, D. Liaw, I. Hennessy, J. Oliner, F. Christians, and R. Parsons. PTEN expression causes feedback upregulation of insulin receptor substrate 2. *Mol Cell Biol* 21: 3947-58., 2001.

26. Singh, A. K., and Y. Jiang. Quantitative chromatographic analysis of inositol phospholipids and related compounds. *J Chromatogr B Biomed Appl* 671: 255-80., 1995.

27. Smith, R. L., P. J. Roach, and J. C. Lawrence, Jr. Insulin resistance in denervated skeletal muscle. Inability of insulin to stimulate dephosphorylation of glycogen synthase in denervated rat epitrochlearis muscles. *J Biol Chem* 263: 658-65., 1988.

28. Taylor, V., M. Wong, C. Brandts, L. Reilly, N. M. Dean, L. M. Cowsert, S. Moodie, and D. Stokoe. 5' phospholipid phosphatase SHIP-2 causes protein kinase B inactivation and cell cycle arrest in glioblastoma cells. *Mol Cell Biol* 20: 6860-71., 2000.

29. Turinsky, J. Dynamics of insulin resistance in denervated slow and fast muscles in vivo. *Am J Physiol* 252: R531-7., 1987.

30. Turinsky, J., and A. Damrau-Abney. Akt1 kinase and dynamics of insulin resistance in denervated muscles in vivo. *Am J Physiol* 275: R1425-30., 1998.

31. Turinsky, J., A. Damrau-Abney, and D. J. Loegering. Blood flow and glucose uptake in denervated, insulin-resistant muscles. *Am J Physiol* 274: R311-7., 1998.

32. Velloso, L. A., F. Folli, X. J. Sun, M. F. White, M. J. Saad, and C. R. Kahn. Cross-talk between the insulin and angiotensin signaling systems. *Proc Natl Acad Sci U S A* 93: 12490-5., 1996.

33. Wilkes, J. J., and A. Bonen. Reduced insulin-stimulated glucose transport in denervated muscle is associated with impaired Akt-alpha activation. *Am J Physiol Endocrinol Metab* 279: E912-9., 2000.

34. Woodcock, E. A. Analysis of inositol phosphates in heart tissue using anion-exchange high-performance liquid chromatography. *Mol Cell Biochem* 172: 121-7., 1997.

4. Conclusões

Os resultados do presente trabalho demonstraram que:

- A desnervação aguda provocou resistência à insulina, reduzindo a utilização de glicose pelo músculo esquelético.

- No músculo desnervado houve redução da fosforilação em tirosina do IR, IRS1 e IRS2 e da fosforilação em serina da Akt1 após estímulo agudo com insulina.

- No músculo desnervado, após estímulo agudo com insulina, ocorreu também redução da associação entre IRS1/IRS2 e p85/PI3kinase e redução da fosforilação em serina da p85/PI3-kinase. Entretanto, houve aumento da atividade da PI3-kinase associada ao IRS1 e IRS2, porém, com redução do conteúdo de fosfoinositóis fosforilados nas frações lipídicas.

- A desnervação aguda não alterou a expressão da fosfatase SHIP2, mas aumentou sua fosforilação após estímulo agudo com insulina. Bloqueio da expressão da SHIP2 pelo tratamento com o oligonucleotídeo antisense resultou na restauração parcial do conteúdo de fosfoinositóis fosforilados e aumento da captação de glicose pelo músculo.

- A modulação do estado funcional da SHIP2 pode ser um dos mecanismos moleculares de instalação da resistência à insulina induzida pela desnervação aguda. Assim, manobras que levam a redução da atividade da fosfatase SHIP2 pode se constituir numa ferramenta para o tratamento de síndromes de resistência à insulina.

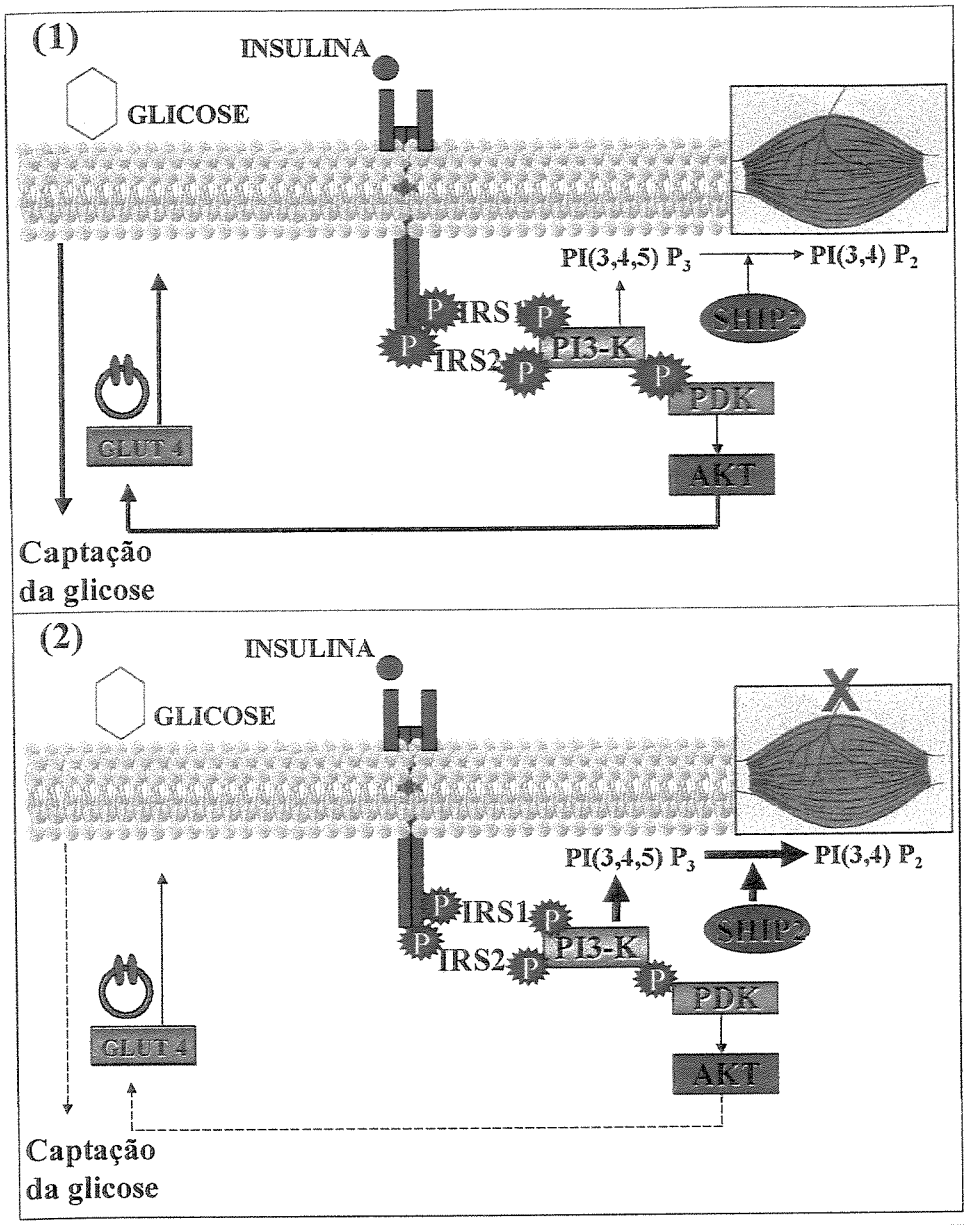

Figura B. Efeito da desnervação na transmissão do sinal da insulina no músculo esquelético

Baseado nos achados do presente estudo propõe-se o modelo acima onde (1) representa os fenômenos moleculares e funcionais que ocorrem após a ligação da insulina ao seu receptor em músculo esquelético não desnervado e em (2) as alterações decorrentes da desnervação. (O calibre das setas representa a magnitude do evento)

5. Referências Bibliográficas

Araki E.; Lipes M.A.; Patti M.E.; Bruning J.C.; Haag B.; Johnson R.S. and Kahn C.R.: Alternative patway of insulin signalling in mice with targeted disruption of the IRS1 gene. **Nature,** 372: 186-190, 1994.

Bornier M.; Laird D.M. and Lane M.D.: Insulin-activated tyrosine phosphorylation of a 15-kilodalton protein in intact 3T3-L1 adipocytes. **Proceedings of the National Academy of Science USA,** 84: 1844-1848, 1987.

Bruning J.C.; Michael M.D.; Winnay J.N.; Hayashi T.; Horsch D.; Accili D.; Goodyear L.J. and Kahn C.R.: A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. **Mol. Cell.,** 2: 559-569, 1998.

Buse M.G. & Buse J.: Glucose uptake and respnsive to insulinof the isolated rat diaphragm. The effect of denervation. **Diabetes,** 8: 218-225, 1959.

Carpenter C.L. & Cantley L.C.: Phosphoinositide kinases. **Biochemistry,** 29: 11147-11156, 1990.

Cheatham B. & Kahn C.R.: Insulin action and the insulin signaling network. **Endocri. Rev.,** 16: 117-142, 1995.

Cuatrecasas P.: Affinity chromatography and purification of the insulin receptor of liver cell membranes. **Proceedings of the National Academy of Science USA,** 69: 1277-1281, 1972.

Day C.S.; Riano F.; Tomaino M.N.; Buranatanitkit B.; Somogyi G.; Sotereanos D. and Huard J.: Growth factor may decrease muscle atrophy secondary to denervation. **J. Reconstru. Microsurg.,** 1: 51-57, 2001.

Dyson J.M.; O`Malley C.J.; Becanovic J.; Munday A.D.; Berndt M.C.; Coghill I.D.; Nandurky H.H.; Ooms L.M. and Micthell C.A.: The SH2-containing inositol polyphosphatase 5-phosphatase, SHIP2, binds filamin and regulates submembraneous actin. **J. Cell. Biol.,** 155: 1065-1079, 2001.

Folli F.; Saad M.J.A.; Backer J.M. and Kahn C.R.: Insulin stimulation of phosphatidylinositol 3-kinase and association with insulin receptor substsrate 1 in liver and muscle of the intact rat. **J. Biol. Chem.,** 267: 22171-22177, 1992.

Freychet P.; Roth J. and Neville D.M.: Insulin receptor in the liver: specific bindidng of [^{125}I] insulin to the plasma membrane and its relation to insulin bioactivity. **Proceedings of the National Academy of Science USA,** 68: 1833-1837, 1971.

Han X.X.; Fernando P.K. and Bonen A.: Denervation provokes greater reduction in insulin-stimulated glucose transport in muscle than severe diabetes. **Mol. Cell. Biochem.,** 210 (1-2): 81-89, 2000.

Henriksen E.J.; Roodnick K.J.; Mondon C.E.; James D.E and Holloszy J.O.: Effect of denervation or unweighting on glut-4 protein in rat soleus muscle. **J. Apll. Physiol.,** 70: 2322-2327, 1991.

Hofmann W.W.: Musculotrophic effects of insulin receptors before and after denervation. **Brain Research,** 401: 312-321, 1987.

Kahn C.R.: Currrent concepts of the molecular mechanism of insulin action. **Annual Review of Medicine,** 36: 429-451, 1985.

Kasuga M.; Karlsson F.A. and Kahn C.R.: Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. **Science,** 215: 185-187, 1982.

Kido Y.; Burks D.J.; Withres D.; Bruning J.C.; Kahn C.R. White M.F. and Accili D.: Tissue-specific insulin resistance in mice with mutations in insulin receptor, IRS-1 and IRS-2. **J. Clin. Invest.,** 105: 199-205, 2000.

Klip A. & Paquet M. R.: Glucose transport and glucose transporters in muscle and their metabolic regulation. **Diabets Care,** 13: 228-243, 1990.

Kohn A.D.; Summers S.A.; Birnbaum M.J. and Roth R.A.: Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. **J. Biol. Chem.,** 271: 31372-31378, 1996.

Krook A.; Roth R.A.; Jiang X.J.; Zierath J.R. and Wallberg-Henriksson H.: Insulin-stimulated Akt kinase activity is reducted in skeletal muscle from NIDDM subjects. **Diabetes,** 47: 1281-1286, 1998.

Lin Y.; Brady M.J.; Wolanske K.; Holbert R.; Ruderman N.B. and Yaney G.C.: Alterations of nPKC distribution, but not Akt/PKB activation in denervation rat soleus muscle. **Am. J. Physiol.,** E318-E325, 2002.

Marion E.; Kaisaki P. J.; Pouillon V.; Gueydan C.; Levy J.C.; Bodson A.; Krzentowski G.; Daubresse J.C.; Mockel J.; Behrends J.; Servais G.; Szpirer C.; Kruys V.; Gauguier D. and Schurmans S. The gene INPPL1, encoding the lipid phosphatase SHIP2, is a candidate for type 2 diabetes in rat and man. **Diabetes,** 51: 2012-2017, 2002.

Markuns J.F.; Wojtaszewski J.F.P. and Goodyear L.J.: Insulin and exercise decrease glycogen synthase kinase-3 activity by different mecanism in rat skeletal muscle. **J. Biol. Chem.,** 274: 24896-24900, 1999.

Muraille E.; Pessese X.; Kuntz C. and Erneux C.: Distribution of Src-homology-2-domain-containing inositol 5-phosphatase SHIP-2 in both non-haemopoietic and haemopoietic cells and possible involvement of SHIP-2 in negative signalling of B-cell. **Biochem. J.,** 342: 697-705, 1999.

Nathan D.M.: Long-term complications of diabetes mellitus. **New Eng. J. Med.,** 328: 1676-1685, 1993.

Paassen-Ijkema J.; Meek M.F: and Gramsbergen A.: Reinnervation of muscle after transection of sciatic nerve in adult rats. **Muscle Nerve,** 6: 891-897, 2002.

Saltiel A.R. & Kahn C.R.: Insulin signalling and the regulation of glucose and lipid metabolism. **Nature,** 414: 799-806, 2001.

Sanches-Margalet V.; Goldfine I.D.; Vlahos C.J. and Sung C.K.: Role of PI 3-kinase insulin receptor signaling: studies with inhibitor LY294002. **Biochem. Biophys. Res. Comm.,** 204: 336-452, 1994.

Sasaoka T.; Hori H.; Wada T.; Ishiki M.; Haruta T.; Ishihara H. and Kobayashi M. SH2-containing inositol phosphatase 2 negatively regulates insulin-induced glycogen synthesis in L6 myotubes. **Diabetologia,** 44: 1258-1267, 2001.

Schurmans S.; Carrió R.; Behrends J.; Pouillon V.; Merino J. and Clement S.: The mouse SHIP2 (INPPL1) gene: complementary DNA, genomic struture, promoter analysis, and gene expression in the embryo and adult mouse. **Genomics,** 62: 260-271, 1999.

Smith R.L.; Rach P.J. and Lawrence J.C.: Insulin Resistence in denervated skeletal muscle. **J. Biol. Chem.,** 263: 658-665, 1988.

Sun X.J.; Rothenberg P.; Kahn C.R.; et al.: Struture of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. **Nature,** 352: 73-77, 1991.

Sun X.J.; Wang L.M.; Patti M.A.; Bruning J.C.; Haag B.; Johson R.S. and Kahn C.R.: Role of IRS2 in insulin and cytikine signalling. **Nature,** 337: 173-177, 1995.

Sunderland S.: **Nerves and Nerve Injuries,** 2nd ed. Edinburgh, Churchill Livingston, 1978.

Tamemoto H.; Kadowaki T.; Tobe K., Yagi T.; Sakura H., Hayakawa T.; Terauchi Y.; Ueki K.; Kaburagi Y.; Satoh S.; et al.: Insulin resistance and growth retardtion in mice lacking insilin receptor substrate-1. **Nature,** 372: 182-186, 1994.

Taylor S.I.: Deconstructing type 2 diabetes. **Cell,** 97: 9-12, 1999.

White M.F.; Maron R. and Kahn C.R.: Insulin rapidly stimulates tyrosine phosphorylation of a Mr=185,000 protein in intact cells. **Nature,** 318: 183-186, 1985.

Withers D.J.; Gutierrez J.S.; Towery H.; Burks D.J.; Ren J.M.; Previs S.; Zhang Y.; Bernal D.; Pons S.; Shulman G.I.; Bonner-Weir S. and White M.F.: Disruption of IRS2 causes type 2 diabetes in mice. **Nature,** 391: 900-904, 1998.

Zhou M.; Vallega G., Kandror K.V. and Pilch P.: Insulin-mediated translocation of GLUT4-containing vesicles is preserved in denervated muscles. **Am. J. Physiol.,** E1019-E1026, 2000.