UNIVERSIDADE ESTADUAL DE CAMPINAS

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NOVOS SUBSTRATOS DO RECEPTOR DE INSULINA: REGULAÇÃO DA PROTEÍNA SHC EM MODELOS ANIMAIS DE RESISTÊNCIA À INSULINA E DO IRS-3 EM CÉLULAS BETA PANCREÁTICAS

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para obtenção do título de Doutor em Biologia Funcional e Molecular na área de Fisiologia

Orientador: Prof. Dr. Mário José Abdalla Saad

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SUMÁRIO

LISTA DE ABREVIAÇÕES

^{125}I	isótopo radioativo do Iodo
AII	angiotensina II
AMPc	monofosfato de adenosina cíclico
ATP	trifosfato de adenosina
BSA	albumina sérica bovina
CaM	calmodulina
DEXA	dexametasona
DNA	ácido desoxirribonucléico
DTT	ditiotreitol
EDTA	ácido etilendinitrilotetracético sal dissódico
EGF	fator de crescimento epidermal
GLUT	transportador de glicose
GRB2	proteína ligante do receptor de fator de crescimento
GTP	trifosfato de guanosina
IGF-1	fator de crescimento -1 semelhante à insulina
IGF-1R	receptor fator de crescimento – 1 semelhante à insulina
IR	receptor de insulina
IRS-1	substrato 1 do receptor de insulina
IRS-2	substrato 2 do receptor de insulina
IRS-3	substrato 3 do receptor de insulina
IRS-4	substrato 4 do receptor de insulina
IRSs	substratos do receptor de insulina
kDa	quilo daltons
μg	micrograma
μU	microunidades

min	minutos
Mr	peso molecular
Nck	molécula adaptadora
ng	nanogramas
PCR	polymerase chain reaction
PDGF	fator de crescimento derivado de plaqueta
pg	picograma
PI 3-kinase	fosfatidilinositol 3-quinase
PKA	proteína quinase A
PKC	proteína quinase C
pp125	proteína fosforilada de 125 kDa
pp160	proteína fosforilada de 160 kDa
pp180	proteína fosforilada de 180 kDa
pp60	proteína fosforilada de 60 kDa
PSMF	fenil-metil-sulfonil-fluoreto
RT	transcriptase reversa
RTKs	receptores tirosina-quinases
SDS-PAGE	sódio dodecil-sulfato/poliacrilamida gel
sec	segundos
SHPTP2	fosfatase tirosina específica
SOS	son of sevenless
Src	oncogene produto do sarcoma
STZ	estreptozotocina
TOG	tolerancia oral à glicose
Tris	Tri(hidroximetil)-aminometano
Tyr	tirosina

RESUMO

A insulina inicia seus efeitos metabólicos e promotores de crescimento através da ligação à subunidade α do seu receptor. Esta ligação promove a fosforilação em tirosina da subunidade β, e dá inicio a uma cascada de eventos intracelulares, mediante a fosforilação de vários substratos endógenos. Entre os primeiros substratos ativados estão o substrato 1 do receptor de insulina, e uma nova proteína, possuidora do domínio SH2, cuja estrutura lembra àquela do colágeno (*Src* homology), denominada Shc.

No presente estudo, determinamos a capacidade do receptor de insulina ativado de induzir a fosforilação em tirosina da proteína Shc, assim como a associação Shc-Grb2 em fígado, músculo e tecido adiposo de ratos normais e ratos submetidos a cinco situações experimentais de resistência à insulina i.e., jejum prolongado, tratamento crônico com dexametasona, envelhecimento, tratamento agudo com adrenalina e diabetes induzida por estreptozotocina.

Os resultados demonstraram que após infusão de concentrações fisiológicas de insulina em ratos normais, a Shc é substrato do receptor de insulina, cujo pico de fosforilação acontece 5 minutos após a infusão de insulina nos três tecidos estudados, e se associa à Grb2.

Ratos em jejum, uma situação aguda de resistência à insulina que cursa com baixos níveis de glicose e insulina plasmáticas, apresentaram significativa diminuição do grau de fosforilação da Shc induzida por insulina em fígado (50%) e gordura (62%), em relação aos seus controles. Por outro lado, a infusão de insulina em ratos portadores de diabetes mellitus induzida por estreptozotocina, estado caracterizado por apresentar elevados níveis de glicose sanguínea associados a baixas concentrações de insulina plasmática, levou a um significativo incremento do grau de fosforilação da Shc em fígado (175%), músculo (180%) e gordura (133%), em relação aos controles (100%). Embora

ambos os modelos experimentais apresentaram baixos níveis de insulina plasmática, as características de fosforilação da Shc foram opostas. Este resultado sugere que em modelos agudos de resistência à insulina, a concentração plasmática de glicose circulante é o principal parâmetro que determina a indução da fosforilação da proteína Shc nos tecidos estudados.

Não foi observada variação nos níveis de fosforilação em tirosina em fígado, músculo ou gordura de ratos tratados agudamente com adrenalina após estímulo com insulina, embora um aumento estatisticamente significativo nos níveis basais de associação Shc/Grb2 foi evidenciada nos três tecidos estudados nestes animais.

Em animais portadores de hiperinsulinemia crônica (ratos velhos ou tratados com dexametasona), houve um aumento da fosforilação em tirosina da Shc induzida por insulina nos tecidos hepático (64% e 58%) e muscular (81% e 64%) de ratos com 20 meses ou tratados com glicocorticoides, respectivamente. Em ratos velhos, incremento foi evidenciado também em tecido adiposo (76% acima do controle). Estes resultados sugerem que, em situações crônicas de resistência à insulina, a hiperinsulinemia pode ser um dos fatores responsáveis do aumento de fosforilação da Shc observada nestes animais.

Para todos os tecidos e modelos experimentais estudados, esta regulação demonstrou ser independente dos níveis protéicos da proteína Shc, que permaneceram inalterados, mas o grau de fosforilação em tirosina parece determinar a capacidade de associação da Shc com a proteína adaptadora Grb2.

Nossos resultados demonstram que, em tecidos animais, a Shc é susceptível de ser tirosino-fosforilada pela insulina através do seu receptor. Esta fosforilação é tempo- e dose- dependente, e sua intensidade determina as capacidade de

associação da Shc com a proteína adaptadora Grb2. Tanto o grau de fosforilação da Shc quanto a associação Shc/Grb2, apresentam regulação diferenciada, dependente do modelo de resistência à insulina e do tecido analisado, mas parece que os níveis de insulina podem contribuir para essa regulação.

A fosforilação da Shc induzida pela insulina nos modelos descritos, apresentou características diferentes daquelas exibidas pelo substrato-1 do receptor de insulina, observadas em estudos prévios realizados em ratos sujeitos a idênticas condições experimentais, sugerindo uma dissociação no padrão de comportamento destas duas proteínas, após serem ativadas pela insulina.

1. INTRODUÇÃO

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, através da modificação da expressão ou atividade de uma série de enzimas e sistemas de transporte intracelulares. Em mamíferos, a insulina é o principal hormônio regulador do metabolismo de nutrientes. Em resposta ao aumento da concentração de glicose sanguínea, as células β das ilhotas pancreáticas liberam a insulina na corrente sanguínea, que é conduzida para os diferentes tecidos alvos, promovendo o influxo de nutrientes e bloqueando a liberação de outras formas de energia estocadas (BIRNBAUM, 1993).

Assim, a insulina estimula a síntese de proteínas e a captação de glicose em músculos estriado e cardíaco, ativa a lipogênese, bloqueia a lipólise no tecido adiposo (MYERS & WHITE, 1996), e inibe a neoglicogênese no tecido hepático (FELIG & BERGMAN, 1990). Estudos diversos têm demonstrado que a insulina intervem ativamente nos processos de transcrição gênica para a codificação de diversas proteínas (O`BRIEN & GRANNER, 1991), e que através do domínio C-terminal do receptor específico, cumpre um importante papel na regulação das sinais mitogênicas destinadas à estimulação da síntese de DNA e da atividade das MAP-quinases (ANDO et al., 1992; DICKENS et al., 1992; PANG et al., 1994).

A insulina inicia seus efeitos metabólicos e promotores de crescimento através da ligação à subunidade α do seu receptor. O receptor de insulina (IR) é uma glicoproteína presente na membrana celular, que pertence à família das tirosina-quinases (RTK) (FREYCHET et al., 1971; CUATRECASAS, 1972; KAHN, 1985). Este receptor comporta-se, funcionalmente, como uma enzima alostérica com uma subunidade α regulatória e uma subunidade β catalítica (KASUGA et al., 1982 a e b). 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; PERLMAN et al., 1989; WHITE et al., 1988), determinando a transferência de grupos fosfato do ATP para aminoácidos tirosina do receptor. Uma vez ativado, o receptor fosforila substratos intracelulares específicos, cumprindo assim o primeiro passo de uma longa cadeia de interações destinadas a produzir os efeitos biológicos da insulina.(VAN OBBERGHEN et al., 1983).

A identificação e clonagem de várias proteínas que interagem com receptores que apresentam atividade tirosina-quinase (RTKs), têm contribuído na compressão dos mecanismos moleculares de transmissão de sinais mediados por este tipo de receptores (ULLRICH & SCHLESSINGER, 1990). A estimulação dos RTKs leva à autofosforilação de regiões específicas na subunidade catalítica, e promove a fosforilação de substratos protéicos intracelulares (CANTLEY et al., 1991; KOCH et al., 1991).

Diferente da maior parte de RTKs, que uma vez fosforilados ligam-se diretamente às proteínas com domínio SH2 para propagar o sinal, o receptor de insulina, uma vez autofosforilado, estimula sua atividade tirosina-quinase e fosforila proteínas intermediárias ou substratos do receptor responsáveis pela associação com proteínas possuidoras do domínio SH2 (CHEATAM & KAHN, 1995).

A porção SH2 (Src homology 2, "counterpart of viral src"), parte importante da estrutura de algumas proteínas citoplasmáticas, é um domínio altamente conservado, constituído por aproximadamente 100 aminoácidos, que se caracteriza por sua capacidade de interagir, através de ligações de alta afinidade, com as sequências tirosina-fosforiladas de receptores e outras fosfoproteínas (ANDERSON et al., 1990; KOCH et al., 1991; McGLADE et al., 1992; REEDIJK et al., 1992). A associação de proteínas com domínio SH2 ao

receptor ativado, é estritamente dependente da fosforilação do receptor (KOCH et al., 1991; MARGOLIS, 1992). Proteínas que possuem este domínio são: subunidade p85 do fosfatidilinositol PI 3-kinase (MYERS et al., 1992; MYERS & WHITE, 1993), a Grb2 (SKOLNIK et al., 1993), ou a proteína SH-P2 (Syp) (FREEMAN et al., 1992).

A primeira evidência da existência de um substrato direto do receptor de insulina, foi obtida em células FAO (hepatoma de rato), onde utilizando anticorpos antifosfotirosina (αPY), foi possível detectar uma banda que migrava a 185 kDa (SUN et al., 1991) a qual, sendo a primeira proteína efetora do IR identificada, foi denominada substrato-1 do receptor de insulina (IRS-1). Novas proteínas efetoras, substratos diretos do receptor de insulina, têm sido identificadas posteriormente em diferentes células e tecidos, e denominadas IRS-2 (MIRALPEIX et al., 1992), IRS-3 (LAVAN & LIENHARD, 1993) e IRS-4 (PERROTI et al., 1987), devido a suas diferentes características estruturais e funcionais.

Os substratos do receptor de insulina (IRSs), são proteínas citoplasmáticas estruturalmente divididas em duas regiões, as quais determinam sua especificidade. Assim, a região amino-terminal, altamente conservada, é a responsável pela ligação dos IRSs com o receptor de insulina ativado, através dos domínios PH e/ou PTB (pleckstrin-homology e phosphotyrosine-binding, respectivamente) localizados nesta região (GUSTAFSON et al., 1995). A porção COOH terminal, pouco conservada, se caracteriza por apresentar sequências de aminoácidos susceptíveis de serem tirosina-fosforilados pelo receptor de insulina ativado, proporcionando dessa forma pontos de ligação com proteínas efetoras possuidoras de domínios SH2 (SUN et al., 1995).

É importante ressaltar o fato que, em células intactas, tanto o IR como o IRS-1 são capazes de ser fosforilados em aminoácidos serina e treonina (KASUGA et al., 1982a-b; SMITH et al., 1993). Isto pode ser estimulado por tratamento prolongado com insulina, ésteres de forbol e análogos do AMP cíclico, e presumivelmente é resultado da fosforilação do receptor pela proteína quinase C ou proteína quinase A (TAKAYAMA et al., 1984). Em contraste à fosforilação em tirosina, a qual ativa a quinase, a fosforilação em serina inativa a capacidade tirosina-quinase do receptor e seu substrato IRS-1 (TAKAYAMA et al., 1988; SMITH et al., 1993). Esta regulação da atividade quinase por fosforilações em aminoácidos distintos, apresenta-se como um importante mecanismo regulador do sinal insulínico em situações fisiológicas e patológicas (MYERS & WHITE, 1996).

Estudos realizados em cultura de células têm demonstrado que a insulina é capaz de induzir a fosforilação de uma nova proteína efetora denominada Shc (PRONK et al., 1993; OKADA et al., 1995). Similar ao IRS-1, a Shc é uma proteína citoplasmática capaz de ser tirosino-fosforilada por uma variedade de fatores de crescimento e citoquinas (OBERMEIER et al., 1993; SEGATTO et al., 1993). Descrita pela primeira vez em 1992 por PELICCI e colaboradores, a proteína Shc (*src* homology 2/α collagen-related), cuja estrutura lembra àquela do colágeno, é possuidora do domínio SH2 na sua molécula, características das quais deriva o seu nome (PELICCI et al., 1992). A família das proteínas Shc consiste em 3 proteínas relacionadas: as espécies de 46 e 52 kDa resultam da utilização de diferentes pontos de iniciação no mesmo transcriptor; entretanto as espécies de 66 kDa parecem ser o resultado de "splicing" alternativo (PELICCI et al., 1992; PRONK et al., 1993; SASAOKA et al., 1994a). Estudos revelaram que a insulina é capaz de fosforilar preferencialmente a $p52^{Shc}$ (OKADA et al., 1995) (Fig. 1).

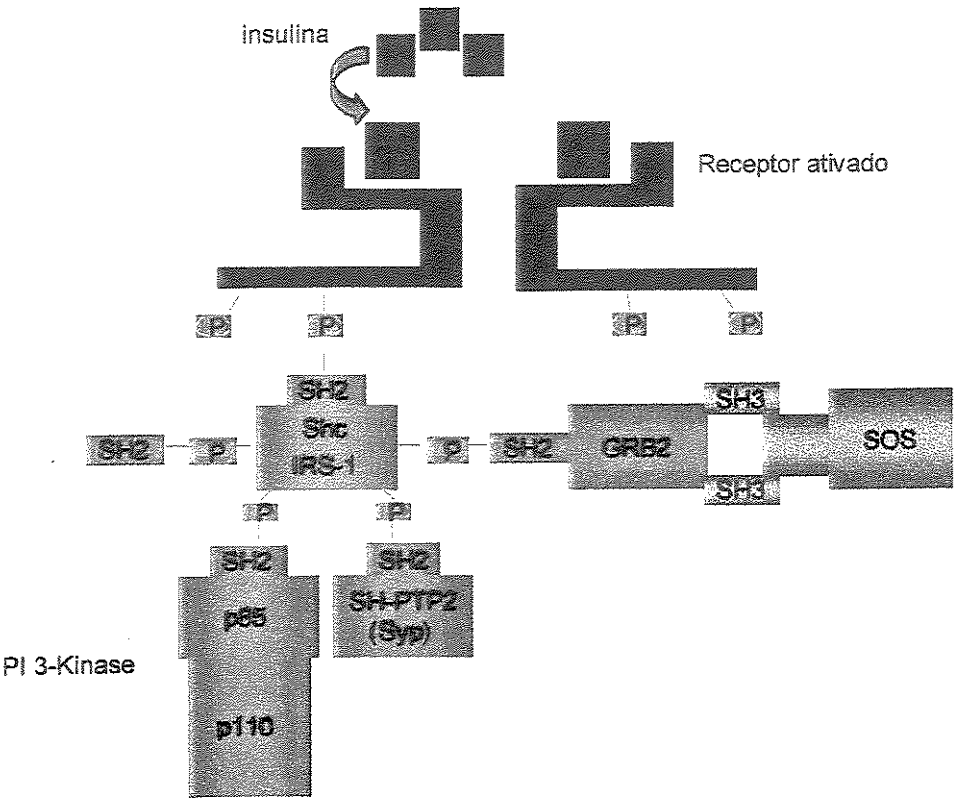

Figura 1: **Vias de sinalização insulínica:** a insulina associa-se ao IR, fosforilando-o. Este por sua vez ativa seus substratos IRS-1 e Shc, favoreciendo sua ligação com proteínas possuidoras de domínios SH2, mediadoras das respostas metabólicas e/ou mitogênicas ao estímulo insulínico.

Com o objetivo de determinar a importância da Shc na mediação das respostas intracelulares iniciadas pela insulina, diversos estudos *in vivo* em cultura de células e em sistemas reconstituídos *in vitro*, têm sido realizados. Embora alguns estudos coincidem em afirmar que as proteínas Shc não co-imunoprecipitam com IR ou IGF-IR ativados (PRONK et al.; 1993; GIORGETTI et al., 1994), pesquisas realizadas utilizando receptores de insulina, IGF-IR e receptores de citoquinas, apontam o IRS-1 e a Shc como substratos diretos destes

vitro usando o sistema duplo-híbrido em levaduras (GUSTAFSON et al., 1995) foram realizados, demonstrando que, pelo menos neste modelo experimental, a p52 Shc interage especificamente com o IR e o IGF-IR, através da ligação dos aminoácidos da porção amino-terminal 46 a 209, com os seus correspondentes localizados no domínio NP*X*Y da região juxtamembrana de ambos os receptores (ISAKOFF et al., 1996).

A tirosil-fosforilação da Shc por insulina, liga esta proteína a uma única molécula efetora, chamada Grb2 (LOWENSTEIN et al.,1992; McGLADE et al.,1992; ROZAKIS-ADCOCK et al., 1992). A Grb2 (growth factor receptor-bound protein-2), é uma pequena proteína citoplasmática de 25 kDa, constituída por dois domínios *Src*-homology (SH)3 e um SH2 (LOWENSTEIN et al., 1992). Esta proteína desempenha um papel fundamental nos processos de crescimento e diferenciação celular, agindo como uma molécula adaptadora que, através do seu domínio SH2, liga receptores tirosino-fosforilados e outras fosfoproteínas como a Shc e o IRS-1, ao fator intercambiador de nucleotídeos da guanina do Ras, mSos (Son of Sevenless) (YOZENAWA et al., 1994; PRUETT et al., 1995). Esta capacidade da Grb2 de ligar-se ao mSos é resultado da presença dos domínios SH3 na sua molécula (LOWENSTEIN et al., 1992).

Estudos orientados a identificar a especificidade das proteínas efetoras ativadas pelas vias mitogênicas e/ou metabólicas, durante os processos de transmissão do sinal em células sensíveis à insulina (SASAOKA et al., 1994b; LAZAR et al., 1995), sugerem que a interação Shc/Grb2/Sos é fundamental no controle da proliferação e/ou transformação celular, assim como na ativação de certos mecanismos de crescimento celular (SASAOKA et al., 1994a-b; OWENS et al., 1994).

Estudos realizados em fibroblastos (GIORGETTI et al., 1994), demonstraram que a expressão de proteínas Shc exógenas pode induzir a estimulação constitutiva de uma via mitogênica, ou podem remover o mecanismo inibitório de iniciação do ciclo celular, resultando em uma resposta mitogênica aumentada. A microinjeção de anticorpos dirigidos contra as proteínas Shc em fibroblastos de rato (rat-1 fibroblasts) super-expressando receptores humanos de insulina, inibe a síntese de DNA induzida por insulina, indicando que a Shc é um componente importante das vias mitogênicas ativadas por este hormônio (SASAOKA et al., 1994b) (Fig. 2).

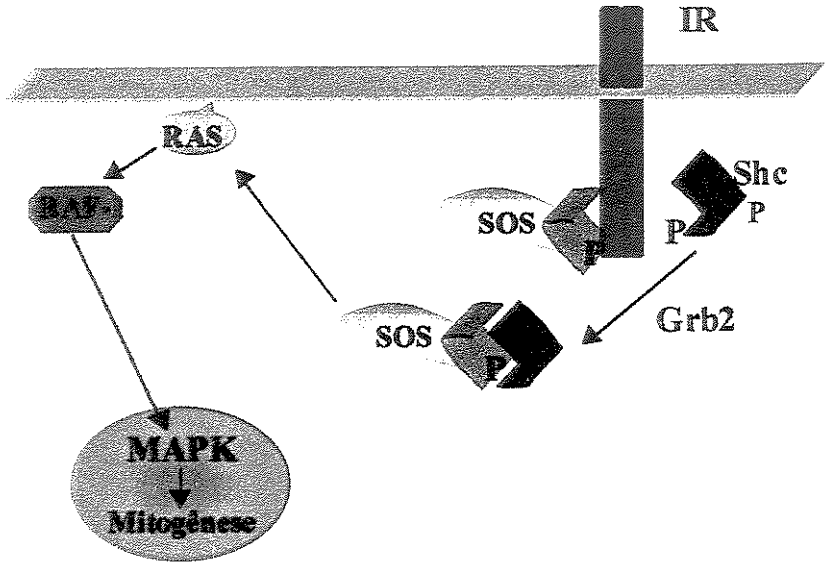

Fig. 2: **Sinalização insulínica através da proteína Shc.**

Estudos prévios sugerem que o complexo Shc/Grb2/Sos ativa a p21ras diferentemente da associação IRS-1/Grb2, e que a homeostase no mecanismo de

mitogênese produzido pela insulina depende de um balanço entre a associação IRS-1/Grb2/Sos e Shc/Grb2/Sos. Parece haver uma especificidade funcional da Shc em relação ao IRS-1, de tal modo que a função desta proteína na ativação das vias mitogênicas, quando associado ao complexo Grb2-Sos, parece ser importante, mas não fundamental, se comparada àquela da Shc (YAMAUCHI & PESSIN, 1995; MYERS et al., 1994; WATERS et al., 1993; ROSE et al., 1994).

Estes dados sugerem que a regulação do sinal insulínico depende, pelo menos em parte, da eficiência na associação do IRS-1 e a Shc com a subunidade β do receptor de insulina. Neste contexto, é importante ressaltar que tanto o IRS-1 como a Shc possuem na sua estrutura molecular o domínio PTB/PI, específico para sua interação com o receptor de insulina, cujo domínio NP*X*-Tyr(P) é reconhecido por ambas as moléculas (YONEZAWA et al.; 1994; O`NEIL et al., 1994; GUSTAFSON et al., 1995).

Esta sequência de aminoácidos está presente na região juxtamembrana do receptor de insulina, e suas interações são mediadas pela Tyr^{972} localizada neste domínio (HERRERA et al., 1985; HERRERA & ROSEN, 1986).

É importante assinalar que no receptor de insulina, nem todas as regiões fosforiladas NP*X*Y interagem com ambas as moléculas (PRONK et al.; 1993; HE et al., 1995). Estas características levariam o IRS-1 e Shc a competir pela Tyr^{972} na sua associação com o receptor fosforilado (WOLF et al., 1995; WARD et al., 1996), sugerindo que as diferenças estruturais que estas proteínas apresentam entre elas, determinariam a especificidade funcional da Shc quando comparada ao IRS-1 (CRAPARO et al., 1995) (Fig. 3).

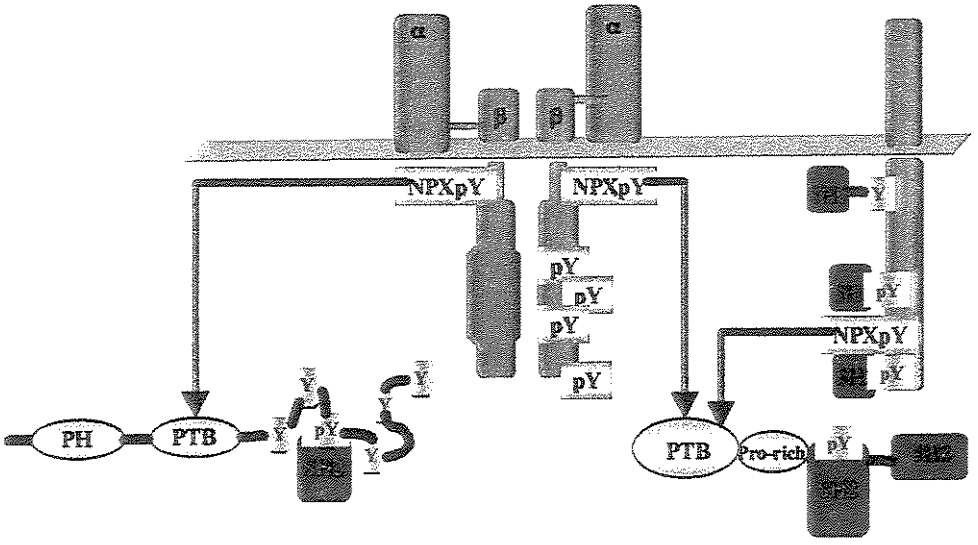

Figura 3: **Associação do IR e do EGFR** com os substratos IRS-1 e Shc, com a proteína Shc: domínios NP*X*-Tyr(P), PH, PI/PTB e SH2.

Assim sendo, a ação insulínica envolve uma cascata de interações covalentes e não covalentes centralizadas, num primeiro nível, no IRS-1 e na Shc. A ligação da insulina ao seu receptor na superfície celular, determina a fosforilação da Shc e do IRS-1. Estes então atuam como proteínas ancoradouras para várias enzimas intracelulares e adaptadores moleculares. Tais ligações determinam múltiplos sinais na cascata da ação insulínica. Como resultado, a Shc se constitui em elemento importante das vias intracelulares de ação insulínica, cujas ligações determinam a multiplicidade dos efeitos biológicos finais deste hormônio.

Entretanto, não foi ainda demonstrada em tecidos animais a fosforilação da proteína Shc induzida pela insulina, nem a interação Shc/Grb2. Nesse sentido, como primeiro objetivo deste estudo, investigamos a capacidade da insulina de estimular a fosforilação da Shc em tecidos de ratos normais. A análise das

características funcionais da interação IR/Shc, foram parte deste objetivo, assim como o foi o estudo da associação Shc/Grb2, e sua relação com o grau de fosforilação da proteína Shc induzido pela insulina.

A compreensão dos mecanismos moleculares de ação da insulina adquire relevância devido à prevalência de doenças que cursam com resistência à insulina, como obesidade, diabetes mellitus, hipertensão e intolerância á glicose associadas a doenças endócrinas. Estudos prévios (SAAD et al., 1992) demonstraram que em animais com resistência à insulina o receptor de insulina, seu substrato IRS-1 e a associação IRS-1/PI 3-kinase, apresentaram uma regulação tecido-específica, capaz de influenciar na etiologia da resistência a este hormônio. Por outro lado, a regulação da fosforilação da proteína Shc e sua interação com a Grb2 após estímulo com insulina, não foram ainda investigados em modelos animais de resistência à insulina. Isto adquire importância porque em cultura de células a proteína Shc parece estar regulada por diferentes fatores que podem estar presentes em modelos animais de resistência à insulina. Assim, citoquinas como o TNFα, e a hiperglicemia também têm se mostrado capazes de regular a fosforilação da Shc em cultura de células (KRODER et al., 1996), sugerindo um possível envolvimento da Shc nos mecanismos moleculares de resistência à insulina.

Estes antecedentes nos levaram ao segundo objetivo, que foi investigar a influência de cinco estados de resistência à insulina no grau de fosforilação da Shc e sua associação com a proteína adaptadora Grb2: jejum prolongado, tratamento agudo com adrenalina, tratamento crônico com dexametasona, diabetes mellitus induzido por estreptozotocina e envelhecimento, em três tecidos insulino-sensitivo; fígado, músculo e gordura.

Contrastando com extensivos estudos realizados em fígado, músculo e tecido adiposo (WHITE, 1997), sabe-se relativamente pouco dos efeitos da insulina nas células beta pancreáticas. A detecção do mRNA do IR e do IRS-1 em células βTC3 produtoras de insulina (HARBECK et al, 1996), deixa aberta a possibilidade da presença de um mecanismo de contra-regulação operando através do IR em células β pancreáticas. Os substratos do receptor de insulina IRS-1 e IRS-2 também têm sido implicados na sinalização através do receptor do IGF-1, presente em linhagens de células produtoras de insulina (VAN SCHRAVENDIJK et al., 1987). Este achado é consistente com a capacidade das células β de responder ao estímulo com insulina ou IGF-1 por meio de vias de sinalização intracelular similares aquelas observadas em tecidos periféricos (VAN SCHRAVENDIJK et al., 1990; LEIBIGER et al., 1988; XU & ROTHEMBERG, 1998; VELLOSO et al., 1995).

Glicose e insulina são capazes de induzir fosforilação do receptor de insulina assim como do IRS-1 e IRS-2 em ilhotas pancreáticas (VELLOSO et al., 1995), restando a pergunta se as proteínas envolvidas no sinal insulínico estão presentes em células β purificadas. Esta pergunta nos levou ao terceiro objetivo do presente trabalho, que foi estudar as primeiras etapas nas vias de sinalização insulínica em células β purificadas de rato.

Estudamos a expressão do substrato-2 do receptor de insulina (IRS-2), um dos primeiros efetores do IR ativado, em células β e não-β pancreáticas purificadas. O IRS-2 tem sido recentemente proposto como uma proteína reguladora importante para o desenvolvimento e sobrevivência das células β (WITHERS et al., 1998). A presença de uma proteína migrando ao mesmo nível da $pp60^{IRS-3}$, constantemente associada ao IRS-2, nos levou a realizar RT-PCR (reverse transcribed-polymerase chain reaction) com o objetivo de revelar a

identidade desta banda, para obter assim mais um elemento que ajude a elucidar a função destas proteínas em células β pancreáticas.

2. DISCUSSÃO

2.1 AVALIAÇÃO CRÍTICA DA METODOLOGIA UTILIZADA

Os métodos utilizados, estimulação *in vivo* com insulina, extração e homogeneização de tecidos hepático, muscular e adiposo em condições desnaturantes ou em condições apropriadas para imunoprecipitação e posterior imunoblotting com anticorpos específicos anti-Shc, anti-Grb2 e anti-fosfotirosina, permitiram uma avaliação das etapas iniciais da ação insulínica após sua ligação ao receptor, numa nova via, em três tecidos com importante papel na utilização de glicose (SAAD et al., 1989). Este método não está sujeito a problemas como artefatos bioquímicos resultantes da homogeneização do tecido e purificação do receptor, protéolise e desfosforilação do receptor fosforilado por fosfatases contaminantes (KATHURIA et al., 1986), bem como da remoção do receptor da membrana plasmática, onde interações com outros componentes celulares podem influenciar sua atividade (FEHLMAN et al., 1985). O método utilizado permite uma determinação direta do grau de fosforilação da proteína Shc e sua associação com o Grb2, assim como da relação destes dois parâmetros com o grau de fosforilação do receptor de insulina, o que implica em uma avaliação da atividade quinase *in vivo* do receptor de insulina, dirigida a um substrato endógeno, a proteína Shc.

Utilizando esta metodologia, estudamos as etapas iniciais da ação insulínica em tecidos muscular, hepático e adiposo dos seguintes modelos animais de resistência à insulina i.e.: jejum prolongado, ratos tratados crônicamente com dexametasona, ratos velhos, ratos tratados agudamente com adrenalina e diabetes induzido por estreptozotocina. Estudamos ainda os efeitos nas etapas iniciais de ação insulínica em células beta pancreáticas de ratos normais e os efeitos da estimulação aguda com insulina e glicose nestas etapas.

2.2 INDUÇÃO DA FOSFORILAÇÃO DA(S) PROTEÍNA(S) SHC POR INSULINA

Publicações 1 e 2

A atividade de tirosina-quinase dos receptores dos hormônios de crescimento, é um evento fundamental na propagação da sinalização dependente da informação dada por estes hormônios (PAWSON & GISH, 1992; SCHLESSINGER & ULLRICH, 1992). Embora muitas combinações de moléculas efetoras têm sido identificadas para numerosos receptores dos fatores de crescimento, todos parecem possuir em comum a fosforilação em tirosina e/ou associação com um novo tipo de proteínas chamadas Shc. (FANTL et al., 1992; MAYER & BALTIMORE, 1993; VAN DER GEER & HUNTER, 1993). Estudos realizados em cultura de células têm demonstrado que a insulina também é capaz de induzir a fosforilação da proteína Shc, mas o significado fisiológico deste processo ainda não foi elucidado (PELICCI et al., 1992; PRONK et al., 1993; OKADA et al., 1995).

Nossos experimentos iniciais tiveram como objetivo determinar a presença da proteína Shc no fígado, músculo e tecido adiposo de ratos, e determinar se esta proteína é capaz de ser fosforilada em tirosina após infusão de insulina. Com o objetivo de investigar uma etapa adicional das vias mitogênicas ativadas pela proteína Shc, estudamos também as modificações da associação que ocorre entre a Shc e a proteína adaptadora Grb2 após tratamento com insulina nestes três tecidos.

Concordante com resultados obtidos em experimentos realizados *in vitro* (PRONK et al., 1993; OKADA et al., 1995) e *in vivo* em cultura de células (GUSTAFSON et al., 1995), mostrando que a isoforma p52 Shc é fosforilada com maior eficiência pela insulina que àquela de 46 kDa, nossos resultados em

músculo e tecido adiposo mostraram que, após tratamento com insulina, a p52 Shc foi a única isoforma fosforilada, e em fígado a isoforma predominantemente fosforilada foi a p52 Shc, com uma mínima fosforilação da espécie $p46^{Shc}$. Este predomínio na fosforilação da p52 Shc em fígado, músculo e gordura de ratos pode ser consequência da presença de altos níveis desta isoforma em tecidos de ratos em relação às outras, embora certas interações com o receptor de insulina podem desempenhar um papel importante neste padrão de fosforilação.

Para determinar se a presença da Shc e sua fosforilação após aplicação de insulina eram funcionalmente importantes, foram realizados experimentos de tempo- e dose-resposta. Foi observada uma estimulação da fosforilação da Shc em fígado, músculo e gordura, capaz de ser modificada em função do tempo após a infusão de insulina, assim como da dose aplicada. Valores máximos de fosforilação da Shc foram obtidos 5 minutos após infusão de insulina nos três tecidos analisados. A dose necessária para se obter a metade dos valores máximos de fosforilação foram obtidos com doses insulínicas de 6 ng em fígado e músculo, e 6 pg em tecido adiposo, valores correspondentes a níveis plasmáticos de insulina de 30 a 120 μU/ml 1 min após infusão. Estas concentrações são similares àquelas obtidas em ratos durante o teste oral de tolerância à glicose. O grau de fosforilação da Shc em músculo e adipócitos foi menos intenso que no fígado, provavelmente devido à menor concentração periférica de insulina.

Tanto a Shc como o IRS-1 são fosforilados com concentrações similares de insulina, mas no experimento tempo-resposta, o máximo nível de fosforilação em tirosina da Shc foi atingido depois que o IRS-1 em fígado, músculo e gordura de ratos, com uma menor intensidade que aquela obtida no IRS-1 (SAAD et al., 1992; CARVALHO et al., 1996). A consequência fisiológica desta diferença não é conhecida, mais parece estar correlacionada com o mecanismo de competição

destas duas proteínas pelo domínio NPX(Y)P do receptor de insulina (ISAKOFF al., 1996). Parece que o receptor de insulina é capaz de utilizar diferentes substratos em tempos diferentes, com a finalidade de induzir uma atividade de tirosina-quinase mais eficiente para cada substrato (Fig.4).

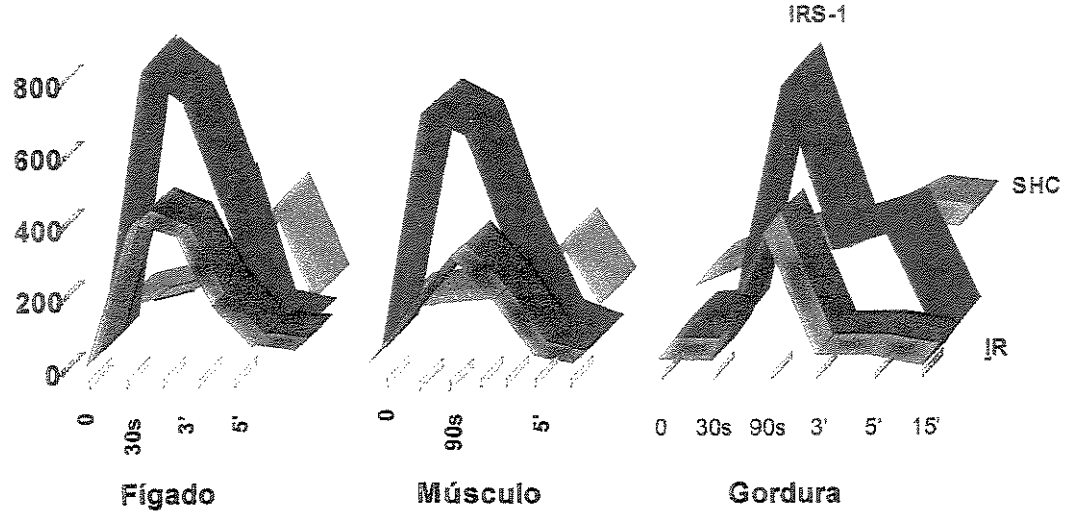

Fig. 4: **Relação temporal** da fosforilação induzida por insulina no IR, IRS-1 e Shc em fígado, músculo e gordura de ratos normais.

É interessante destacar que o IRS-1, que é o primeiro substrato do IR a ser fosforilado, é um importante mediador dos efeitos metabólicos da insulina, enquanto a Shc, que é fosforilada mais tarde, é essencial nas vias mediadoras dos efeitos promotores de crescimento (mitogênese) (SKOLNIK et al., 1993)

Em cultura de células, a fosforilação da Shc é seguida da sua associação a Grb2, uma pequena molécula adaptadora que liga a Shc com outras proteínas encarregadas da transmissão do sinal mitogênico (LOWENSTEIN et al., 1992, WATERS et al., 1996). Nossos estudos demostraram que após estímulo

insulínico a Shc se associa a Grb2 em fígado, músculo e gordura de ratos. Já que a interação Shc-Grb2 leva à ativação das vias mitogênicas da RAS (SKOLNIK et al., 1993), a capacidade da insulina de induzir esta associação sugere um papel importante na ativação e controle das funções mitogênicas da insulina através desta via.

A fosforilação da Shc induzida por insulina foi similar àquela reportada com os hormônios EGF e PDGF (SEGATO et al., 1993; OKADA et al., 1995; OBERMEIER et al., 1994). Embora estudos prévios em cultura de células não encontrassem associação entre as proteínas Shc e o receptor de insulina ativado, experimentos iniciais mostraram que estas duas proteínas co-imunoprecipitam após infusão de insulina. Estudos recentes coincidem em afirmar que a proteína Shc compartilha com o IRS-1 um mesmo grupo de aminoácidos localizados na região amino-terminal de ambas as proteínas, denominado "NP*X*-Y(P)-binding PTB/PI (SAIN) domain" (SHOELSON et al., 1992). Este domínio possui uma sequência de aminoácidos similar aquela do receptor de insulina (WARD et al., 1996; PRONK et al., 1993; HE et al., 1995) sendo capaz de reconhecer e ligar-se à Tyr 972 localizada no segmento NP*X*-Tyr(P) do receptor ativado (SUN et al., 1995; BORK & MARGOLIS, 1995; BATZER et al., 1994, VAN DER GEER et al., 1995; VAN DER GEER & PAWSON, 1995).

Com o objetivo de determinar se no animal intacto o receptor de insulina possui atividade de tirosina-quinase dirigido à proteína Shc, amostras de fígado de ratos com 4 horas de jejum sujeitos a tratamento com 6 pg de insulina ou solução salina (controle) por 30 segundos, foram homogeneizados e imunoprecipitados com anticorpo anti-IR. A atividade de tirosina-quinase destes imunoprecipitados foi determinada mediante incubação dos complexos immunes (anti-receptor de insulina) numa solução tampão contendo ATP. Os complexos foram lavados, suspendidos em solução de Laemmli e analizados por SDS-PAGE

(LAEMMLI, 1970). A incorporação de fosfato ao receptor de insulina e todos os substratos diretamente ligados a ele, foi visualizada por autoradiografia das membranas submetidas a imunobloting com anticorpo anti-fosfotirosina.

As duas primeiras amostras (controles) mostraram uma banda de ~50 kDa (p50) levemente fosforilada tanto na primeira (sem insulina nem ATP), como na segunda (sem insulina com ATP) condições. Não houve mudança na fosforilação desta banda em nenhuma destas situações. A terceira amostra, tratada com insulina mas sem adição de ATP, apresentou uma banda de ~95 kDa, correspondente ao receptor de insulina, além da banda de ~50 kDa já descrita, esta última com níveis de fosforilação similares àqueles dos controles. A autofosforilação do IR obtida pela adição de ATP exógeno foi observada na última amostra (com insulina e ATP), acompanhado de importante incremento na fosforilação da p50, assim como a presença de três novas bandas parcialmente fosforiladas a uma altura aproximada de 125 kDa, 160 kDa e 180 kDa,. Após imunobloting com os anticorpos específicos, a identidade destas bandas foi confirmada como $p52^{Shc}$, IRS-1 (160 kDa) e IRS-2 (180 kDa). Não foi identificada a proteína correspondente à banda de 125kDa (Fig. 5)

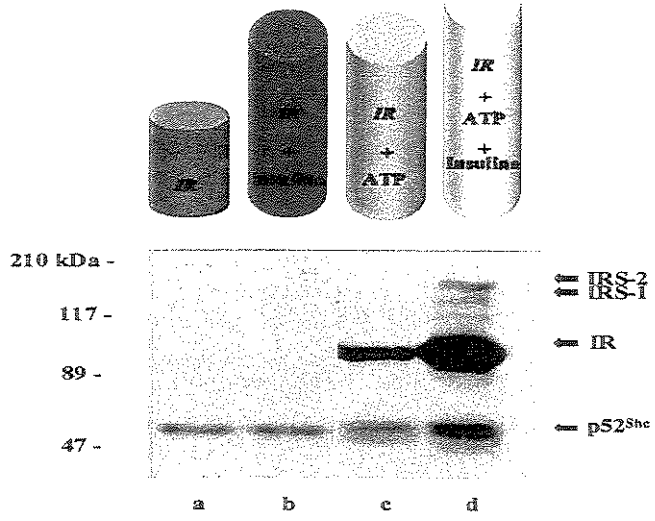

Fig. 5: **Atividade de Tirosino-Quinase do IR em fígado de ratos.** 30 sec após estímulo insulínico, o fígado é extraído e a atividade de tirosino-quinase do receptor de insulina *in vitro* dirigida à proteína Shc determinada e visualizada por autoradiografia.

Num segundo experimento, idênticas condições experimentais àquelas descritas foram aplicadas, mas o tempo de exposição à insulina foi incrementado em 3 minutos. Embora foi observada a presença das mesmas bandas que aquelas observadas com 30 segundos de exposição à insulina, estes resultados mostraram uma significativa diminuição no grau de fosforilação do IR, IRS-1 e IRS-2, associado à considerável aumento do nível de fosforilação da Shc.

É importante considerar elementos deste resultado tais como a importante fosforilação basal da Shc nos controles, a qual aumentou unicamente após autofosforilação do IR por adição de insulina e ATP. Também é interessante ressaltar a diminuição no grau de fosforilação da banda correspondente ao IR fosforilado, assim como das duas bandas de 160 e 180 kDa (IRS-1 e IRS-2, respectivamente), observado três minutos após a infusão de insulina, quando comparadas àquelas apresentadas com 30 segundos de tratamento. Em efeito, o máximo grau de fosforilação do IR, IRS-1 e IRS-2 em fígado de ratos ocorre apenas 30s após infusão de insulina via intraportal (SAAD et al., 1992). Entretanto, a proteína Shc, cujo máximo nível de fosforilação no fígado está entre 3 e 5 minutos após injeção de insulina apresentou, 3 minutos após tratamento com insulina, uma intensa fosforilação.

Esta resposta nos permitiu observar não só a relação entre tempo- e dose-respostas da Shc em relação ao receptor de insulina, mas também em relação com os substratos 1 e 2 do receptor de insulina. O aumento no nível de fosforilação da Shc como resposta à ativação do IR após infusão de insulina, demonstra que a proteína Shc interage com o receptor de insulina após a estimulação com insulina

Shc como resposta à ativação do IR após infusão de insulina, demonstra que a proteína Shc interage com o receptor de insulina após a estimulação com insulina em fígado de ratos normais. Demonstra também que existe uma actividade de tirosina quinase do receptor de insulina hacia a proteína Shc imunopurificada, e nos leva à conclusão que a Shc, como os IRSs, é substratos diretos do receptor de insulina *in vitro*, e que a diferença entre o tempo de aplicação do estímulo e fosforilação da proteína específica, representa a especificidade da interação receptor de insulina com seus substratos.

Com estes dois trabalhos demonstramos que, em fígado de ratos tratados com insulina, o receptor de insulina estimula a proteína Shc immunopurificada. Esta interação determina a fosforilação em tirosina da proteína Shc de maneira tempo e dose dependente, assim como a associação da Shc à Grb2. Esta especificidade na interação IR-Shc abre a possibilidade de se estudar a regulação da insulina na Shc em animais sujeitos a diversas situações de resistência à insulina.

2.3 REGULAÇÃO DA PROTEÍNA SHC EM MODELOS ANIMAIS DE RESISTÊNCIA À INSULINA

Resistência à insulina é definida como uma resposta biológica subnormal a uma dada concentração de insulina. Embora os efeitos da insulina sejam pleiotrópicos, a resistência á insulina tipicamente faz referência à ação da insulina na homeostase de glicose. Os mecanismos moleculares responsáveis pela resistência não são bem conhecidos e podem envolver diferentes vias. Em modelos animais de resistência à insulina existe evidência que a regulação do IRS-1 pode ter um papel importante na modulação da homeostase de glicose (SAAD, *et al.*, 1992). Além disto, citoquinas como a TNFα e a hiperglicemia são capazes de regular a fosforilação da Shc em cultura de células (KRODER et al., 1996), sugerindo que a proteína Shc pode estar envolvida nos mechanismos moleculares de resistência à insulina. Estes antecedentes unidos a capacidade da insulina de estimular a atividade da Shc, nos levaram a investigar a regulação da Shc e sua associação com a Grb2 em tecidos sensitivos à insulina, em cinco modelos experimentais de resistência à insulina: jejum prolongado, hipercortisolismo, envelhecimento, tratamento agudo com adrenalina e diabetes mellitus induzida por estreptozotocina.

Sendo que o elemento básico a ser considerado foi a obtenção de animales comprovadamente resistentes a insulina, o primeiro e fundamental procedimento realizado tanto nos animais controle quanto em àqueles sujeitos aos diferentes tratamentos orientados a induzir resistência à insulina, foi a extracção de amostras sanguíneas, para posterior determinação dos valores de glicose e insulina plasmáticas destes animales.

A tabela 1 expressa os valores de peso corporal, glicose e insulina plasmática dos animais tratados, assim como os níveis de significação destes valores em relação com os respetivos controles.

Tabela 1. Valores de Peso Corporal, Glicose e Insulina Plasmática nos Animais Estudados

Grupos	*Peso Corporal (g)*	*Glicose Plasmática (mg%)*	*Insulina Plasmática (mU/ml)*
Alimentado	*211±6*	*165±8*	*38±4*
Jejum	*179±5*****	*74±2****	*13±4***
Controle	*238±5*	*122±6*	*29±4*
Dexa	*202±4***	*224±2****	*46±4***
2 meses	*157±1*	*123±5*	*29±4*
20 meses	*401±2***	*124±1*	*44±5**
Controle	*148±3*	*115±3*	*29±2*
Adrenalina	*144±3*	*117±3*	*41±4*
STZ	*102±3**	*452±2***	*6±6**

* $p \leq 0.05$
** $p \leq 0.01$
*** $p \leq 0.001$

2.3.1 EFEITOS DO JEJUM PROLONGADO, DO HIPERCORTISOLISMO E DO ENVELHECIMENTO

Publicação 3

Jejum prolongado, hipercortisolismo e envelhecimento em ratos são caraterizados por resistência à insulina (ALMIRA & REDDY, 1979; OKAMOTO et al., 1986; FRIEDENBERG et al., 1985; PENICAUD et al., 1985). No caso de jejum prolongado descreve-se redução da ação insulínica tanto em tecidos periféricos quanto em tecido hepático (ALMIRA & REDDY, 1979; OKAMOTO et al., 1986). Resultados de nosso laboratório têm demonstrado um discreto aumento (20% e 40%) na autofosforilação do receptor de insulina em tecidos hepático e muscular de rato respectivamente (SAAD et al., 1992), associado com um incremento no grau de fosforilação do IRS-1 de aproximadamente 100% em ambos os tecidos após jejum prolongado (SAAD et al., 1992). Os níveis protéicos de IRS-1 também apresentaram uma regulação similar nos dois modelos animais. Houve uma redução na concentração de IRS-1 em tecido muscular de ratos em jejum de 72 horas, e um aumento em tecido hepático. Estes dados evidenciaram a presença de uma regulação tecido específica para esta proteína (SAAD et al., 1992).

Concordando com estudos prévios segundo os quais existe uma relação competitiva de associação com o IR entre Shc e IRS-1 (ISAKOFF et al., 1996), a Shc apresentou, no caso dos animais sujeitos a 72 horas de jejum, um nível de fosforilação significativamente diminuído em fígado e tecido adiposo (~50% fígado, ~40% gordura) em relação aos controles, mas não teve alteração no músculo destes animais (Fig. 6). Em contraste com o IRS-1, as concentrações protéicas da Shc não foram modificadas no fígado, músculo ou gordura dos animais submetidos a jejum prolongado. Estes dados são a primeira evidência

que a fosforilação da Shc induzida pela insulina apresenta uma regulação tecido-específica. Embora esta regulação não possa explicar a resistência à insulina vista no jejum, pode representar um importante mecanismo regulador que dirige sua ação para uma via específica (a do IRS-1) que cumpre um papel definido na homeostase de glicose.

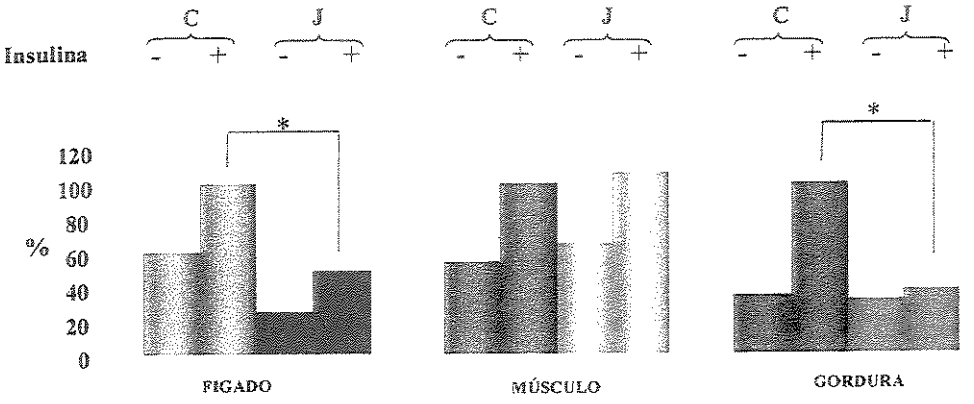

Fig. 6: **Características da fosforilação da proteína Shc pela insulina em animais sujeitos a jejum.**

Com o objetivo de investigar o efeito do jejum nas vias insulínicas ativadas pela proteína Shc, estudamos a associação que ocorre entre a Shc e a proteína adaptadora Grb2 nestes animais. Nossos resultados mostraram uma clara correlação entre o grau de fosforilação da Shc e a associação com a proteína adaptadora Grb2 em todos os tecidos estudados. A diminuição da fosforilação da Shc e sua associação com a Grb2 no fígado dos ratos em jejum, é consistente com a redução na síntese de DNA observada em estudos similares (RIDRAY et al., 1991). Isto fala a favor de especificidade entre as vias de transmissão ativadas pelo IRS-1 ou a Shc, após o estímulo com insulina.

O tratamento de ratos com dexametasona induz resistência à insulina, caracterizada por alterações no metabolismo de glicose em tecidos hepático e periférico, redução na captação de aminoácidos estimulada pela insulina em músculo, e uma diminuição da lipogênese em adipócitos (KAHN et al., 1978; CARO & AMATRUDA, 1982; AMATRUDA et al., 1985; KNUTSON, 1986; MOLLER & FLIER, 1991). A dexametasona também melhora a estimulação da síntese de DNA mediada por insulina (GERMINARIO & McQUILLAN, 1985). Sob condições de hipercotisolismo similares as estudadas no presente trabalho, foi observada uma diminuição na fosforilação do receptor de insulina em fígado e músculo dos animais tratados, assim como uma menor ativação da PI-3 kinase pelo IRS-1 após injeção de insulina (SAAD et al., 1993). Parece que esta ativação possui um papel na resistência à insulina observada em ratos tratados crônicamente com dexametasona, considerando a estreita correlação entre atividade de PI-3 kinase, transporte e síntese de glicogênio (SANCHES-MARGALET et al., 1994; HARUTA et al., 1995; WELSH et al., 1994; CHEATHAM et al., 1994), mas não explica os mecanismos moleculares de ação utilizados pela dexametasona para incrementar a indução insulínica de síntese de DNA nestes tecidos.

Após tratamento com dexametasona (1mg/kg/dia/5dias), os níveis de fosforilação da Shc em tecidos hepático e muscular apresentaram incrementos em 64% e 81% respectivamente, incremento mantido nos mesmos níveis em relação ao grau de associação Shc/Grb2 nesses tecidos. Estes aumentos de fosforilação não foram acompanhados por mudanças no nível protéico da Shc em fígado, músculo ou gordura. Neste último tecido também não houve diferença de fosforilação nos animais tratados em comparação aos seus controles (fig. 7).

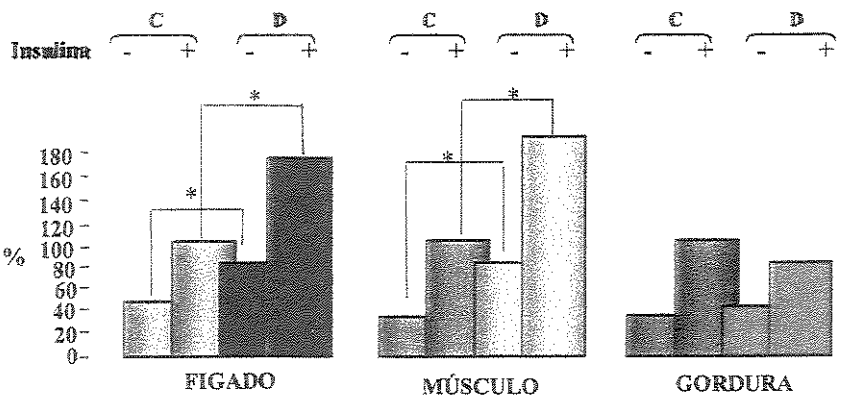

Fig. 7: **Características da fosforilação da proteína Shc pela insulina em animais tratados cronicamente com dexametasona.** *p≤0.05

O incremento nos níveis de tirosil-fosforilação da Shc e sua associação com o Grb2 após injeção de insulina em fígado e músculo de ratos tratados com dexametasona, e a evidência de efeitos opostos no IRS-1 em fígado e músculo de ratos submetidos a idêntico tratamento, demonstram que há uma preservação da via de transmissão específica destinada a otimizar a resposta mitogênica à insulina após sua sensibilização por dexametasona. A falta de resposta desta via em tecido adiposo de ratos submetidos a idêntico tratamento, sugere uma modulação tecido específica na regulação da tirosil-fosforilaçaõ do Shc induzida por insulina em ratos tratados com dexametasona.

Diversos estudos têm mostrado que após sobrecarga oral de glicose (teste de tolerância oral à glicose - TTOG) existe uma queda na tolerância à glicose em pessoas acima de 60 anos de idade (YOSHIMASA et al., 1988; DAVIDSON & KARJALA, 1978; DEFRONZO, 1979), associada a uma secreção normal o aumentada de insulina. Porém, em testes utilizando-se a via endovenosa para a sobrecarga de glicose (teste de tolerância endovenosa a glicose), pessoas idosas

apresentaram uma diminuição na primeira fase de secreção da insulina (FINK et al., 1983). A presença de níveis insulinêmicos normais ou aumentados com glicemias elevadas durante os testes de tolerância oral, sugeriu a existência de um sindrome de resistência à insulina associado ao envelhecimento (GOODMAN et al., 1983). A falta de diferenças na ligação da insulina (binding) a seu receptor isolado em monócitos circulantes e adipócitos (FINK et al., 1983; CARRASCOSA et al., 1989), sugeriu que o envelhecimento poderia estar associado a um defeito pós-receptor na ação insulínica manifestado pela diminuição da sensibilidade a insulina sem mudanças no "binding".

Estudos em receptores isolados de adipócitos (CARRASCOSA et al., 1989) e de músculo esquelético e fígado (KONO et al., 1990) de ratos de diferentes idades, mostraram diferenças na autofosforilação dos receptores dependentes da idade, do tecido e do substrato utilizado. Nos receptores isolados de adipócitos de animais com 24 meses de idade houve uma diminuição na autofosforilação dos receptores, após o estímulo insulínico. Nos receptores extraídos das membranas de fígado ou músculo não foram detectadas alterações na autofosforilação ou na fosforilação dos substratos exógenos utilizados (KONO et al., 1990; BARNARD et al., 1992). A desvantagem destes estudos foram terem sido feitos *in vitro* utilizando-se substratos exógenos para inferir sobre a atividade tirosina-quinase do receptor de insulina.

Nosso trabalho estudou os níveis protéicos da Shc, seu grau de fosforilação e associação da Shc com a Grb2 em fígado, músculo e gordura de ratos *in vivo*, após o estímulo insulínico em animais de 20 meses de idade, tendo como controle ratos Wistar de 2 meses.

Em estudos recentes em ratos sujeitos a idênticas condições experimentais, observou-se uma diminuição na tirosil-fosforilação do IRS-1 e IRS-2 assim como na sua associação com PI3-kinase em fígado e músculo, sugerindo que estes eventos poderiam contribuir na redução da resposta à insulina característica do rato velho (CARVALHO et al., 1996). Nosso estudo mostrou um incremento significativo no grau de fosforilação da Shc e da associação Shc/Grb2 nos três tecidos analisados, demonstrando que os IRSs e a Shc têm regulação oposta no envelhecimento. A correlação existente entre estes dados e seus efeitos na ação da insulina não estão estabelecidos até o momento, já que a ação da insulina na síntese de DNA nestes tecidos de ratos com 20 meses não foi ainda descrita (Fig.8).

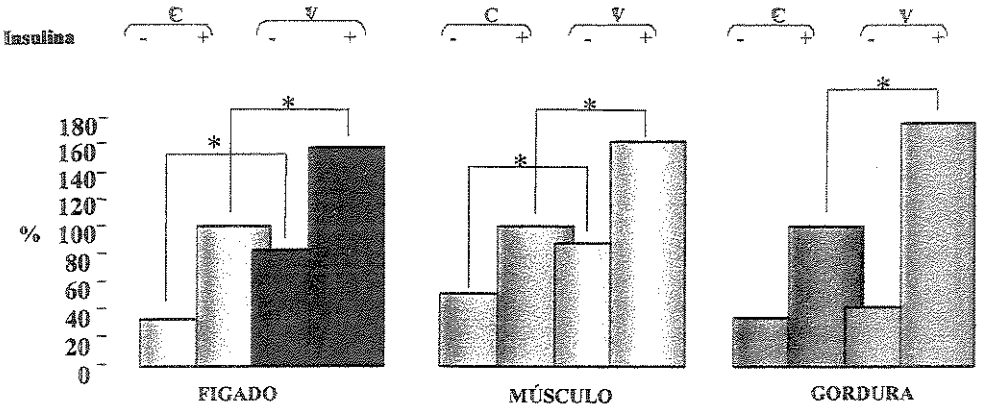

Fig. 8: **Características da fosforilação da proteína Shc pela insulina em animais envelhecidos (20 meses).** *p≤0.05

Se consideramos que tanto a tirosil-fosforilação da Shc induzida após injeção de insulina como a associação Shc/Grb2, aumentaram de maneira significativa em dois dos três modelos experimentais de resistência à insulina estudados, e que estas respostas são opostas àquelas obtidas com o IRS-1 e a PI3-kinase, nossos resultados são consistentes com dados prévios sugerindo uma

dissociação entre os mecanismos moleculares de ação que levam à metabolismo da glicose e a via da Shc após estímulo da insulina (SASAOKA et al., 1994a-b;. LAZAR et al., 1995; OUWENS et al., 1994; GIORGINO & SMITH, 1995; LI & GOLDSTEIN, 1996).

Estes resultados sugerem que a atividade da proteína Shc está relacionada aos níveis de insulina circulantes nos animais estudados. Assim, em modelos animais de resistência à insulina com hiperinsulinemia, foi observado um incremento na fosforilação da Shc e sua associação com a Grb2, enquanto que em nosso modelo compatível com hipoinsulinemia houve uma diminuição da atividade desta via.

Com estes resultados demonstramos, aliás, que jejum prolongado, tratamento crônico com dexametasona e envelhecimento podem modular a indução da insulina na tirosil-fosforilação da Shc e sua associação com a Grb2, e que esta modulação, embora diferente daquela já descrita para IRS-1 (SAAD et al., 1992; 1993), está intimamente relacionada aos níveis plasmáticos de insulina.

É importante assinalar que para todos os modelos experimentais utilizados, a associação Shc/Grb2 foi diretamente proporcional ao grau de fosforilação da Shc (Fig. 9).

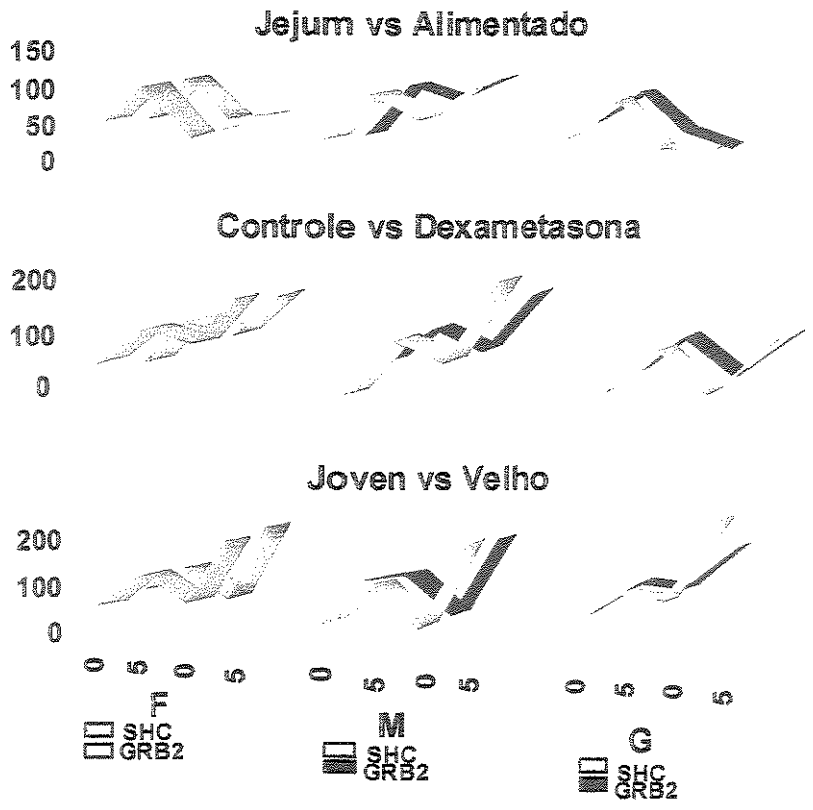

Fig. 9. **Fosforilação** da **Shc** ***vs*** **associação Shc/Grb2.** A comparação da curva de fosforilação da proteína Shc, com àquela que mostra sua associação à Grb2, revela idêntica tendência, indicando que, para todos os tecidos estudados, essa associação é dependente do grau de fosforilação da Shc. F = fígado, M = músculo, G = gordura.

2.3.2 TRATAMENTO AGUDO COM ADRENALINA E DIABETES INDUZIDO POR ESTREPTOZOTOCINA.

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A influência de múltiplos fatores na atividade das proteínas envolvidas no processo de transmissão do sinal insulínico, assim como dos diferentes estados de resistência à insulina nos níveis e grau de fosforilação das proteínas envolvidas nas vias de transmissão do sinal, nos levou ao estudo da proteína Shc, substrato direto do receptor de insulina ativado, em ratos sujeitos a tratamento agudo com adrenalina, uma conhecida situação de resistência à insulina (CHIASSON et al., 1981; PESSIN et al; 1983; BONEN et al., 1992).

O estado de resistência à insulina foi evidenciado pela significativa diminuição da velocidade de desaparecimento da glicose plasmática (k_{itt}), observada após injeção de insulina em ratos previamente tratados com adrenalina. A adrenalina antagoniza a ação da insulina, estimulando a neoglicogênese e glicogenólise (HUANG et al., 1997). Estes efeitos resultam em inibição da captação periférica de glicose mediante mecanismos β-adrenérgicos que determinam a diminuição do transporte de glicose (PESSIN et al., 1983).

Nossos resultados não demonstraram mudanças nos níveis de fosforilação da Shc no fígado, músculo e gordura de ratos tratados agudamente com adrenalina, após injeção de insulina. Demonstrou-se, contudo, um importante aumento na capacidade de associação da Shc com a Grb2 nestes três tecidos (Fig. 10)

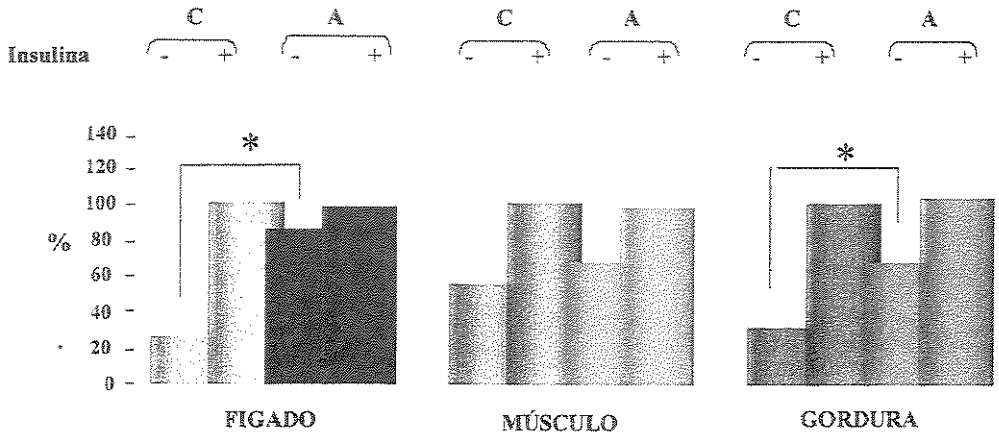

Fig. 10: **Associação Shc/Grb2 em animais sujeitos a tratamento agudo com adrenalina.** *p≤0.05

Comparando estes resultados com àqueles relacionados à proteína IRS-1, obtidos sob idênticas condições experimentais, é possível observar uma clara dissociação nas respostas da Shc e o IRS-1 neste modelo experimental. De fato, enquanto o grau de fosforilação do IRS-1 em fígado e músculo dos ratos tratados com adrenalina apresentou significativa diminuição (SAAD et al., 1995a), a fosforilação da Shc nos mesmos tecidos não sofreu alterações. A diminuição do grau de fosforilação do IRS-1 observado após infusão de insulina em fígado e músculo dos ratos tratados com adrenalina é similar àquele do IR (SAAD et al., 1995a).

Um possível mecanismo que pode desempenhar um papel importante na dissociação das respostas da Shc e o IRS-1 nestes animais, pode estar ligado ao aumento dos níveis de cAMP, que estimularia a fosforilação do IRS-1 em aminoácidos serina e/ou treonina (KIRSCH et al., 1983; JOOST et al.; 1986; KURODA et al., 1987). A dissociação da fosforilação em tirosina do IRS-1 e a

Shc após estímulo insulínico têm sido descrita em linhagens celulares com mutações em dois pontos de fosforilação em tirosina (Y1158, Y1162F, Y1163F-YFF) do receptor de insulina (OUWENS et al., 1994). Estas células apresentaram fosforilação adequada em tirosina da Shc induzida por insulina, assim como formação dos complexos Shc/Grb2 e p21Ras-GTP, acompanhada de reduzida fosforilação em tirosina do IRS-1 e redução da sua associação com a Grb2 e a PI 3-kinase (OUWENS et al., 1994).

Estes resultados conjuntamente com os nossos, sugerem que a diminuição no grau de fosforilação do receptor de insulina pode induzir processos pós-receptor de maneira diferencial, graças à preservação da fosforilação de alguns substratos e vias, mas não de outras. A dissociação nos níveis de fosforilação em tirosina do IRS-1 e a Shc sem nenhuma alteração no receptor de insulina também têm sido observada após tratamento com dexametasona (GIORGINO & SMITH, 1995) e wortmanina (LI & GOLDSTEIN, 1996).

O aumento da associação Shc-Grb2 em condições basais nos animais tratados com catecolaminas é interessante e merece destaque, especialmente pelo fato destes modelos não terem apresentado incremento no grau de fosforilação da Shc após a infusão de insulina, em nenhum dos três tecidos estudados. Existem estudos demonstrando a capacidade da noradrenalina de estimular a atividade da $p52^{Shc}$ e das MAPKs, através do seu receptor adrenérgico alfa-1 em células vasculares humanas (HU et al., 1999). Esta capacidade, unida à ativação do AMP cíclico, que estimula a fosforilação das IRSs em serina e treonina (KIRSCH et al., 1983; JOOST et al.; 1986; KURODA et al., 1987) exerceria efeitos opostos, resultando em inibição da fosforilação da Shc, sem alterar a associação Shc/Grb2, e regulando assim a via mitogênica das MAPKs nesta situação de resistência à insulina.

Estudamos ainda o efeito do diabetes mellitus induzido por STZ na fosforilação da Shc e sua associação com a Grb2. O diabetes induzido por estreptozotocina (STZ) é outro modelo experimental utilizado para o estudo da resistência à insulina. Similar ao que acontece com animais submetidos a situações de jejum prolongado, o diabetes induzido por STZ em ratos é caracterizado por diminuição da captação e metabolização de glicose, que leva estes animais a um estado de resistência à insulina, tanto em tecidos periféricos quanto em tecido hepático (ALMIRA & REDDY, 1979; OKAMOTO et al., 1986; FRIEDENBERG et al., 1985; PENICAUD et al., 1985). O mecanismo molecular deste tipo de resistência ainda não foi esclarecido, embora alterações na fosforilação de substratos do receptor de insulina tenham sido descritos (SAAD et al., 1992).

Utilizando ratos tratados com estreptozotocina, foram analisadas as características da fosforilação da p52 Shc quando induzida por insulina, assim como sua relação com a posterior associação Shc-Grb2 em fígado, músculo e tecido adiposo destes animais. A presença de diabetes nos ratos tratados, foi verificada pelos elevados níveis de glicose que eles apresentaram. Nestes experimentos, os níveis de fosforilação da Shc, assim como da associação Shc-Grb2, apresentaram aumento significativo após infusão de insulina nos três tecidos estudados. A fosforilação da Shc mostrou-se incrementada também no estado basal em músculo dos ratos diabéticos (Fig. 11).

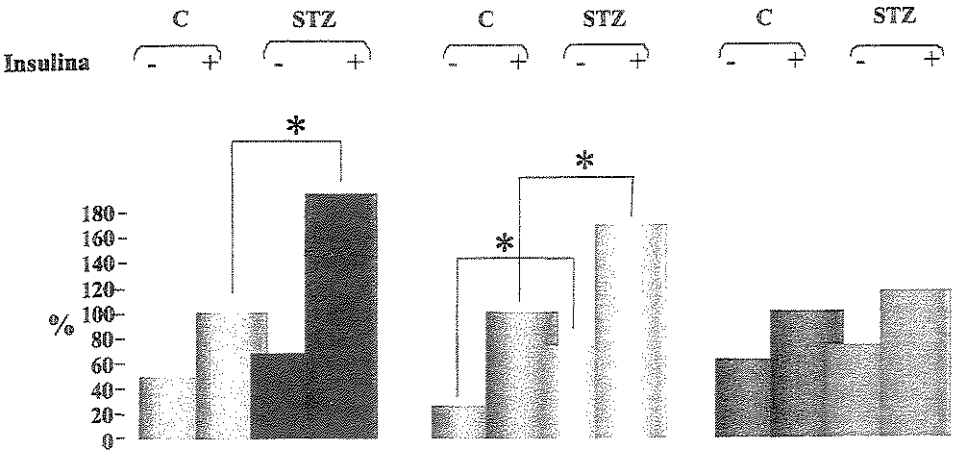

Fig. 18: **Características da fosforilação da proteína Shc pela insulina em Fígado (vermelho), Músculo (amarelo) e Gordura (verde) de animais diabéticos.** *p≤0.05

É interessante assinalar que, as características opostas de fosforilação entre Shc e IRS-1 apresentadas em outros modelos experimentais de resistência à insulina (SAAD et al., 1992; CARVALLO et al., 1996; PÁEZ-ESPINOSA et al., 1999), não se confirmaram em fígado e músculo de ratos tratados com STZ. Parece que as alterações presentes na fosforilação do IRS-1 e as subsequentes associações protéicas são consequência de um fenômeno de "up-regulation" do IRS-1 no diabetes induzido por STZ (GIORGINO et al., 1992). Este fenômeno foi específico deste modelo experimental e não esteve presente em outros modelos experimentais de diabetes como o camundongo *ob/ob* (SAAD et al., 1992; 1993) ou em ratos Goto-Kakizaki (KROOK et al., 1997).

Tomando como base nossos resultados, pode-se dizer que em fígado, músculo e gordura de ratos tratados com STZ existe uma associação competitiva entre a Shc e o IRS-1 pelo receptor de insulina fosforilado. A hiperfosforilação do IRS-1, por um mecanismo de "up-regulation" produto da hipoinsulinemia, assim como o incremento da atividade da PI 3-kinase ligada ao IRS-1, podem em parte ser explicadas pelo aumento do grau de fosforilação do IR. Essa maior fosforilação do receptor, poderia justificar parcialmente a maior fosforilação da Shc induzida por insulina, mas outros dados tornam essa hipótese pouco provável.

Os resultados obtidos em animais diabéticos foram opostos àqueles em ratos com 72 horas de jejum, outro modelo experimental de resistência à insulina que cursa com baixos níveis deste hormônio. A diferença destes dois modelos

está relacionada fundamentalmente aos níveis de glicose, sugerindo que as elevadas concentrações de glicose circulante nos animais diabéticos, pode contribuir para elevar os níveis de fosforilação da Shc em fígado, músculo e tecido adiposo dos ratos tratados com STZ (TEMARU et al., 1997; ASAKAWA et al., 1997; WELHAM et al., 1997; SHARFE & ROIFMAN, 1997).

Existe evidencia que a indução do gene da *c-fos,* precisa da ativação da tirosina-quinase do receptor de insulina (CHANG et al., 1995), seguido de posterior tirosil-fosforilação da proteína Shc (HARADA et al., 1996). Altas concentrações de glicose têm-se mostrado capazes de ativar as MAPK em células glomerulares de ratos diabéticos por STZ (HANEDA et al., 1997). Estes dados sugerem que a hiperglicemia, através da proteína Shc, desempenha um importante papel como reguladora das vias mitogênicas utilizadas pela insulina.

Os resultados deste estudo demonstram que em animais tratados agudamente com adrenalina, há um aumento basal da associação Shc/Grb2 em relação aos controles. A infusão de insulina não induziu modificação no nível de fosforilação da Shc, nem incrementou a associação Shc/Grb2. Foi demostrado ainda que em animais tratados com estreptozotocina, a fosforilação em tirosina da Shc induzida por insulina, assim como a formação do complexo Shc/Grb2, aumentam em relação ao grupo controle e apresentam uma regulação tecido-específica (Fig. 12).

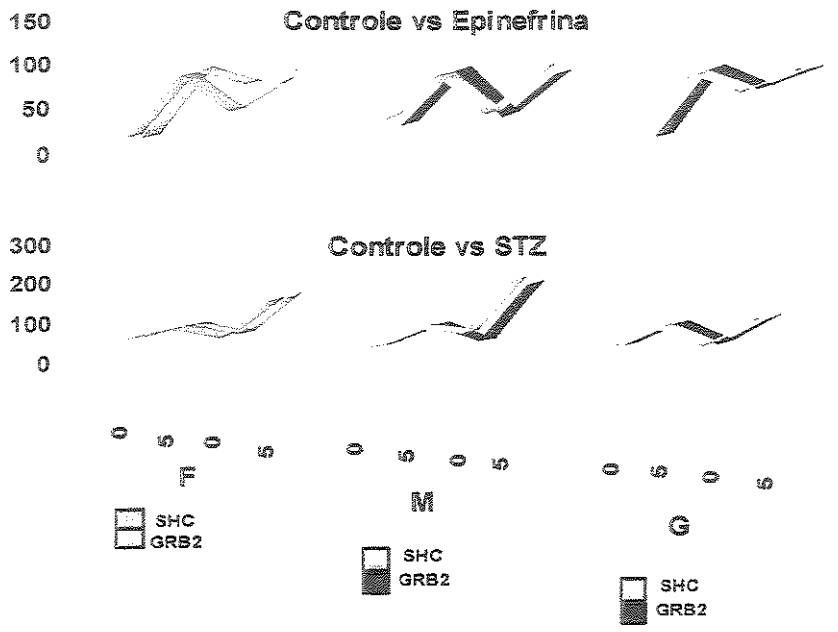

Fig. 12. **Fosforilação da Shc *vs* associação Shc/Grb2.** A comparação da curva de fosforilação da proteína Shc, com àquela que mostra sua associação à Grb2, revela idêntica tendência, indicando que, para os tecidos estudados, essa associação é dependente do grau de fosforilação da Shc. F = fígado, M = músculo, G = gordura.

2.4 NOVOS SUBSTRATOS DO RECEPTOR DE INSULINA

2.4.1 EXPRESSÃO DO mRNA EM CÉLULAS BETA PANCREÁTICAS DE RATO

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Têm sido recentemente demonstrado que células β pancreáticas expressam o mRNA do receptor de insulina (HARBECK et al., 1996). A insulina induz atividade tirosina-quinase do receptor de insulina e induz a ativação das suas vias intracelulares de sinalização, em linhagens de células β como são as células βTC3 (ROTHEMBERG et al., 1995). A superexpressão de receptores de insulina (wild type) em células produtoras de insulina estimula sua atividade tirosina-quinase e incrementa a expressão do mRNA de insulina (XU & ROTHEMBERG, 1998). Estas observações, junto àquelas segundo as quais uma deficiência na expressão de IRS-2 (IRS-2 -/- mice) produz defeito das células β pancreáticas e diabetes mellitus (WHITERS et al., 1998), assim como que camundongos manipulados geneticamente para não expressar o receptor de insulina nas suas células β pancreáticas (IR-/-), apresentam defeitos na secreção de insulina e intolerância à glicose (KULKARNI et al., 1999), sugerem que a sinalização insulínica é importante para uma adequada função das células β.

No presente estudo investigamos a expressão do IRS-2 em células β purificadas de pâncreas de ratos Wistar adultos normais, e comparados com a expressão em células não-β dos mesmos animais. Nossos resultados indicaram a presença de IRS-2 em células β, cuja expressão apresentou-se notavelmente diminuída em células não-β. Detectamos também a presença de uma importante banda na altura de 60 kDa em células β e em tecido adiposo, utilizado como controle, mas não em fígado ou em células não-β. A expressão desta proteína de

60 kDa em "immunobloting" com anticorpo anti-IRS-2 em células β, levantou a possibilidade de uma co-imunoprecipitação do IRS-2 com o substrato 3 do receptor de insulina ($p60^{IRS-3}$), previamente descrito em tecido adiposo periepididimal de rato (LAVAN & LIENHARD, 1993; ROSS et al., 1998). Mediante RT-PCR demonstramos a presença de IRS-3 mRNA em células β e não-β pancreáticas, mas não foi possível realizar sua identificação protéica.

A presença de IRS-2 em células β purificadas comprova os resultados já obtidos em linhagens celulares, e abre interessantes perspectivas no campo da sinalização intracelular em células β, onde a presença de proteínas capazes de interagir com receptores ativados pela insulina mostra não somente que a insulina cumpre um importante papel na regulação da atividade das células β pancreáticas, mas também que ela pode desempenhar suas funções através de vias de sinalização similares àquelas dos tecidos periféricos, embora com significado diferente.

Os substratos do receptor de insulina são caracterizados pela sua capacidade de interagir com a PI 3-kinase. Em tecidos sensíveis à insulina, a atividade de PI 3-kinase está implicada na estimulação da síntese protéica (MENDEZ et al., 1996), a estimulação da síntese de DNA (WANG et al., 1993), e a regulação do tráfego vesicular e o transporte de glicose (CHEATAM & KHAN, 1995). Estudos realizados em células produtoras de insulina HIT-T15 (LEIBIGER et al., 1998) e βTC6-F7 (XU & ROTHEMBERG, 1998), demonstraram que a PI 3-kinase poderia estar envolvida na "up-regulation" da transcripção do gene de insulina mediada por insulina, através das vias do IRS-2/PI 3-kinase/p70 ribosomal s6 protein kinase e CaM kinase (LEIBIGER et al., 1998; MYERS et al., 1994).

O significado fisiológico da presença de diferentes isoformas de IRS no mesmo tecido não está plenamente explicada, mas pode refletir uma redundância que mantém um adequado mecanismo de transmissão da sinal. Nesse sentido, o IRS-2 aparentemente compensa a ausência do IRS-1 em hepatócitos do camundongo IRS-1 -/-, mas não em músculo esquelético ou adipócitos (MENDEZ et al., 1997; YAMAUCHI et al., 1996; ARAKI et al., 1994). Alternativamente, estas isoformas podem ter evoluído para mediar funções específicas associadas ao estímulo insulínico (JONES & PERSAUD, 1998). Novos estudos são necessários para esclarecer as possíveis funções dos substratos do receptor de insulina para a transmissão da sinal mediada por insulina em células β pancreáticas.

O presente estudo, que teve como objetivo investigar a regulação da Shc em modelos animais de resistência à insulina, e dos IRSs em células beta pancreáticas, levou às seguintes conclusões:

1. O receptor de insulina ativado pelo seu hormônio específico se liga à proteína Shc, levando à fosforilação em tirosina desta proteína e subsequente associação da Shc a Grb2.

2. A fosforilação em tirosina da Shc pelo receptor de insulina nos tecidos hepático, muscular e adiposo de ratos, apresentou-se dependente do tempo de duração do estímulo insulínico assim como da dose de insulina aplicada.

3. Tanto a fosforilação em tirosina da Shc quanto sua associação à Grb2 estão regulados de maneira específica segundo o modelo de resistência à insulina estudado.

4. A interação Shc/Grb2 depende e acompanha o grau de fosforilação em tirosina da Shc.

5. Em situações de hipoglicemia com hipoinsulinemia existe uma diminuição da fosforilação da Shc assim como da associação Shc/Grb2.

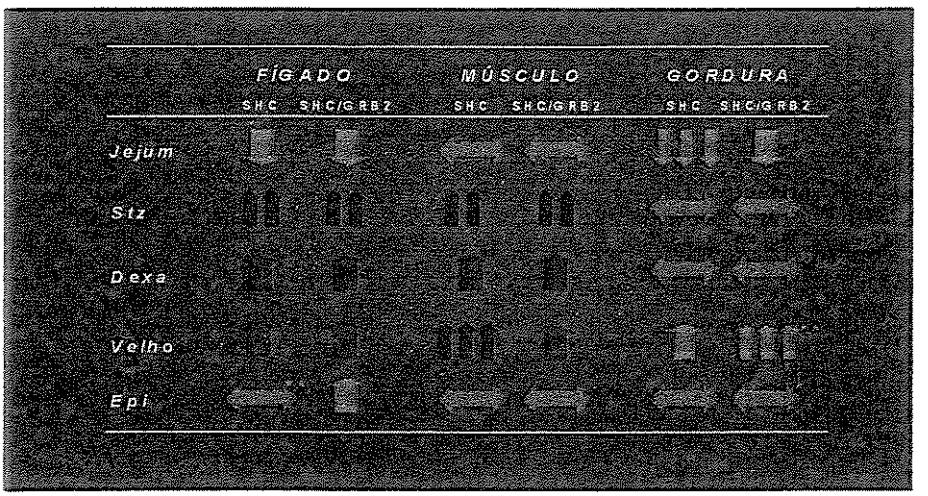
6. Situações de resistência à insulina acompanhadas de hiperglicemia produzem aumento do grau de fosforilação da Shc e da associação Shc/Grb2.

7. A administração aguda de hormônios que aumentam o AMPc não afeta o grau de fosforilação em tirosina da Shc, nem a sua interação com o Grb2.

8. Estes resultados são opostos àqueles obtidos em idênticas situações experimentais com o substrato-1 do receptor de insulina.

9. A presença do IRS-2 e mRNA IRS-3 em células beta pancreáticas abre um novo campo para o estudo das vias de transmissão insulínica nestas células.

- A figura 13 mostra de maneira esquemática o grau de fosforilação da Shc assim como a associação Shc/Grb2 observadas após infusão de insulina nos tecidos analisados. *p≤0.05; **p≤0.01, ***p≤0.001.



3. CONCLUSÕES

4. ABSTRACT

Shc is a novel type of tyrosine-phosphorylated protein activated in response to a wide variety of polypeptide ligands. In this report, we used immunoprecipitation and immunoblotting to examine the effect of insulin on Shc tyrosine phosphorylation and Shc/Grb2 association in insulin-sensitive tissues of the intact rat.

Following an infusion of insulin, Shc was tyrosine phosphorylated in the liver, skeletal muscle and adipose tissue in a time- and dose-dependent fashion, which peaked 5 min after exposure to the hormone and, except in the case of adipose tissue, returned to basal values after 15 min. There was coimmunoprecipitation of Shc and the insulin receptor after stimulation with insulin. Receptor tyrosine kinase activity toward Shc was also observed. Following an infusion of insulin, Shc was found to associate with Grb2.

Insulin-induced Shc phosphorylation and Shc/Grb2 association were also investigated in five animal models of insulin resistance (72-h starvatation, chronic dexamethasone treatment, aging, acute epinephrine treatment and STZ-induced diabetes mellitus). There were no differences in Shc protein expression between tissues from control and insulin resistant animals. In all the tissues and animal models studied, the Shc/Grb2 association were directly correlated with the levels of Shc phosphorylation reached after insulin infusion.

In fasted hypoinsulinemic rats there was a decrease in insulin-induced Shc phosphorylation in liver and adipose tissue. However, a significant increase in Shc phosphorylation was observed in liver, muscle and fat from STZ-treated rats, another insulin-resistant state which courses with low insulin levels, but high plasmatic glucose concentrations.

Other insulin-resistant states which courses with hyperglicemic levels like dexamethasone-treated rats and aging, showed similar results to those observed in diabetic rats. Liver and muscle of hypercortisolemic rats showed a significant increase in insulin-induced Shc tyrosine phosphorylation, so as liver, muscle and adipose tissue of 20 months-old rats.

Interestingly, acute stimulus with epinhephrine, a normoglicemic condition, did not display changes in insulin-induced Shc tyrosil phosphorylation nor Shc/Grb2 association in the three tissues studied.

These results indicate that Shc tyrosil phosphorylation and Shc/Grb2 association are regulated in the different type of insulin resistance and that this regulation seems to be related to the plasmatic glucose and insulin levels found in these animals.

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6. PUBLICAÇÕES

Publicação 1

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Insulin Induces Tyrosine Phosphorylation of Shc and Stimulates Shc/GRB2 Association in Insulin-Sensitive Tissues of the Intact Rat

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Shc is a novel type of tyrosine-phosphorylated protein activated in response to a wide variety of polypeptide ligands. In this study, we used immunoprecipitation and immunoblotting to examine the effect of insulin on Shc tyrosine phosphorylation and Shc/GRB2 association in insulin-sensitive tissues of the intact rat. Following an infusion of insulin, Shc was tyrosine-phosphorylated in the liver, skeletal muscle, and adipose tissue in a time- and dose-dependent fashion, which peaked 5 min after exposure to the hormone and, except in the case of adipose tissue, returned to basal values after 15 min. There was coimmunoprecipitation of Shc and the insulin receptor after stimulation with insulin. Receptor tyrosine kinase activity toward Shc was also observed. Following an infusion of insulin, Shc was found to associate with GRB2. These results demonstrate that after stimulation of rat tissues with insulin, Shc binds to the insulin receptor, is tyrosine-phosphorylated, and subsequently associates with GRB2.

Key Words: Shc; GRB2; tyrosine phosphorylation; tyrosine kinase activity.

Introduction

The insulin receptor (IR) is the principal mediator of insulin action in cellular mitogenic and metabolic processes. The IR β-subunit, which contains an intrinsic tyrosine kinase, undergoes tyrosyl autophosphorylation and is activated in response to insulin binding to the extracellular α-subunit *(1–3)*. The discovery of tyrosine kinase activity in the IR suggested that the mechanism of insulin action involves tyrosyl phosphorylation of intracellular substrates *(4,5)*. Using anti-phosphotyrosine antibodies, an insulin-stimulated phosphoprotein called pp185 has been identified in many cells and tissues *(6,7)*. One component of pp185, known as insulin receptor substrate 1 (IRS-1) band, has been purified and cloned from several sources *(7–9)*. Recently, another constituent of the pp185 band termed IRS-2 was also purified, and its cDNA sequence determined *(10)*.

In 1992, Pelicci et al. characterized a cDNA clone that encodes a new protein termed Shc (for *src* homology 2/α-collagen-related). Shc is an ubiquitously expressed cytoplasmic protein, capable of being phosphorylated after stimulation by a wide variety of growth factors and cytokines *(11–14)*. The mammalian *Shc* gene encodes three overlapping proteins of 46, 52, and 66 kDa. In cell cultures, insulin has been shown to induce the phosphorylation of the p46 and p52 isoforms of Shc *(14,15)*. In contrast to IRS-1 and IRS-2, which can associate with a wide variety of downstream effector molecules, the tyrosine phosphorylation of Shc protein leads to a specific association with a small 23-kDa adapter protein, the growth factor receptor-bound protein-2, GRB2 *(16–19)*. GRB2 is constitutively associated with the proline-rich domain of son-of-sevenless (SOS), a guanylnucleotide exchange factor for the p21 GTP-binding protein Ras *(20,21)*.

Once stimulated, the Shc-Grb2/SOS complex induces the activation of a specific mitogenic pathway by removing the mechanism that inhibits initiation of the cell cycle *(13,22,23)*. The latter action represents a crucial step in the transmission of the mitogenic signal within the cell.

Although these findings indicate that Shc has an important role in cellular growth *(24,25)*, there has not been a physiological demonstration of the above pathway of insulin action in animal tissues. For this reason, we have investigated the ability of insulin to stimulate Shc phosphorylation and Shc/GRB2 association in insulin-sensitive tissues of intact rats.

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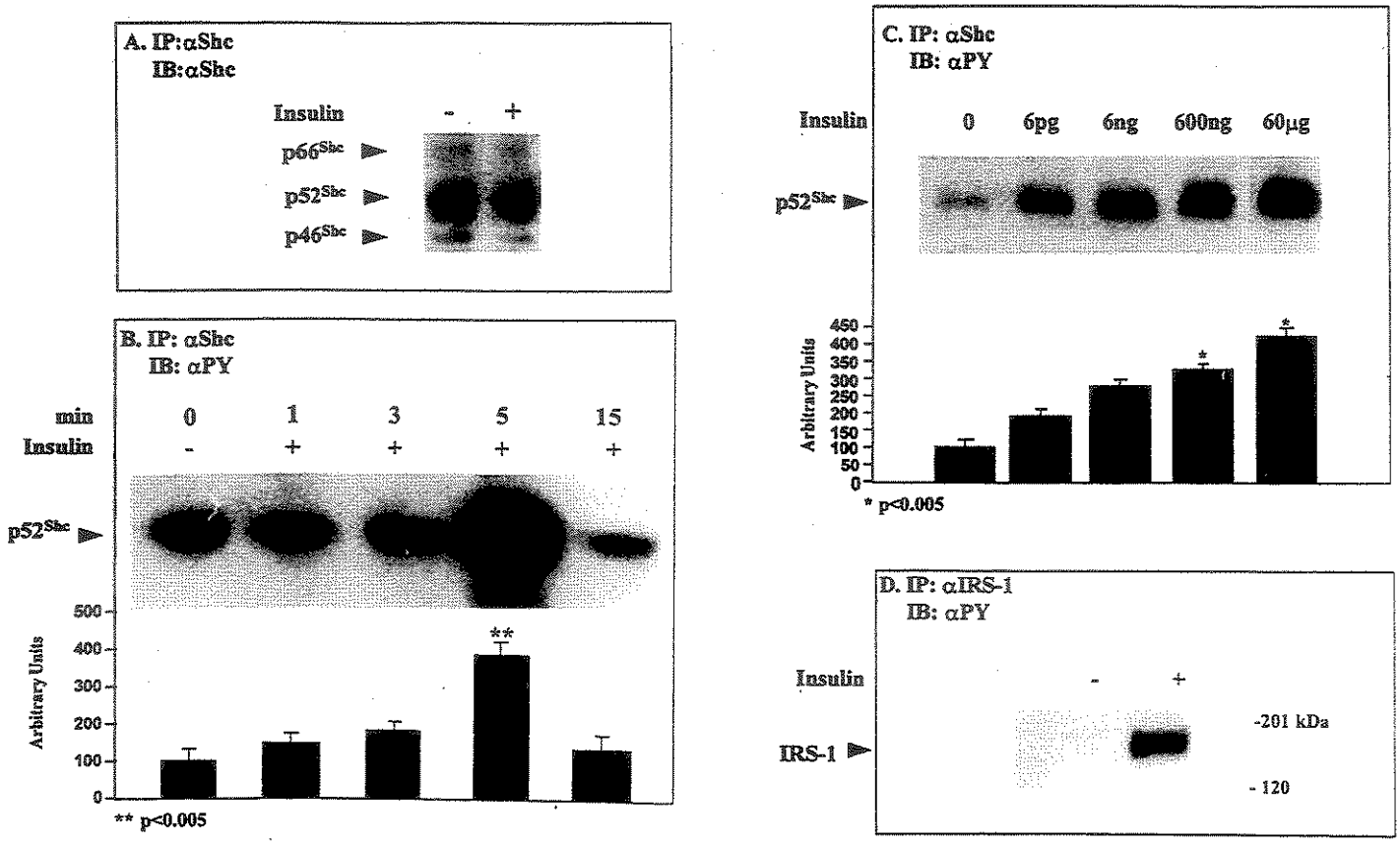

Fig. 1. Shc protein levels (**A**), time-course (**B**), and dose–response (**C**) of insulin-stimulated Shc phosphorylation in rat liver. Insulin-stimulated IRS-1 phosphorylation (**D**) in rat liver. Six-week-old male rats were anesthetized and the abdominal wall was incised to expose the viscera. Saline or insulin (at the time and dose indicated) was administered as a bolus injection via the portal vein. In the dose–response experiments, 5 min after insulin infusion, a sample of liver was excised and homogenized in extraction buffer A at 4°C as described in Materials and Methods. After centrifugation, aliquots from the supernatant were immunoprecipitated with anti-Shc antibody (A, B, and C) or anti-IRS-1 antibody (D) and protein A-Sepharose 6MB, and then resolved on 10% SDS-polyacrylamide gels. The protein bands were subsequently transferred to a nitrocellulose membrane, and detected with anti-Shc antibody (A) or anti-phosphotyrosine antibody (B, C, and D) and ^{125}I-protein A. Scanning densitometry was performed on the autoradiograms of 12 experiments for Shc protein levels, 51 samples from 8 course experiments, and 8 dose–response experiments.

Results

Following stimulation with insulin, Shc was rapidly tyrosine-phosphorylated, regardless of the tissue tested. The predominant isoform observed in liver was p52^{Shc}. In muscle and fat, p52^{Shc} was the only detectable Shc isoform (Figs. 1A, 2A, and 3A, respectively). Using nonimmunized rabbit sera in immunoprecipitation and/or immunoblotting, no bands were detected at 52 kDa.

The effect of insulin on Shc protein levels in liver was examined by immunoprecipitation and immunoblotting techniques, using anti-Shc antibody. As shown in Fig. 1A, there was no change in the level of this protein after acute insulin stimulation.

To estimate the rate of insulin-induced Shc phosphorylation in liver, we performed a time-course experiment after the administration of insulin into the portal vein. Figure 1B shows that there was detectable immunoreactivity in the basal state, indicating that Shc was already tyrosine-phosphorylated before the infusion of insulin in portal vein of fasted rats. Five minutes after the infusion of insulin, there was an increase on Shc tyrosine phosphorylation levels, which declined to basal levels over the following 15 min. An analysis of 51 samples of 8 experiments yielded a mean increase of 380% ($p < 0.005$) in Shc tyrosine phosphorylation 5 min after insulin stimulation.

By immunoblotting the same membranes with anti-Shc antibody, there was no change in the level of Shc protein during the time-course experiments (data not shown).

The insulin-stimulated phosphorylation of Shc in liver was dose-dependent (Fig. 1C). The level of Shc phosphorylation after an infusion of 6 pg of insulin, was nearly half of the maximal stimulation seen with 6 ng of the hormone, and the levels of Shc proteins did not change (data not shown).

We also used the same protocol to perform IRS-1 immunoprecipitation in the rat liver, and compare with Shc phosphorylation levels after insulin infusion. As shown in Fig. 1D, the insulin-induced tyrosine phosphorylation level in liver extracts is higher than the increase in Shc phosphorylation.

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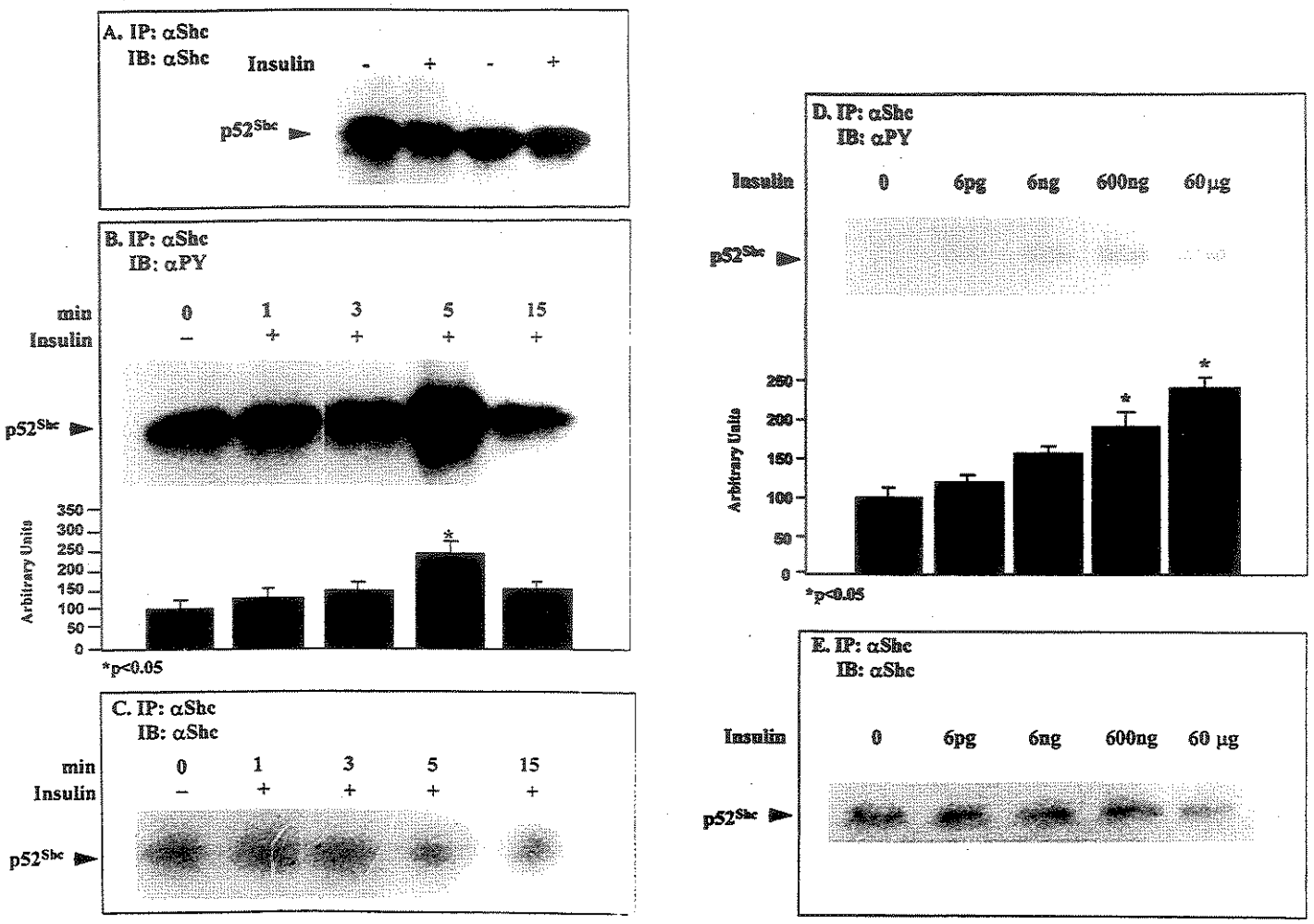

Fig. 2. Shc protein levels (**A**), time-course (**B** and **C**), and dose–response (**D** and **E**) of insulin-stimulated Shc phosphorylation in rat muscle. Six-week-old male rats were anesthetized, and the abdominal wall was incised to expose the portal vein. Saline or insulin (at the time and dose indicated) was then administered as a bolus injection. In the dose–response experiments, 5 min after insulin infusion, a sample of a gastrocnemius muscle was excised and homogenized in extraction buffer A at 4°C as described in Materials and Methods. Muscle samples were immunoprecipitated with anti-Shc antibodies, and immunoblotted with antiphosphotyrosine antibody (B and D) or anti-Shc antibody (A, C, and D). The data are representative of nine experiments for Shc protein levels, 10 time-course experiments, and 7 dose–response experiments.

The time-course of insulin-induced Shc phosphorylation in muscle was similar to that in liver. Some basal immunoreactivity was also detected, indicating that Shc was tyrosine-phosphorylated in rat muscle before insulin stimulation. The level of phosphorylation increased to 232% ($p < 0.05$) at 5 min, returning to basal levels after 15 min (Fig. 2B).

To determine the expression of Shc in muscle during the time-course, we studied the Shc protein levels before and after insulin injection. Figure 2C shows that there was no change in protein levels following the administration of the hormone.

The insulin-induced phosphorylation of Shc in muscle was dose-dependent (Fig. 2D). The presence of phosphorylated Shc was detectable after the injection of 6 pg of insulin. Half-maximal stimulation of this phosphorylation was obtained with an insulin dose between 6–600 ng. Shc protein levels in dose–response experiments remained unchanged (Fig. 2E).

The time-course of insulin-induced Shc tyrosil phosphorylation in adipose tissue was slightly different from that of liver and muscle. In adipose tissue, Shc phosphorylation increased to 204% 1 min after insulin infusion, and reached a maximum at 280% ($p < 0.05$) 5 min later. However, the level of phosphorylation was sustained for at least 15 min (Fig. 3B).

As with liver and muscle, there was no change in the levels of Shc protein in adipose tissue after insulin stimulation during the time-course experiments (Fig. 3C).

The insulin-stimulated phosphorylation of Shc in adipocyte extracts was dose-dependent. The levels of phosphorylated Shc reached a half-maximum value with 6 pg of insulin (Fig. 3D). When these membranes were blotted with anti-

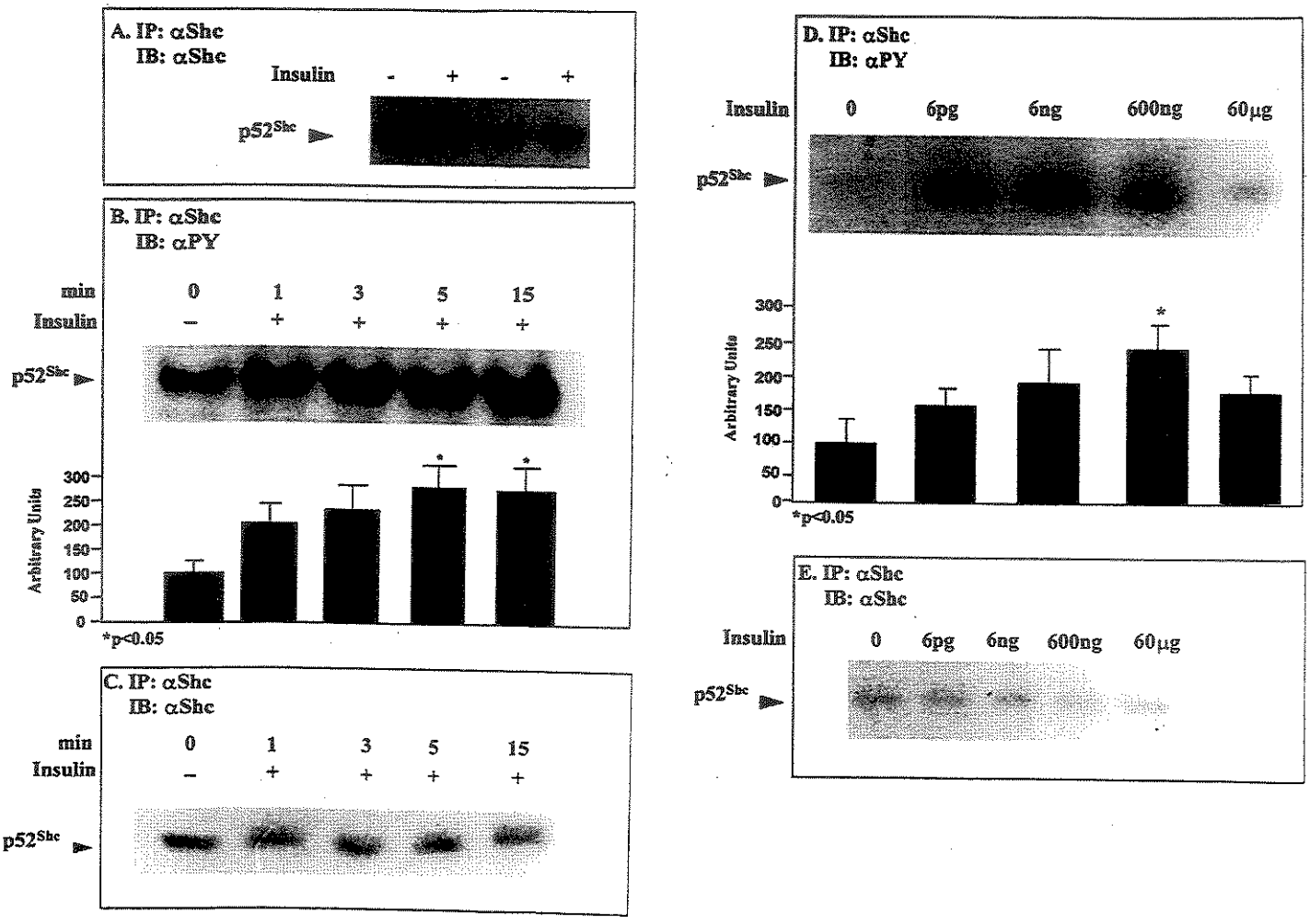

Fig. 3. Shc protein levels (**A**), time-course (**B** and **C**), and dose–response (**D** and **E**) of insulin-stimulated Shc phosphorylation in rat adipose tissue. Six-week-old male rats were anesthetized, and the abdominal wall was incised to expose the portal vein. Saline or insulin (at the time and dose indicated) was administered as a bolus injection via the portal vein. In the dose–response experiments, 5 min after insulin infusion, a sample of adipose tissue was excised and homogenized in extraction buffer A at 4°C as described in Materials and Methods. Adipose tissue samples were immunoprecipitated with anti-Shc antibodies, and immunoblotted as described in legend for Fig. 2. The data are representative of 6 experiments for Shc protein levels, 9 time-course experiments, and 8 dose–response experiments.

Shc antibody, the levels of Shc protein remained unchanged (Fig. 3E).

The rapid induction of Shc tyrosine phosphorylation by insulin suggested that Shc may associate with the insulin receptor. To test this possibility, liver extracts were immunoprecipitated with anti-Shc antibody before and after insulin stimulation, and the precipitated proteins then probed with antireceptor antibody. Shc was found to coprecipitate with the insulin receptor after stimulation with the hormone (Fig. 4A). Anti-insulin receptor coimmunoprecipitation was also performed with the same tissue extracts. As shown in Fig. 4B, Shc was coimmunoprecipitated by anti-insulin receptor antibody following insulin stimulation.

To determine whether insulin-induced insulin receptor kinase activity was able to phosphorylate Shc, we measured enzyme autophosphorylation and kinase activity in vitro. Following the infusion of saline or a low dose of insulin (6 pg) into the portal vein to obtain limited tyrosine phosphorylation of the insulin receptor, liver extracts were immunoprecipitated with antibody to the insulin receptor. The pellet was incubated at room temperature with ATP to permit IR autophosphorylation. Figure 4C demonstrates that after infusion of a low dose of insulin, there is a limited autophosphorylation of insulin receptor and also a discrete increase in tyrosine phosphorylation of a lower band (~55 kDa). After infusion of a low dose of insulin in vivo and the addition of ATP in vitro, there is a clear increase in insulin receptor autophosphorylation and in tyrosine phosphorylation of at least four bands (the same lower-molecular-mass band and three bands higher than insulin receptor). By stripping and immunoblotting the same membrane with specific antibodies, we demonstrate that the two higher bands are IRS-2 and IRS-1, and that the lowest band of ~55 kDa is Shc. We have not identified the band appearing between IRS-1 and insulin receptor band.

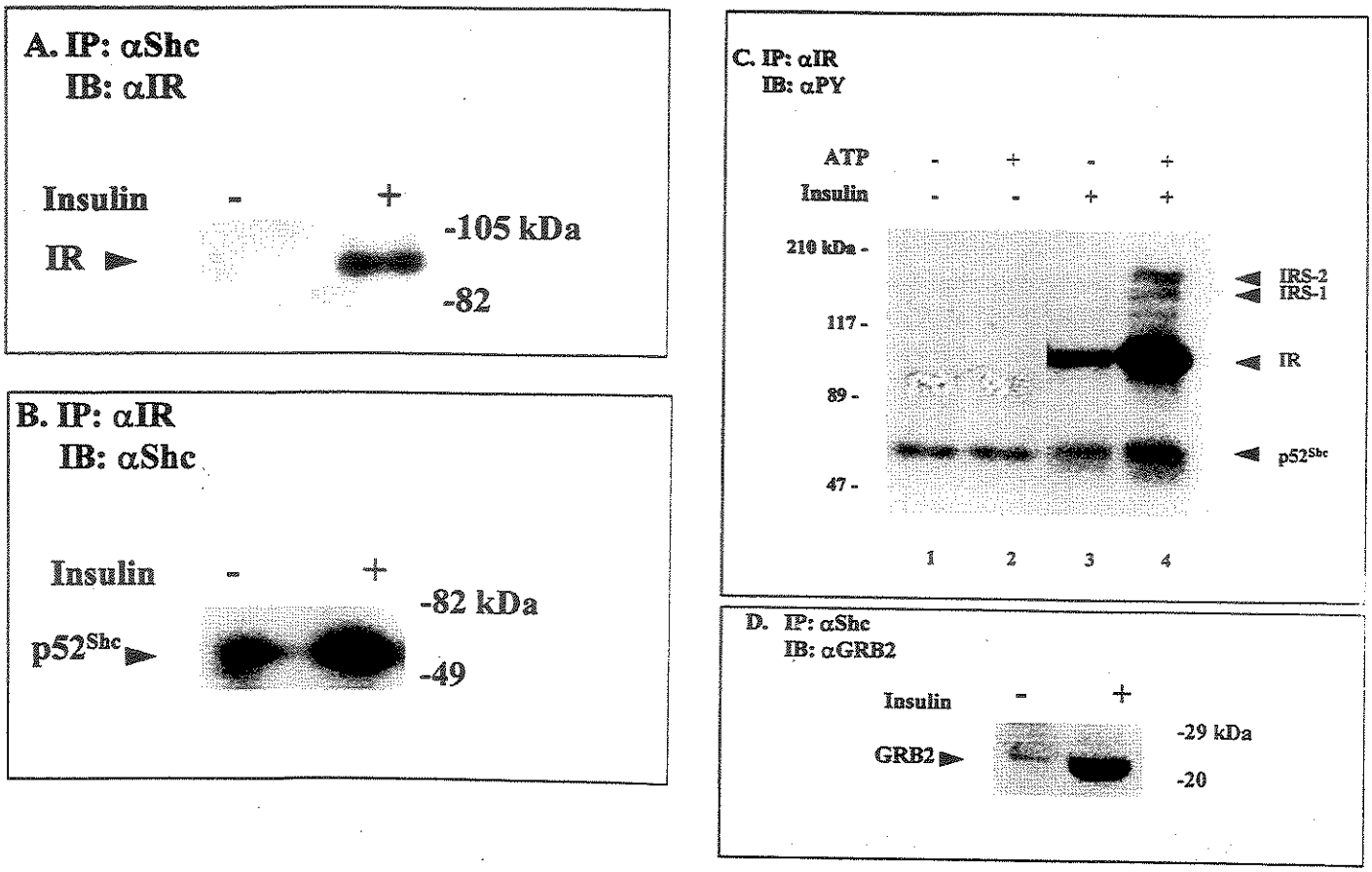

Fig. 4. The association of Shc with the insulin receptor (**A** and **B**), IR tyrosine kinase activity measured by autophosphorylation (**C**), and Shc-GRB2 association in rat liver (**D**). The rats were anesthetized and the abdominal wall was incised to expose the viscera. Saline or 60 μg of insulin (as indicated in the figure) were administered as a bolus injection into the portal vein. Five minutes later, a sample of the tissue were excised and homogenized in extraction buffer at 4°C as described in Materials and Methods. After centrifugation, aliquots containing equal amounts of protein were immunoprecipitated (IP) with anti-Shc or anti-insulin receptor (αIR) antibodies as indicated in the figure. The immunoprecipitates were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were then immunoblotted (Blot) sequentially with anti-insulin receptor (A), anti-Shc (B), or anti-GRB2 (D) antibodies as indicated in the figure. The data are representative of five experiments for each antibody. To measure the tyrosine kinase activity of IR (C), 6 pg of insulin were injected into the portal vein of the rat, and the liver was excised 30 s (the maximal time of IR tyrosine phosphorylation in liver) after the insulin injection. To stimulate partial IR autophosphorylation, IR was then immunoprecipitated and allowed to autophosphorylate in vitro in the presence of exogenous ATP (lane 4). Tyrosine phosphorylation was measured by immunoblotting the ATP stimulated pellet with an antiphosphotyrosine antibody. Control conditions are shown in lanes 1 and 2. In lane 1, the liver extract was not exposed to insulin or ATP. In lane 2, the liver extract was not exposed to insulin, but ATP was added to the in vitro autophosphorylation reaction. In lane 3, insulin (6 pg) was infused into the portal vein and the liver extracted after 30 s, but no exogenous ATP was added during the in vitro phosphorylation step.

To determine the presence of Shc/GRB2 association in liver extracts, samples of this tissue were immunoprecipitated with anti-Shc antibodies before and 5 min after insulin stimulation, and blotted with anti-GRB2 antibodies. Shc/GRB2 association was observed only when the Shc protein was phosphorylated (Fig. 4D).

Discussion

It is well established that the hormone-stimulated tyrosine kinase activity of growth factor receptors is a necessary event in the propagation of growth factor-dependent downstream signaling *(26–30)*. Although several combinations of proximal effectors have been identified for numerous growth factor tyrosine kinase receptors, they all appear to have in common tyrosine phosphorylation and/or association with Shc proteins *(11,15,18)*. Insulin has also been shown to phosphorylate this protein in cultured cells *(11,14,15)*, although the physiological significance of this process has not yet been elucidated. We have demonstrated here that in liver, skeletal muscle, and, to a lesser extent in adipose tissue, Shc undergoes tyrosine phosphorylation after insulin administration in intact rats.

Di Guglielmo et al. *(31)* demonstrated in rat liver that epidermal growth factor (EGF) administration clearly induced Shc tyrosine phosphorylation, whereas insulin infusion led to insulin receptor tyrosine phosphorylation,

but little detectable tyrosine phosphorylation of Shc and no recruitment of GRB2. In the study of Di Guglielmo et al., preparative subcellular fractionation was used to address signal transduction in vivo, whereas our work experiments were conducted in whole-tissue extracts. In addition, the composition of homogenization buffers we used was different. Thus, in our studies, the concentration of vanadate was 100-fold greater than that used in the studies of Di Guglielmo et al. These methodological differences might explain why Di Guglielmo et al. found little detectable Shc tyrosine phosphorylation, whereas we observed a moderate increase in Shc tyrosine phosphorylation in liver after insulin stimulation.

In vitro binding experiments have shown that the 52-kDa Shc isoform associates more efficiently with the insulin receptor than the 46-kDa Shc isoform *(14,15)*. In previous studies using cultured cells, the 52-kDa Shc isoform was also shown to have a higher level of tyrosine phosphorylation than the 46-kDa species when stimulated with insulin *(14,15,23)*. Our results for rat muscle and adipose tissue demonstrate that after treatment with insulin, only the 52-kDa Shc isoform was tyrosine-phosphorylated. In liver, this isoform of the protein presents high levels of phosphorylation with little phosphorylation of the 66- and 46-kDa isoforms. This predominance may be a consequence of the high levels of the 52-kDa Shc isoform in rat tissues compared with other isoforms, although interactions with the insulin receptor may also play a role in this pattern of phosphorylation.

To our knowledge, this is the first demonstration of insulin-induced Shc tyrosine phosphorylation in intact animals. Acute, half-maximal stimulation of Shc phosphorylation in liver occurred after the infusion of 6 ng of insulin, which corresponds to plasma insulin levels of 40 μU/mL, 1 min later (data not shown). This concentration is similar to that which is achieved in rats during an oral glucose or meal tolerance test. Shc tyrosine phosphorylation in muscle and adipocytes following the intraportal injection of insulin was less sensitive than in liver, probably because of the lower peripheral insulin concentrations.

The insulin-induced Shc phosphorylation was similar to that reported for EGF and platelet-derived growth factor (PDGF) signaling *(14,15,32)*. Shc proteins have also been shown to associate with the activated EGF receptor *(11,33–35)*. Although previous studies in cell cultures found no association between Shc proteins and the activated insulin receptor, we have shown that these two proteins coimmunoprecipitate after an infusion of insulin. Recent studies have demonstrated that Shc must bind directly to the activated insulin receptor via its PTB/PI domain in order to be tyrosine-phosphorylated in vivo *(10,36–38)*. Studies using cells expressing insulin receptor mutants support a model in which both Shc and IRS-1 recognize a core NPX-Tyr(P) motif on the insulin receptor, with the amino acids surrounding this motif being critical for binding either IRS-1 or Shc *(39)*. Our results also showed that insulin receptor has tyrosine kinase activity toward Shc after insulin stimulation.

Both Shc and IRS-1 are phosphorylated at similar insulin concentrations, but maximal Shc phosphorylation occurs later than IRS-1 maximal phosphorylation in rat tissues *(40,41)*. We have previously demonstrated that maximal insulin-induced IRS-1 phosphorylation occurs at 30 and 90 s in liver and muscle, respectively *(40,41)*. The present study is showing that in both tissues, maximal insulin-induced Shc tyrosine phosphorylation occurs at 5 min postinjection of insulin. The physiological consequence of this differential time-course is not known, but since Shc and IRS-1 compete for the same binding site at the insulin receptor, it is tempting to speculate that the receptor uses different substrates at different times, in order to serve as a more effective tyrosine kinase toward each substrate. It is also interesting that IRS-1, which is phosphorylated first, mediates the metabolic and growth-promoting effects of insulin, whereas Shc, which is phosphorylated later, is mainly a component of growth-signaling pathways.

In cultured cells, insulin-induced tyrosine phosphorylation of Shc is followed by the association of Shc with a small 23-kDa adapter protein known as GRB2 *(16–19)*. Our demonstration that Shc associates with GRB2 in rat tissues after insulin stimulation supports the idea that tyrosine phosphorylation of Shc, and the subsequent interaction of this protein with GRB2, may be an essential component of a common pathway used by many tyrosine kinases. The Shc/GRB2 interaction may play a crucial role in the activation of $p21^{ras}$ and in the control of downstream effector molecules *(22)*.

It is interesting that in Chinese hamster ovary cell lines with mutations at two tyrosine phosphorylation sites on the insulin receptor there is a dissociation between insulin-induced IRS-1 and Shc tyrosine phosphorylation *(42,43)*. These cell lines show normal insulin-induced tyrosine phosphorylation of Shc, Shc–GRB2 complex formation, and $p21^{ras}$ activation, but have reduced tyrosine phosphorylation of IRS-1, as well as reduced IRS-1 association with GRB2 and PI3-kinase *(42)*. A dissociation between IRS-1 and Shc tyrosine phosphorylation has also been observed in other cell lines, without any change in the insulin receptor phosphorylation level, following treatment with dexamethasone *(44)* or wortmannin *(45)*. In this regard, it would be of interest to investigate, in parallel insulin-induced insulin receptor, IRS-1, and Shc tyrosine phosphorylation in the tissues of animal models of insulin resistance.

In summary, we have shown that following the stimulation of rat tissues with insulin, Shc binds to the insulin receptor, is tyrosine-phosphorylated, and subsequently associates with GRB2.

Materials and Methods

Materials

The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonylfluoride (PSMF), aprotinin, silicone, and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium amobarbital and human recombinant insulin (Humulin R) were purchased from Eli Lilly Co. (Indianapolis, IN). Adenosine 5'-triphosphate (ATP) from equine muscle was from Sigma Chemical Co. Protein A-Sepharose 6 MB was purchased from Pharmacia (Uppsala, Sweden). [^{125}I] protein A was obtained from Amersham (Aylesbury, UK), and nitrocellulose (BA85; 0.2 μm) was obtained from Schleicher and Schuell (Keene, NH). Male Wistar rats were from the UNICAMP Central Animal Breeding Center. Monoclonal antiphosphotyrosine antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-Shc, anti-GRB2, anti-IRS-1, and anti-insulin receptor antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

Six-week-old male Wistar rats (mean body wt of 131 g ± 6 g) were used. The animals were fed standard rodent chow and water ad libitum. Food was withdrawn 12–14 h before the experiments (except for animals used to determine IR tyrosine kinase activity, which were fasted for only 4 h). All animal experiments were approved by the Ethical Committee of the State University of Campinas (UNICAMP).

Methods

The rats were anesthetized with sodium amobarbital (15 mg/kg body wt, ip) and used 10–15 min later, as soon as anesthesia was assured by the loss of foot and corneal reflexes. The abdominal cavity was opened, the portal vein exposed, and 0.5 mL of saline (0.9% NaCl) with or without 6 μg of insulin (or the concentration described for the dose–response experiments) was injected. Samples of liver, skeletal muscle, and adipose tissue were collected at 0, 1, 3, 5, and 15 min after insulin infusion, minced coarsely, and homogenized immediately in approx 10 vol of solubilization buffer A at 4°C, using a Polytron PTA 20Ss homogenizer (Brinkmann Instruments, model PT 10/35), operated at maximum speed for 30 s. Buffer A (for solubilization) consisted of 1% Triton X-100, 50 m*M* HEPES (pH 7.4), 100 m*M* sodium pyrophosphate, 100 m*M* sodium fluoride, 10 m*M* EDTA, 10 m*M* sodium vanadate, 2 m*M* PSMF, and 0.1 mg of aprotinin/mL. The extracts were centrifuged at 30,000*g* in a Beckman 70.1 Ti rotor at 4°C for 20 min in order to remove insoluble material, and the resulting supernatant was used for immunoprecipitation with 15 μL of polyclonal anti-Shc. The immune complexes were precipitated with protein A-Sepharose 6MB and were washed three times with 50 m*M* Tris, pH 7.4, 2 m*M* sodium vanadate, and 0.1% Triton X-100.

Protein Analysis by Immunoblotting

After washing, the pellet was suspended in Laemmli sample buffer with 100 m*M* dithiothreitol and heated in a boiling water bath for 5 min. The samples were subjected to SDS-PAGE (10% Tris-acrylamide) in a Bio-Rad miniature slab gel apparatus.

Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin et al. *(46)*. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4°C in blocking buffer (3% BSA, 10 m*M* Tris, 150 m*M* NaCl, and 0.02% Tween-20). The prestained molecular-mass standards used were phosphorylase B (105 kDa), bovine serum albumin (82 kDa), ovalbumin (49 kDa), carbonic anhydrase (33.3 kDa), soybean trypsin inhibitor (28.6 kDa), and lysozyme (19.4 kDa). The nitrocellulose filter thus treated was then incubated for 4 h at 22°C with antiphosphotyrosine antibody, anti-insulin receptor antibody, or GRB2 antibody (0.5 μg/mL each, diluted in blocking buffer), and then washed for 30 min in blocking buffer without BSA. The blots were then incubated with ^{125}I-protein A (30 μCi/μg) in 10 mL of blocking buffer for 1 h at 22°C and washed again. ^{125}I-protein A bound to the antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at –70°C for 12–48 h. Band intensities were quantified by optical densitometry (Molecular Dynamics) of the developed autoradiogram.

IR Tyrosine Kinase Activity Toward Shc

Following the infusion of a very low dose of insulin (6 pg) or saline into the portal vein of 4-h fasted rats, liver extracts were immunoprecipitated as described above, using anti-insulin receptor (β-subunit) antibody. The immune complexes were collected on protein A/G plus.

The protein kinase activity of the immunoprecipitates was measured by incubating the immune complexes (anti-insulin receptor) in 100 μL of buffer containing 50 m*M* Tris, pH 7.5, 0.2 m*M* sodium vanadate, 0.1% Triton X-100, 3 m*M* $MnCl_2$, and 15 μ*M* ATP for 30 min at room temperature. The complexes were washed twice with cold buffer, and then suspended in Laemmli sample buffer and analyzed by SDS-PAGE according to the method of Laemmili *(47)*. The separated proteins were transferred to nitrocellulose membranes. The incorporation of phosphate into insulin receptor and other proteins that might be substrates bound to IR was visualized by autoradiography of antiphosphotyrosine immunoblots *(48)*.

Statistical Analysis

Time-course and dose–response data are presented as the mean ± SE of *n* experiments for liver, muscle, and adipose tissue. To check the fit of data for the assumption of parametric analysis of variance, Bartlett's test for the

homogeneity of variances was used. When necessary, the data were log-transformed to correct for variance heterogeneity or nonnormality *(49)*. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey (equal n) or Tukey-Kramer (unequal n) test for individual differences between times or insulin concentrations. Protein Shc levels were analyzed by Student's t-test. The level of significance employed was $p < 0.05$.

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Publicação 2

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Insulin receptor has tyrosine kinase activity toward Shc in rat liver

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Abstract

Insulin induces tyrosine phosphorylation of Shc in cell cultures and in insulin-sensitive tissues of the intact rat. However, the ability of insulin receptor (IR) tyrosine kinase to phosphorylate Shc has not been previously demonstrated. In the present study, we investigated insulin-induced IR tyrosine kinase activity towards Shc. Insulin receptor was immunoprecipitated from liver extracts, before and after a very low dose of insulin into the portal vein, and incubated with immunopurified Shc from liver of untreated rats. The kinase assay was performed *in vitro* in the presence of exogenous ATP and the phosphorylation level was quantified by immunoblotting with antiphosphotyrosine antibody. The results demonstrate that Shc interacted with insulin receptor after infusion of insulin, and, more important, there was insulin receptor kinase activity towards immunopurified Shc. The description of this pathway in animal tissue may have an important role in insulin receptor tyrosine kinase activity toward mitogenic transduction pathways.

Key words
- Tyrosine kinase activity
- Insulin receptor
- Shc
- Insulin action

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Considerable evidence has been accumulated to indicate that activation of tyrosine kinase activity is essential for most intracellular signaling pathways (1,2). Many polypeptides like insulin, IGF-1, PDGF or EGF, acting through the tyrosine kinase capacity of their own receptors, lead to the transfer of phosphate groups of ATP to multiple tyrosine residues on the receptor itself, a mechanism known as autophosphorylation (3). Autophosphorylation of tyrosine-kinase growth factor receptors is required for the full kinase activity toward their own substrates. Interaction of tyrosine phosphorylated receptors with various SH2 domain-containing effector molecules is responsible for downstream signal transduction leading to biological action (4).

The insulin receptor (IR) tyrosine kinase is activated by two separate events, where three residues, Tyr_{1158}, Tyr_{1162} and Tyr_{1163}, located in the so-called regulatory loop, appear to play a central role in this process (5). First, insulin binding partially increases the activity of the insulin receptor tyrosine kinase and stimulates its ß-subunit autophosphorylation. Second, autophosphorylation of the insulin receptor fully activates the en-

zyme toward specific intracellular substrate proteins (6). The substrate specificity of the purified insulin receptor tyrosine kinase has been extensively examined *in vitro*. The receptor prefers substrates in which the tyrosine residue is preceded by an acidic amino acid (Y^{-1} or Y^{-2} position) and is followed by a hydrophobic residue (Y^{-1}) (7,8).

These results are consistent with many studies showing that both insulin receptor substrate-1 (IRS-1) and Shc (src homology 2/α collagen related), a ubiquitously expressed intracellular signaling protein, share a related amino-terminal region called Shc and the IRS-1 NPXY-binding (SAIN) domain, also known as PTB/PI domain (9), which displays an amino acid sequence similar to that of the IR specificity, and is able to recognize and bind to the phosphorylated Tyr 972 located within the NPX-Tyr(P) motif of IR (10).

Although the PTB/PI domain of Shc appears to interact with the insulin receptor (11), the tyrosine kinase activity of the insulin receptor towards Shc has not been previously demonstrated. The aim of the present study was to investigate the tyrosine kinase activity of the insulin receptor towards Shc, using partially activated IR and immunopurified Shc from animal tissues.

Six-week-old male Wistar rats (130-150 g) were allowed access to standard rodent chow and water *ad libitum*. Food was withdrawn 4 h before the experiments. The rats were anesthetized with sodium thiopental (25 mg/kg body weight, *ip*) and used 10-15 min later, as soon as anesthesia was assured by the loss of foot and corneal reflexes. The abdominal cavity was opened, the portal vein exposed, and 0.5 ml of saline (0.9% NaCl) with or without 6 pg of insulin was injected. Three min after insulin injection, the liver was removed, minced coarsely, and homogenized immediately in approximately 10 volumes of solubilization buffer A (50 mM Tris, pH 7.4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF, 0.1 mg/ml aprotinin, and 1% Triton-X 100), at 4°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35; Westbury, NY) operated at maximum speed (setting 10) for 30 s. After extraction the samples were centrifuged at 12,000 rpm at 4°C in a Beckman 70.1 Ti rotor (Beckman, Palo Alto, CA, USA) for 30 min to remove insoluble material, and the resulting supernatant was used for immunoprecipitation with anti-insulin receptor or anti-Shc antibodies. The immune complexes were precipitated with protein A Sepharose 6MB. After immunoprecipitation Shc protein was subjected to immunopurification techniques by washing three times with a buffer containing 300 mM NaCl, 0.03% (v/v) Tween 20, 2 mM PMSF and 0.1 mg/ml aprotinin. The complexes were then resuspended in 30 µl of 0.2 M Na_2CO_3, pH 11, and incubated in 20 µl of a buffer containing 1 M Tris, pH 6.8, 0.02% (v/v) Tween 20, 3 mM PMSF and 0.1 mg/ml aprotinin. The protein kinase activity was measured by adding immunoprecipitated IR and immunopurified Shc in 100 µl of buffer containing 50 mM Tris, pH 7.4, 0.2 mM sodium vanadate, 0.1% Triton-X 100, 3 mM $MnCl_2$ with or without 15 µM ATP for 30 min at room temperature (12). The complexes were washed once with the same kinase buffer and then resuspended in 20 µl of Laemmli sample buffer with 200 mM DTT (13), and subsequently separated by 8% SDS-PAGE. Proteins in the gel were electrophoretically transferred to nitrocellulose in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin et al. (14). The incorporation of phosphate into insulin receptor and Shc was visualized by autoradiography of anti-phosphotyrosine immunoblots. The prestained molecular weight standards used were myosin (194 kDa), ß-galactosidase (116 kDa), bovine serum albumin (85 kDa), and ovalbumin (49 kDa).

When Shc immunopurified from 4-h

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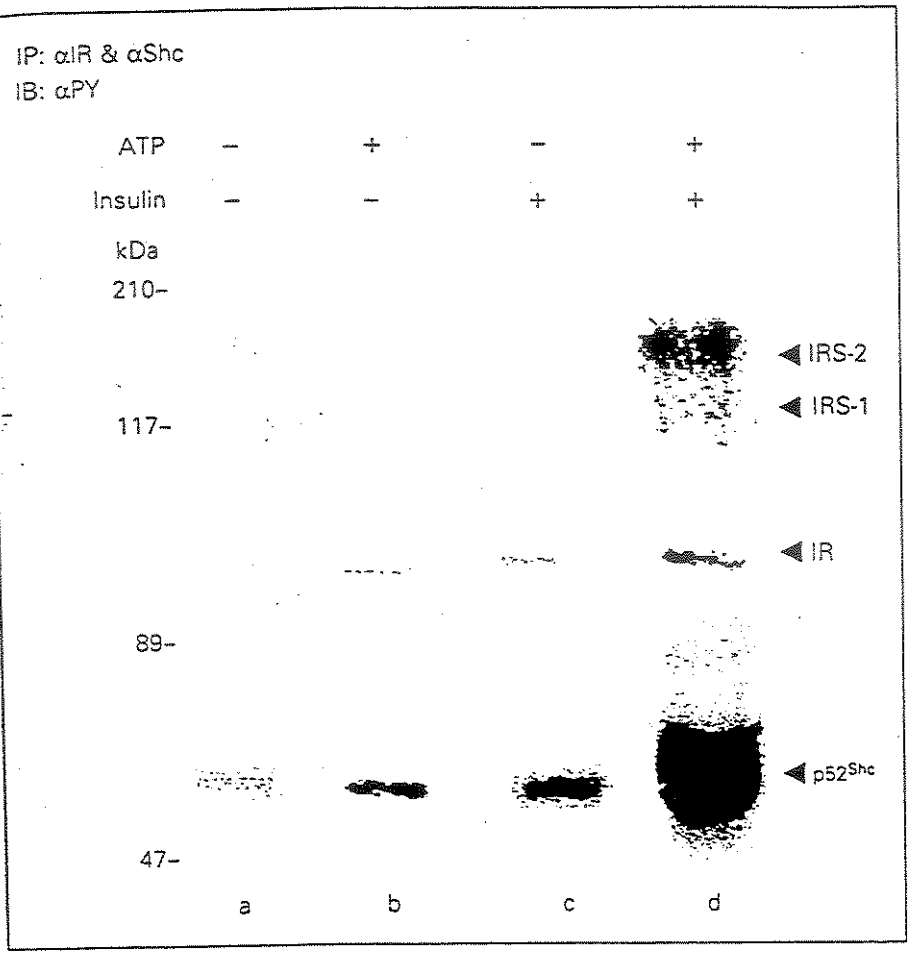

Figure 1 - IR tyrosine kinase activity measured by autophosphorylation with immunopurified complex. The rats were anesthetized and the abdominal wall was incised to expose the viscera. Saline or 6 pg of insulin (as indicated in the figure) was administered as a bolus injection into the portal vein of the rat and the liver was excised 3 min (the intermediate between maximal times of IR and Shc tyrosine phosphorylation in liver) after insulin injection to stimulate partial IR autophosphorylation. IR was then immunoprecipitated and Shc was immunopurified as described in Methods, and then allowed to react *in vitro* in the presence of exogenous ATP (lane d). Tyrosine phosphorylation was measured by immunoblotting the ATP-stimulated pellet with an antiphosphotyrosine antibody. Control conditions are shown in lanes a and b. In lane a, the liver extract was not exposed to insulin or ATP. In lane b, the liver extract was not exposed to insulin, but ATP was added to the *in vitro* autophosphorylation reaction. In lane c, insulin (6 pg) was infused into the portal vein and the liver extracted after 3 min, but no exogenous ATP was added during the *in vitro* phosphorylation step. In lane d, insulin was infused and ATP was added to the *in vitro* phosphorylation reaction. This experiment was performed 5 times with closely similar results.

fasted non-insulin stimulated rat liver were added to immunoprecipitated IR complexes from liver of rats exposed to 6 pg of insulin stimulus, a lower basal level of $p52^{Shc}$ phosphorylation independent of the ATP *in vitro* assay was observed (Figure 1, lane a). After 3 min, a time between maximal IR and Shc phosphorylation levels in liver after insulin infusion *in vivo*, a weak IR tyrosyl phosphorylation was also observed, with little increase in the intensity of the lower band (~55 kDa) (Figure 1, lane c). After *in vitro* cold ATP addition, the IR and $p52^{Shc}$ bands became stronger than their respective non-ATP-stimulated bands (Figure 1, lanes b and d), but IR remained phosphorylated to a lesser extent than Shc. Two upper weak bands also appeared. It was previously demonstrated that these bands are IRS-1 and IRS-2 (15).

Over the past few years, considerable progress has been made in elucidating the signaling pathways downstream from tyrosine phosphorylation. The data of the present study show an already known pattern of insulin receptor tyrosine phosphorylation occurring during the early intracellular steps of insulin signaling in rat liver, which could be crucial for the insight into the molecular basis for insulin receptor tyrosine kinase mitogenic transduction pathways.

The insulin-stimulated phosphorylation of Shc occurs at physiological post-prandial insulin levels as shown in Figure 1, and leads to association with the Grb2-SOS complex to activate $p21^{ras}$ (16,17), which activates mitogenesis by activating the mitogen-activated protein kinase (18). Therefore, a key to understanding the mechanism of how acti-

vated insulin receptors increase the amount of $p21^{ras}$-GTP lies in the clarification of the mechanism of linkage between insulin receptor and Shc.

Shc can interact with tyrosine-phosphorylated growth factor receptors in three different ways, either through its SH2 domain or its amino-terminal domain, or both (16-18). Increasing evidence indicates that the amino-terminal domain of Shc is responsible for the binding to phosphotyrosine residues of the growth factor receptors. Such evidence was based on cell culture *in vitro* assays and also on the sensitive two-hybrid assay of protein-protein interaction which demonstrated that Shc interacts directly with IR (19,20).

Unlike EGF and PDGF receptors, coimmunoprecipitation studies have not shown interaction between Shc and the IR *in vivo*. It seems that the interaction of Shc with the IR, in spite of being of sufficient affinity to localize these molecules adjacent to the IR kinase to promote phosphorylation, may be of a low enough affinity to allow these molecules to rapidly dissociate from the activated receptor following phosphorylation, causing a reduced binding affinity of Shc to the IR, and impairing the analysis of the association of these two proteins (18).

Our results show that Shc interacts with the insulin receptor after insulin stimulation in rat liver. Furthermore, there is a kinase activity of the insulin receptor towards immunopurified Shc. We have previously demonstrated that insulin induces Shc tyrosine phosphorylation in rat tissues, and a kinase activity of the insulin receptor towards Shc was also suggested (15). The results presented here clearly demonstrate that the insulin receptor is able to induce tyrosine phosphorylation of immunopurified Shc.

It is interesting to note that besides the insulin receptor and Shc, that was added to the reaction, two other bands appeared and became phosphorylated after the addition of ATP. By immunoblotting with specific antibodies it was previously demonstrated that these bands correspond to IRS-1 and IRS-2 which probably were bound to the insulin receptor after insulin stimulation (15).

In summary, we have demonstrated that after an infusion of very low doses of insulin in the intact rat, it is possible to demonstrate that the insulin receptor has tyrosine kinase activity toward immunopurified Shc.

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Publicação 3

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Insulin-induced tyrosine phosphorylation of Shc in liver, muscle and adipose tissue of insulin resistant rats

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Abstract

Insulin stimulates rapid tyrosine phosphorylation of the protein Shc, which subsequently binds to Grb2, resulting in the activation of a complex mitogenic signaling network. In this study, we examined the levels of Shc protein, its phosphorylation state and Shc–Grb2 association in liver, muscle and adipose tissue before and after insulin administration in three animal models of insulin resistance (chronic dexamethasone treatment, 72-h starvation and aging). There were no differences in Shc protein expression between tissues from control and insulin resistant animals. In fasted hypoinsulinemic rats, there was a decrease in insulin-induced Shc phosphorylation in liver and adipose tissue. However, a significant increase in Shc phosphorylation was observed in liver and muscle from dexamethasone-treated hyperinsulinemic rats and in liver, muscle and adipose tissue of hyperinsulinemic 20-month-old rats. Alterations in Shc phosphorylation correlated well with the level of Shc–Grb2 association. These results indicate that Shc tyrosyl phosphorylation and Shc–Grb2 association are regulated in the different types of insulin resistance and that this regulation is apparently related to the animals' plasma insulin levels. The Shc–Grb2 association is directly related to the insulin-induced tyrosyl phosphorylation of Shc.

Keywords: Diabetes; Fasting; p52Shc; Shc–Grb 2 association; Hypoinsulinemia; Hyperinsulinemia

1. Introduction

Upon binding insulin, the insulin receptor undergoes autophosphorylation at tyrosine residues, resulting in increased kinase activity that leads to the phosphorylation of other intracellular proteins (White and Kahn, 1994). One of these phosphorylated proteins, insulin receptor substrate 1 (IRS-1), is critical for the mitogenic and metabolic effects of insulin (Waters et al., 1993). The Shc protooncogene product also appears to be phosphorylated on tyrosine in response to insulin (Pronk et al., 1993; Giorgetti et al., 1994). The phosphorylation of Shc leads to its association with Grb2, an adapter protein containing both SH2 and SH3 domains (Lowestein et al., 1992; Rozakis-Adock et al., 1992). Shc–Grb2 association is upstream to the activation of an important signaling pathway leading ultimately to the stimulation of mitogen-activated protein kinase (MAPK) (Pelicci et al., 1992; Egan et al., 1993; Sasaoka et al., 1994). We have recently reported that insulin can induce time- and dose-dependent Shc tyrosine phosphorylation and association with Grb2 in rat tissues (Páez-Espinosa et al., 1998).

Previous studies have demonstrated that in insulin resistant rats, the insulin receptor, its substrate IRS-1 and its association with phosphatidylinositol 3-kinase (PI 3-kinase) are regulated in a tissue-specific manner, and that a reduction in the level of IRS-1 tyrosine phosphorylation may play a role in the resistance to this hormone (Saad et al., 1992). Also, it was recently suggested that fatty acid-induced insulin resistance is associated with a decrease in insulin-stimulated MAP kinase (Usui et al., 1997). In addition, TNF-α and hyperglycemia regulate Shc phosphorylation in cultured

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cells (Kroder et al., 1996), which suggests that Shc may be involved in the molecular mechanisms of insulin resistance.

To further understand the molecular events involved in insulin-regulated intracellular pathways and to examine the interactions between Shc and the mechanisms of insulin resistance in vivo, we have investigated the influence of three insulin-resistant states (chronic dexamethasone treatment, 72-h fasting and aging) on the level of tyrosine phosphorylation of the protein p52Shc after stimulation with insulin, as well as the interaction between Shc and Grb2 in rat liver, muscle and adipose tissue.

2. Materials and methods

2.1. Materials

The reagents and apparatus for SDS–PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA). Tris-hydroxymethylamino methane (Tris), phenylmethylsulfonylfluoride (PSMF), aprotinin, silicone, dithiothreitol and streptozotocin (*N*-[methylnitrosocarbamoyl]-D-glucosamine) were obtained from Sigma Chemical (St. Louis, MO). Sodium amobarbital was purchased from Eli Lilly (Indianapolis, IN), and regular highly purified insulin (Iolin R) was from Biobrás (São Paulo, Brazil). Protein A-Sepharose 6 MB was purchased from Pharmacia (Uppsala, Sweden). [^{125}I]-Protein A was obtained from Amersham (Aylesbury, UK) and nitrocellulose membrane (BA85; 0.2 μm) was obtained from Schleicher and Schuell (Keene, NH). Monoclonal antiphosphotyrosine antibodies (100 μg/ml), anti-Shc (antibody against three Shc isoforms, p46, p52 and p66) and anti-GRB2 rabbit polyclonal antibodies (100 μg/ml) were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Animals

Male Wistar rats 8–10 weeks old provided by the university's Animal Breeding Center were fed standard rodent chow and water ad libitum. Food was withdrawn 12–14 h before the experiments with old and dexamethasone-treated rats and their respective controls. For fasting experiments, the rats were fasted for 72 h, while their matching controls were not fasted. Chronic hypercortisolemia was induced with dexamethasone (1 mg/kg per day, ip, for 5 days). Twenty-month-old rats were used in the aging experiments and were compared with young (2-month-old) rats. The experiments were approved by the Ethics Committee of the State University of Campinas (UNICAMP).

2.3. Methods

The rats were anesthetized with sodium thiopental (100 μg/kg body weight, ip) and used 10–15 min later, as soon as anesthesia was assured by the loss of pedal and corneal reflexes. Blood samples for their glucose and insulin levels were collected from a caudal vein and assayed using a glucose dye test (Labtest, Brazil) and a standard radioimunoassay, respectively.

The abdominal cavity was then opened, the portal vein exposed and 6 μg of insulin was injected. This dose ensured a plasma insulin level at the order of 100 times greater than the post-prandial level in control rats. Samples of liver, skeletal muscle and adipose tissue were collected 5 min after insulin infusion. The tissues were minced coarsely and homogenized immediately in approximately 10 volumes of solubilization buffer A at 4°C, using a Polytron PTA 20S homogenizer (Brinkmann Instruments, model PT 10/35), operated at maximum speed for 30 s. Buffer A (for solubilization) consisted of 1% Triton X-100, 50 mM Hepes (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PSMF and 0.1 mg of aprotinin/ml.

The tissues were extracted in an identical fashion, for each condition studied. The extracts were centrifuged at 30,000 × *g* in a Beckman 70.1 Ti rotor at 4°C for 20 min to remove insoluble material. Protein quantification done by the biuret dye method, and equal amounts of protein were used for immunoprecipitation with 10 μl of polyclonal anti-Shc antibody. The immune complexes were precipitated with protein A-Sepharose 6MB and were washed three times with 50 mM Tris (pH 7.4) containing 2 mM sodium vanadate, and 0.1% Triton X-100.

2.4. Immunoblotting

After washing, the immunoprecipitate obtained as described above was resuspended in Laemmli sample buffer (Laemmli, 1970) containing 100 mM dithiothreitol and was heated in a boiling water bath for 5 min. The samples were run in 10% polyacrylamide gels containing SDS using a Bio-Rad miniature slab gel apparatus. Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as previously described (Towbin et al., 1979), except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high molecular mass proteins. Nonspecific protein binding to the nitrocellulose was reduced by pre-incubating the filter overnight at 4°C in blocking buffer (5% non-fat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The prestained molecular mass standards used were phosphorylase B (105 kDa), bovine serum albu-

min (82 kDa), ovalbumin (49 kDa), carbonic anhydrase (33.3 kDa), soybean trypsin inhibitor (28 kDa) and lysozyme (19 kDa). The nitrocellulose filter was then incubated for 4 h at 22°C with anti-phosphotyrosine antibody, anti-Shc antibody or anti-Grb2 antibody (40 ng/ml), diluted in blocking buffer (3% non-fat dry milk) and then washed for 30 min in 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 1 h at 22°C and washed again. [^{125} I]-Protein A bound to the antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at −70°C for 12–48 h. Band intensities were quantified by optical densitometry (Hoefer Scientific Instruments, CA; model GS 300) of the developed autoradiographs.

Extensive optimization procedures were performed to confirm ideal time and doses for the normal rats, used as controls in these experiments (2 months old and mean body weight of 200 g)(Páez-Espinosa et al., 1998). In addition, assays were performed to ensure autoradiographic readings in the linear range.

2.5. Statistical analysis

The experiments were always performed by studying chronic dexamethasone-treated, fasted or aging rats in parallel with a control group. Comparisons between control and dexamethasone-treated, fed (0 h) or fasted (72 h) rats and between young and aging rats, were done using Student's unpaired *t*-test. Densitometric values are expressed as a percentage of the values for the insulin-stimulated control (100%) of each experimental group. The level of significance used was $P < 0.05$.

3. Results

3.1. Characteristics of dexamethasone, fasted and 20-month-old rats

As described previously, dexamethasone induced a state of insulin resistance characterized by a two-fold increase in blood glucose and serum insulin levels (Haber and Weinstein, 1992). In addition, dexamethasone-treated rats lost or failed to gain weight, during the 5 days of treatment. Twenty-month-old rats had elevated body weights and serum insulin levels compared with the 2-month-old controls. There were no significant differences in blood glucose levels. Fasting for 72 h resulted in a significant loss in body weight, and a reduction in blood glucose levels and serum insulin levels compared to fed rats (Table 1).

3.2. Shc protein expression and phosphorylation

Shc protein expression in liver, muscle and adipose tissue of dexamethasone-treated, 20-month-old, and 72-h-fasted rats was not significantly different from that seen in the respective controls, before and after insulin stimulation (data not shown). The predominant isoform phosphorylated in muscle was p52Shc, whereas in liver and fat, p52Shc was the only phosphorylated isoform detected.

3.3. Effect of dexamethasone on Shc phosphorylation and Shc–Grb2 association

Basal tyrosyl phosphorylation of hepatic Shc from rats treated chronically with dexamethasone increased by 25% ($P < 0.02$), and was further potentiated by insulin (control 100 ± 9% vs. dexamethasone 164 ±

Table 1
Body weight and blood glucose and insulin serum levels in the rats studied[a]

Groups	N	Body weight (g)	Plasma glucose (mg%)	Plasma insulin (μU/ml)
Control	12	238 ± 47	122 ± 6	29 ± 4
Dexamethasone	12	202 ± 39*	224 ± 17**	46 ± 4***
Fed	12	211 ± 62	165 ± 8	38 ± 4
Fasted	12	179 ± 51****	74 ± 2†	13 ± 4‡
2-Month-old	12	157 ± 13	123 ± 5	29 ± 4
20-Month-old	12	401 ± 148††	124 ± 11	44 ± 5‡‡

[a] The data are expressed as the mean ± S.E.M.
* $P < 0.005$ compared to the control group.
** $P < 0.001$ compared to the control group.
*** $P < 0.005$ compared to the control group.
**** $P < 0.005$ compared to the fed group.
† $P < 0.001$ compared to the fed group.
‡ $P < 0.005$ compared to the fed group.
†† $P < 0.005$ compared to the 2-month-old group.
‡‡ $P < 0.05$ compared to the 2-month-old group.

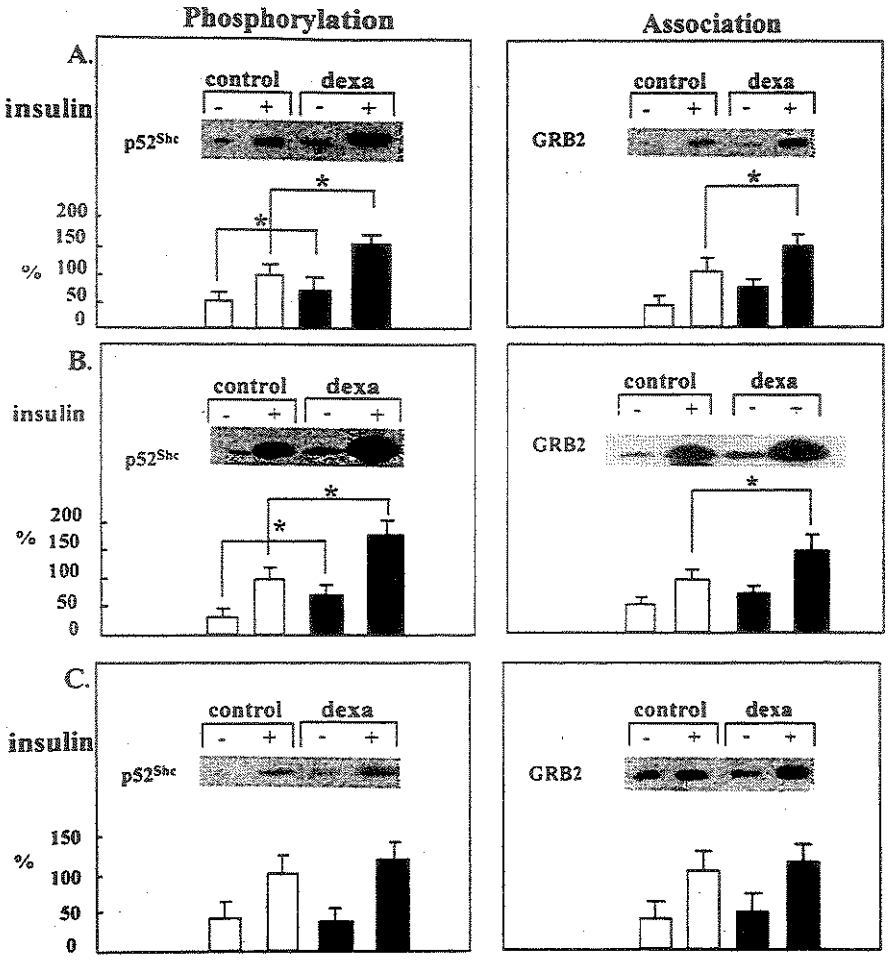

Fig. 1. Insulin stimulates tyrosine phosphorylation of Shc and Shc/Grb2 association in tissues from control and dexamethasone-treated rats. The proteins were isolated and processed as described in Section 2.3 and were detected with anti-Shc/antiphosphotyrosine antibodies (left panels) and anti-Shc/anti-Grb2 antibodies (right panels). (A) Liver, (B) muscle, and (C) adipose tissue. Shc tyrosyl phosphorylation and Shc–Grb2 association were determined by scanning densitometry of 4, 5 and 7 experiments for muscle, liver and adipose tissue respectively, and are shown by bars. The values represent the mean ± S.E.M. and are expressed as a percentage of the insulin-stimulated control (100%). * $P \leq 0.05$.

10%; $P < 0.03$) (Fig. 1A, left panel). A significant increase in Shc–Grb2 association was also observed in liver from dexamethasone-treated rats after the administration of insulin (control 100 ± 16% vs. dexamethasone 153 ± 13%; $P < 0.04$) (Fig. 1A, right panel). In muscle of dexamethasone-treated rats, the basal level of Shc tyrosyl phosphorylation was higher than in the controls ($P < 0.02$), and insulin-induced Shc tyrosyl phosphorylation was further increased in dexamethasone-treated rats (control 100 ± 9% vs. 181 ± 13%; $P < 0.03$) (Fig. 1B, left panel). This increase was accompanied by a significant rise in Shc–Grb2 association (control 100 ± 6% vs. 152 ± 12% for insulin-stimulated Shc–Grb2 association, $P < 0.04$) (Fig. 1B, right panel). Basal and insulin-induced Shc tyrosyl phosphorylation levels, as well as Shc–Grb2 association, were similar in the adipose tissue of dexamethasone-treated and control rats (Fig. 1C, left and right panels).

3.4. Effect of fasting on insulin-induced Shc tyrosine phosphorylation and Shc–Grb2 association

Contrary to that observed for tissues from dexamethasone-treated rats, there was a significant decrease in the level of insulin-induced Shc tyrosine phosphorylation (fed 100 ± 6% vs. fasted 49 ± 9%; $P < 0.05$) (Fig. 2A, left panel) and in Shc–Grb2 association in the liver of 72-h-fasted rats compared to the controls (fed 100 ± 6% vs. fasted 51 ± 16%; $P < 0.05$) (Fig. 2A, right panel). These observations suggested a clear relationship between Shc phosphorylation levels and Shc–Grb2 association following stimulation with insulin. There was no significant increase in Shc protein phosphorylation (fed 100 ± 10% vs. fasted 107 ± 15%) and Shc–Grb2 association (control 100 ± 22% vs. fasted 112 ± 14%) in the muscle of 72-h-fasted rats (Fig. 2B, left and right panel). There was a significant decrease in the insulin-induced Shc phosphorylation levels (fed 100 ± 6% vs. fasted 38 ± 8%; $P < 0.001$) (Fig. 2C, left

panel) and in Shc–Grb2 co-immunoprecipitation in adipose tissue from fasted compared to fed rats (fed 100 ± 21% vs. fasted 29 ± 2%; $P < 0.05$) (Fig. 2C, right panel).

3.5. Effect of aging on Shc phosphorylation and Shc–Grb2 association

The basal phosphorylation of Shc in liver from 20-month-old rats was higher than in 2-month-old rats ($P < 0.001$). The increase in Shc phosphorylation in liver after insulin infusion was greater in old rats (control 100 ± 15% vs. aging 158 ± 7%; $P < 0.02$) (Fig. 3A, left panel) and was followed by an increase in Shc–Grb2 association (control 100 ± 5% vs. aging 166 ± 1%, $P < 0.05$) (Fig. 3A, right panel). Fig. 3B (left panel) shows that there was an increase in the basal (control 48 ± 4% vs. aging 86 ± 10%, $P < 0.01$) and insulin-induced (control 100 ± 7% vs. aging 164 ± 16%, $P < 0.001$) Shc tyrosyl phosphorylation in muscle from aging rats. After insulin injection, there was also a greater increase in Shc–Grb2 association in muscle of aging rats (control 100 ± 5% vs. aging 159 ± 10%, $P < 0.05$) (Fig. 3B, right panel). The insulin-induced tyrosyl phosphorylation of Shc in adipose tissue from aging rats was significantly greater than in young controls (young 100 ± 12% vs. 20-month-old 176 ± 2%; $P < 0.05$) (Fig. 3C, left panel). In adipose tissue from aging rats, basal (control 50 ± 5% vs. aging 92 ± 14%, $P < 0.01$), and insulin-stimulated (control 100 ± 9% vs. aging 156 ± 7%, $P < 0.001$) Shc–Grb2 association was increased (Fig. 3B, left panel).

4. Discussion

Insulin resistance is defined as a subnormal biological response to a given concentration of insulin. Although

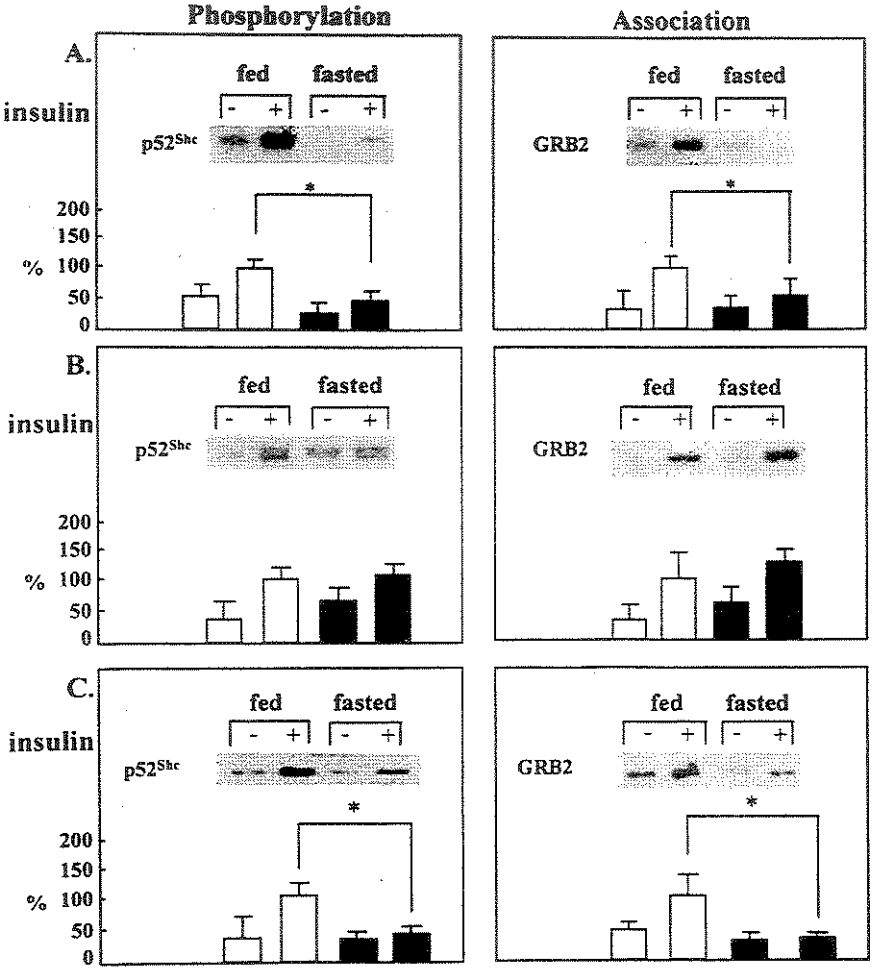

Fig. 2. Insulin stimulates tyrosine phosphorylation of Shc and Shc/Grb2 association in tissues from fed and fasted rats. The proteins were isolated and processed as described in Section 2.3 and were detected with anti-Shc/antiphosphotyrosine antibodies (left panels) and anti-Shc/anti-Grb2 antibodies (right panels). (A) Liver, (B) muscle, and (C) adipose tissue. Shc tyrosyl phosphorylation and Shc–Grb2 association were determined by scanning densitometry of 5, 6 and 8 experiments for liver, adipose tissue and muscle, respectively, and are shown by bars. The values represent the mean ± S.E.M. and are expressed as a percentage of the insulin-stimulated control (100%). * $P \leq 0.05$.

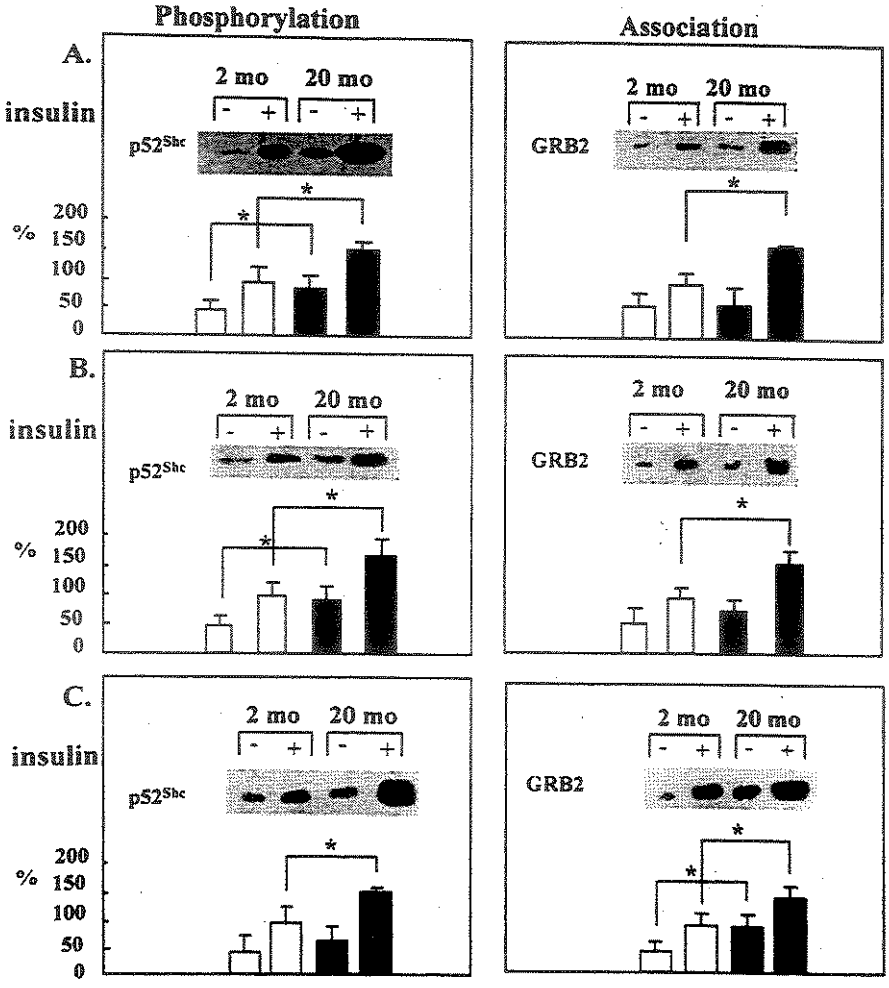

Fig. 3. Insulin stimulates tyrosine phosphorylation of Shc and Shc/Grb2 association in tissues from young and old rats. The proteins were isolated and processed as described in Section 2.3 and were detected with anti-Shc/antiphosphotyrosine antibodies (left panels) and anti-Shc/anti-Grb2 antibodies (right panels). (A) Liver, (B) muscle, and (C) adipose tissue. Shc tyrosyl phosphorylation and Shc–Grb2 association were determined by scanning densitometry of 5 experiments for each tissue, and are shown by bars. The values represent the mean ± S.E.M. and are expressed as a percentage of the insulin-stimulated control (100%). * $P \leq 0.05$.

the effects of insulin are pleiotropic, insulin resistance typically refers to the action of insulin on glucose homeostasis. The molecular mechanisms responsible for selective resistance are not known and may be reflected in other pathways. In animal models of insulin resistance, there is evidence that regulation of IRS-1 may have a role in the modulation of glucose homeostasis (Saad et al., 1992). In the present study, we investigated the regulation of Shc, which is also an insulin receptor substrate, in insulin sensitive tissues of two animal models of insulin resistance with hyperinsulinemia (aging and dexamethasone-treated rats), and in insulin resistance with hypoinsulinemia (72-h-fasted rats).

Dexamethasone induces insulin resistance, as shown by the altered glucose metabolism in peripheral tissues and liver, decreased insulin-stimulated amino acid uptake in muscle, and decreased lipogenesis in adipocytes (Rannels and Jefferson 1980; De Pirro et al., 1981; Amatruda et al., 1985; Haber and Weinstein 1992; Saad et al., 1997). In contrast, dexamethasone enhances the insulin-mediated stimulation of DNA synthesis (Germinario and McQuillan 1985). Under conditions of hypercortisolisemia similar to those used in this study, dexamethasone has been reported to reduce insulin-induced insulin receptor autophosphorylation in the liver and muscle of rats in vivo and also IRS-1 activation of PI 3-kinase in these tissues (Saad et al., 1993). Such alterations in IRS-1/PI 3-kinase activation may play a role in the insulin resistance of dexamethasone-treated rats considering the correlation between PI3-kinase activity, glucose transport and glycogen synthesis (Cheatham et al., 1994; Sanches-Margalet et al., 1994; Welsh et al., 1994; Haruta et al., 1995), but does not explain the signaling mechanisms that mediate the ability of dexamethasone to enhance insulin-simulated DNA synthesis.

Tyrosine phosphorylation of Shc proteins may be involved in the control of cell proliferation and/or

transformation. The microinjection of antibodies to Shc proteins into rat-1 fibroblasts overexpressing human insulin receptors inhibits DNA synthesis induced by insulin, indicating that Shc is an important component of a mitogenic insulin signal transduction pathway (Sasaoka et al., 1994). Studies aimed at examining the effects of insulin on Shc-activated MAP kinase activity and glucose metabolism in insulin-sensitive cell lines have successfully dissociated the activation of MAP kinase by Shc from the stimulation of glucose transport and glycogen synthase activity (Sasaoka et al., 1994; Lazar et al., 1995).

The present study has shown that chronic corticosteroid treatment of rats significantly increased Shc tyrosine phosphorylation levels and the association between Shc–Grb2 following stimulation with insulin, without changing hepatic and muscle Shc protein expression. The increase in Shc tyrosine phosphorylation and association with Grb2 after the injection of insulin in dexamethasone-treated rats may represent a pathway leading to an enhanced mitogenic response. In contrast to liver and muscle, chronic treatment with dexamethasone did not affect the insulin-stimulated Shc phosphorylation or the level of Shc–Grb2 association in adipose tissue. These findings suggest a tissue-specific regulation of insulin-induced Shc tyrosine phosphorylation in dexamethasone-treated rats.

Prolonged fasting in rats is characterized by insulin deficiency and insulin resistance (Penicaud et al., 1985; Balage et al., 1990). Both peripheral and hepatic insulin resistance to glucose metabolism have been observed in vivo (Ridray et al., 1991). Fasting is associated with decreased DNA synthesis in various tissues (Penicaud et al., 1985; Ridray et al., 1991). Our results showed a significant decrease in insulin-induced Shc protein phosphorylation and Shc–Grb2 association in liver and adipose tissue, but not in muscle, of 72-h-fasted rats. The regulation of Shc in insulin-sensitive tissues of fasted rats was completely different from that of IRS-1. In liver and muscle of 72-h-fasted rats, there was an increase in insulin-induced IRS-1 tyrosine phosphorylation (Saad et al., 1992). These results suggest that the administration of insulin to 72-h-fasted rats leads to a shift in the early steps of insulin signal transduction, which consequently increases IRS-1 tyrosine phosphorylation and decreases (liver and adipocytes) or does not change (muscle) the level of Shc tyrosine phosphorylation. Although this regulation may not explain the insulin resistance seen in fasting, it may represent an important regulatory mechanism for directing the action of insulin to a pathway with a definite role in glucose homeostasis. The decreased insulin-induced Shc tyrosine phosphorylation in the liver of fasted rats is consistent with the reduced DNA synthesis observed in these animals (Ridray et al., 1991).

Aging has been shown to impair insulin action on glucose metabolism. There is an impaired insulin-stimulated glucose uptake and a decrease in the inhibition of hepatic glucose production by physiological concentrations of insulin (Kono et al., 1990; Barnard et al., 1992). We have reported that in old rats there is a decrease in IRS-1 and IRS-2 tyrosine phosphorylation and in the association with PI3-kinase, and that these events may contribute to the reduced insulin responsiveness in these animals (Carvalho et al., 1996). In this study, we observed an increase in Shc tyrosine phosphorylation and in the association with Grb2 in the three tissues examined. The correlation between these data and the effects of insulin cannot be established here since the action of insulin on DNA synthesis in the tissues of old rats was not investigated.

Since Shc tyrosine phosphorylation and Shc–Grb2 association increased in two out of the three situations of insulin resistance studied,, and since previous reports have shown a dissociation between the insulin-induced Shc pathway and glucose metabolism (Sasaoka et al., 1994; Lazar et al., 1995), we suggest that Shc may not have any role in contributing to these forms of insulin resistance. The interesting point in these three animal models of insulin resistance is the correlation between insulin levels and the regulation of Shc tyrosine phosphorylation and Shc–Grb2 association. In animal models of insulin resistance with hyperinsulinemia, there was an increase in Shc phosphorylation and Grb2 association, and in the hypoinsulinemic model there was a decrease in this pathway. This regulation is contrary to the regulation demonstrated for IRS-1 in the same animal models (Saad et al., 1992). This implies that in most tissues of the above three models of insulin resistance there was a clear dissociation between Shc tyrosine phosphorylation and IRS-1 tyrosine phosphorylation. A similar dissociation has been described in cells transfected with a mutant insulin receptor (Ouwens et al., 1994), and in cells treated with dexamethasone (Giorgino and Smith, 1995) or wortmannin (Li and Goldstein, 1996).

The basis for this dissociation is unclear, although changes in the total tissue content of Shc and/or IRS-1 are apparently not involved. The amino acid sequence NPXY has been shown to be a consensus binding site for Shc (Gustafson et al., 1995). Interestingly, this sequence is present in the juxtamembrane region of the insulin receptor and Tyr960 in this motif is required for interaction with IRS-1 (Ward et al., 1996). This observation is consistent with the suggestion that both Shc and IRS-1 compete for this tyrosine residue in their association with the phosphorylated receptor (Wolf et al., 1995; Thirone et al., 1998).

Although IRS-1 is best known as a substrate for tyrosine phosphorylation, it is essentially a phosphoserine-containing protein. IRS-1 contains over 30 potential

serine/threonine phosphorylation sites in motifs recognized by various kinases such as PKC, MAP kinases and cyclic AMP- and cyclic GMP-dependent protein kinases (Sun et al., 1991; Wang et al., 1993; Van der Kuur et al., 1995; Myers and White, 1996). Serine phosphorylation also plays a role in the regulation of IRS-1 signaling. Okadaic acid, a serine phosphatase inhibitor, increases the serine phosphorylation of IRS-1 in 3T3-LI adipocytes which in turn appears to inhibit insulin-stimulated tyrosine phosphorylation (Tanti et al., 1994).

It is possible that serine phosphorylation of IRS-1 induced by PKC in hyperinsulinemic situations reduces the substrate's affinity for the insulin receptor, thereby allowing Shc to be more competitive. In support of this hypothesis, previous work has demonstrated that insulin activates PKC in various rat tissues (Cooper et al., 1990; Arnold et al., 1993; Nivet et al., 1993). In addition, Li and Goldstein (1996) have recently shown that reducing IRS-1 serine phosphorylation results in increased IRS-1 tyrosine phosphorylation and decreased Shc tyrosine phosphorylation.

We have shown that chronic dexamethasone treatment, fasting and aging can modulate insulin-induced Shc tyrosine phosphorylation and Shc–Grb2 association, and that this modulation is closely correlated with the plasma insulin levels. In summary, our results demonstrated that in fasted hypoinsulinemic rats, there was a decrease in insulin-induced Shc phosphorylation in liver and adipose tissue. However, a significant increase in Shc phosphorylation was observed in liver and muscle from dexamethasone-treated hyperinsulinemic rats and in liver, muscle and adipose tissue of hyperinsulinemic 20-month-old rats. Alterations in Shc phosphorylation correlated well with the level of Shc–Grb2 association and was different from that previously described for IRS-1 in the same animal models (Saad et al., 1992, Saad et al., 1993).

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Publicação 4

Regulation of insulin-stimulated tyrosine phosphorylation of Shc and IRS-1 in the muscle of rats: effect of growth hormone and epinephrine

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Abstract **Insulin receptor substrate-1 (IRS-1) and Shc protein have the same binding site at the insulin receptor and compete in their association with the phosphorylated receptor. The present study demonstrates that a decrease in the level of muscle insulin receptor phosphorylation induced by chronic growth hormone (GH) treatment or acute epinephrine infusion is accompanied by a reduction in the level of IRS-1 phosphorylation and in the association with phosphatidylinositol 3-kinase. In contrast, no change is observed in insulin-stimulated Shc tyrosine phosphorylation, or in the association of this substrate with Grb2. These data suggest that a reduction in insulin receptor phosphorylation may affect post-receptor processes differentially by preserving the phosphorylation of some substrates and pathways, but not of others.**

Key words: Insulin signal transduction; Growth hormone; Epinephrine

1. Introduction

The insulin receptor is the principal mediator of insulin action in cellular mitogenic and metabolic processes. The insulin receptor β-subunit, which contains an intrinsic tyrosine kinase, undergoes tyrosyl autophosphorylation and is activated in response to insulin binding to the extracellular α-subunit [1,2]. This interaction further enhances the tyrosine kinase activity of the receptor towards other intermediate molecules, including insulin receptor substrate-1 (IRS-1), IRS-2 and Shc [3–9]. These molecules, rather than the insulin receptor itself, then couple to a downstream signaling pathway by serving as binding sites for SRO homology 2 (SH2) domain-containing signaling molecules [10]. IRS-1 binds to the 85 kDa subunit of phosphatidylinositol 3-kinase (PI 3-kinase), Grb2 and other SH2-containing proteins [11]. Shc protein has been shown to directly induce the association with Grb2 [12]. Recent studies indicate that Shc and IRS-1 have the same binding site at the insulin receptor, a finding consistent with the suggestion that both Shc and IRS-1 compete in their association with the phosphorylated receptor. As yet, there has been no comparison of insulin-induced IRS-1 and Shc tyrosine phosphorylation in situations of insulin resistance accompanied by a reduction in insulin receptor phosphorylation.

We have demonstrated elsewhere that there are common molecular events in the muscle of rats treated chronically with growth hormone (GH) [13] or acutely with epinephrine [14], including a decrease in insulin-induced insulin receptor and IRS-1 tyrosine phosphorylation and in the association of the latter with PI 3-kinase. These reductions correlate with the insulin resistance and reduced glucose uptake described in the muscle of these animals [15,16]. In this study, we investigated Shc and IRS-1 tyrosine phosphorylation in the muscle of the above two animal models of insulin resistance, both of which show reduced insulin-induced insulin receptor autophosphorylation.

2. Materials and methods

2.1. Materials

The reagents and apparatus for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad (Richmond, CA, USA). Tris[hydroxymethyl]amino-methane (Tris), phenylmethylsulfonyl fluoride (PMSF), aprotinin, silicone, and dithiothreitol (DTT) were from Sigma Chemical Co. (St. Louis, MO, USA). Sodium amobarbital and human recombinant insulin (Humulin R) were from Lilly. Human biosynthetic GH (Norditropin) was purchased from Novo Nordisk (Bagsvaerd, Denmark). [^{125}I]Protein A was from Amersham (Amersham, UK) and protein A Sepharose 6 MB from Pharmacia (Uppsala, Sweden). Nitrocellulose (BA85, 0.2 μm) was from Schleicher&Schuell. Male Wistar rats were from the UNICAMP Central Animal Breeding Center. Monoclonal antiphosphotyrosine antibody (1 μg/ml), polyclonal anti-Shc, polyclonal anti-Grb2 and polyclonal anti-PI 3-kinase (p85) antibody were from Upstate Biotechnology Incorporated (UBI, Lake Placid, NY, USA). Anti-IRS-1 antibodies were raised in rabbits using a synthetic peptide (Pep 80) derived from the amino acid sequence (YIPGATMGTSPALTGDEAA) corresponding to residues 489–507 of the rat protein and purified by affinity chromatography on a column prepared by coupling the synthetic peptide to Affi-Gel 10 (Bio-Rad Laboratories) as previously described [17]. Anti-insulin receptor antibody was raised in rabbits using a synthetic peptide derived from the amino acid sequence (KKNGRILPRSNPS) corresponding to the C-terminus of the rat protein [17].

2.2. Animals

Male Wistar rats, 6 weeks old, were divided into two groups and the studies were performed in parallel using the control and treated rats. All groups received standard rodent chow and water ad libitum. An excess of GH was induced by the s.c. injection of human GH at a dose of 1 mg twice a day for 5 days. The control group received the equivalent amount of normal saline (0.9% NaCl). The experiments were performed on the morning of the fifth day. In another series of experiments, anesthetized rats were injected i.p. with epinephrine (25 μg/100 g body weight) or an equal volume of saline (control group) and the animals used 5 min later. In all groups, the rats were fasted for 12 h before being used as described below.

2.3. Methods

Rats were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.), and were used 10–15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the vena cava exposed, and 6 μg of insulin diluted in normal saline (0.9% NaCl) was injected. Ninety seconds later, the hindlimb muscle was removed, minced coarsely and homogenized immediately in extraction buffer (1% Triton X-100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and

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0.1 mg of aprotinin/ml) at 4°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 s. For immunoprecipitation with anti-Shc, hindlimb muscle was removed 5 min after the injection of insulin.

The extracts were centrifuged at 15000 rpm and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA, USA) for 45 min to remove insoluble material, and the resulting supernatant was used for immunoprecipitation with anti-IRS-1, anti-Shc or anti-IR (insulin receptor) antibody and protein A Sepharose 6 MB.

2.4. Protein analysis by immunoblotting

Proteins were denatured by boiling in Laemmli [18] sample buffer containing 100 mM DTT, run on SDS-PAGE and transferred to nitrocellulose membranes in Towbin [19] buffer containing 0.02% SDS and 20% methanol. The membranes were blocked, probed, and developed as described previously [20]. Blots were exposed to preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at −80°C for 12–48 h. Band intensities were quantitated by optical densitometry (Hoefer Scientific Instruments, San Francisco, CA; model GS 300) of the developed autoradiographs that were used at exposures in the linear range.

2.5. Statistical analysis

The experiments were performed by studying all groups of animals in parallel. For comparisons, Student's unpaired t-test was used as appropriate. The level of significance employed was $P < 0.05$.

3. Results

3.1. Animal characteristics

Seven animals from each group of rats underwent an insulin tolerance test. The glucose disappearance rate (K_{itt}) of the control group was 4.40 ± 0.39%/min. Chronic GH treatment and acute epinephrine treatment induced similar levels of insulin resistance, as reflected in their glucose disappearance rates which were 1.94 ± 0.81%/min and 1.89 ± 0.63%/min, respectively. Both of these values were significantly ($P < 0.05$) lower that the control K_{itt}.

3.2. Characteristics of insulin-stimulated insulin receptor, IRS-1 and Shc phosphorylation in the hindlimb muscle of GH-treated rats

There was no change in the insulin receptor level of muscle from rats treated with GH, as determined by immunoblotting with an antibody to the COOH-terminus of the insulin receptor (Fig. 1A). In muscle samples previously immunoprecipitated with anti-insulin receptor antibody and immunoblotted with antiphosphotyrosine antibody, there was a decrease to 43 ± 11% ($P < 0.05$, $n = 6$) in the insulin-stimulated phosphorylation of the 95 kDa β-subunit of the insulin receptor in GH-treated rats when compared to the controls (Fig. 1B).

Using a specific anti-peptide antibody against IRS-1, the level of this protein was found to be unchanged in the muscle of rats treated chronically with GH (Fig. 2A), although there were changes in the level of phosphorylation of this protein (Fig. 2B). Following the administration of insulin, the intensity of this band increased in both groups of rats. However, comparison of the bands stimulated by insulin revealed that the extent of IRS-1 phosphorylation was reduced to 38 ± 11% ($P < 0.05$, $n = 6$) in GH-treated rats compared to the controls. Previous studies [6,10,21–24] have suggested that there is a relatively stable, high affinity interaction between IRS-1 and the 85 kDa subunit of PI 3-kinase such that both proteins can be co-precipitated by antibodies to either protein. In muscle samples previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted for the 85 kDa subunit of PI 3-

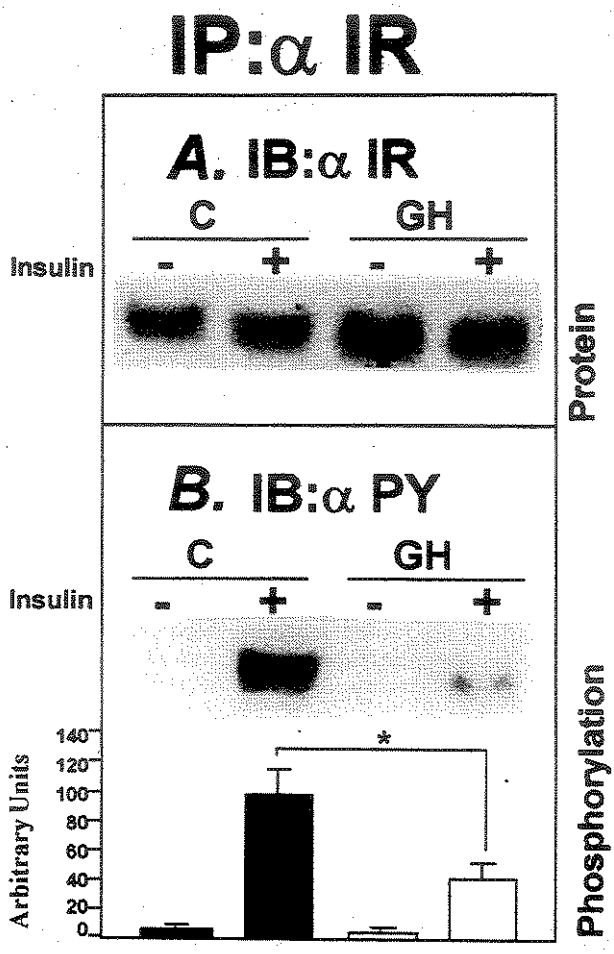

Fig. 1. Insulin-stimulated tyrosine phosphorylation of the insulin receptor in muscle from GH-treated rats. Saline (0.9%, lanes 1 and 3 or insulin 6 μg, lanes 2 and 4) was administered into the portal vein as a bolus injection and 90 s later the muscle was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-insulin receptor antibody and immunoblotted with the same antibody (A). The same samples were immunoprecipitated with anti-insulin receptor antibody and immunblotted with antiphosphotyrosine antibody. (B) Scanning densitometry of autoradiograms was performed in six experiments. The black bars represent the control group and the white bars represent the GH-treated group. The data are expressed as the mean ± S.E.M. *$P < 0.05$.

kinase (Fig. 2C), there was little basal PI 3-kinase immunoreactivity in the control and GH-treated rats. After stimulation with insulin, a band with the expected molecular mass (85 kDa) of the PI 3-kinase regulatory subunit was observed in anti-IRS-1 antibody immunoprecipitates of muscle from rats in both groups. This finding is consistent with a stable association between IRS-1 and PI 3-kinase. However, the amount of PI 3-kinase associated with IRS-1 was reduced to 45 ± 7% ($P < 0.05$, $n = 4$) in GH-treated rats, thus suggesting a diminished association between IRS-1 and PI 3-kinase. This reduction was probably a consequence of reduced IRS-1 tyrosyl phosphorylation since the level of PI 3-kinase did not change after GH treatment.

Chronic GH treatment did not significantly change the level of Shc protein (Fig. 2D). To define better the extent of Shc phosphorylation, we performed a Western blot analysis of

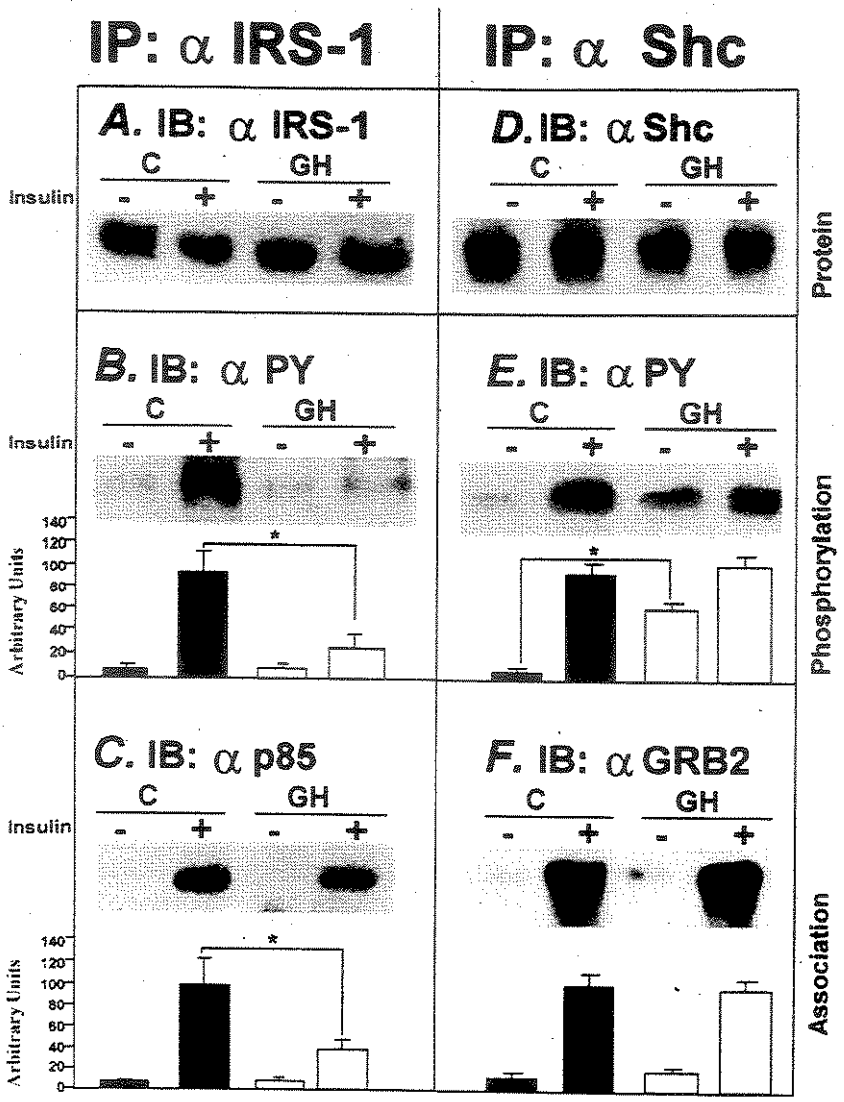

Fig. 2. Insulin-stimulated tyrosine phosphorylation of IRS-1 and Shc in intact muscle from GH-treated rats. Saline (0.9%, lanes 1 and 3 or insulin 6 μg, lanes 2 and 4) was administered into the portal vein as a bolus injection and 90 s later the muscle was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-IRS-1 and immunoblotted with the same antibody (A). The same samples were immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antiphosphotyrosine antibody (B). C: The immunoblotting results of PI 3-kinase in anti-IRS-1 immunoprecipitates from the muscle of control and GH-treated rats. Five minutes after the bolus injection of saline or insulin (6 μg), the muscle was excised and aliquots immunoprecipitated with anti-Shc antibody and blotted with the same antibody (D). The same samples were immunoprecipitated with anti-Shc antibody and blotted with antiphosphotyrosine antibody (E). F: The immunoblotting results of Grb2 in anti-Shc immunoprecipitates from the muscle of control and GH-treated rats. Scanning densitometry of autoradiograms was performed in six experiments. The black bars represent the control group and the white bars represent the GH-treated group. The data are expressed as the mean ± S.E.M. $^*P < 0.05$.

tyrosyl-phosphorylated proteins in anti-Shc immunoprecipitates before and after stimulation with insulin in both groups of animals. Basal Shc phosphorylation was higher in GH-treated rats (138 ± 7%, $P < 0.05$, $n = 4$) than in the control group. After stimulation with insulin, the intensity of this band increased in both groups of animals, although the extent of Shc phosphorylation in GH-treated rats was not significantly different from that in the controls (Fig. 2E). Since Shc can associate with Grb2, after insulin stimulation, blots with samples which had been previously immunoprecipitated with anti-Shc were incubated with anti-Grb2 and no change was observed in this association (Fig. 2F).

3.3. *Characteristics of insulin-stimulated insulin receptor, IRS-1 and Shc phosphorylation in the muscle of epinephrine-treated rats*

As in the rats treated with GH, acute epinephrine treatment did not significantly change the insulin receptor protein level (Fig. 3A). However, following stimulation with insulin, phosphorylation of the insulin receptor was reduced to 47 ± 4% ($P < 0.001$, $n = 6$) in epinephrine-treated rats compared to the controls (Fig. 3B).

Similarly, no significant change occurred in the level of IRS-1 protein in the muscle of rats treated with epinephrine when compared to the controls (Fig. 4A). To define better the

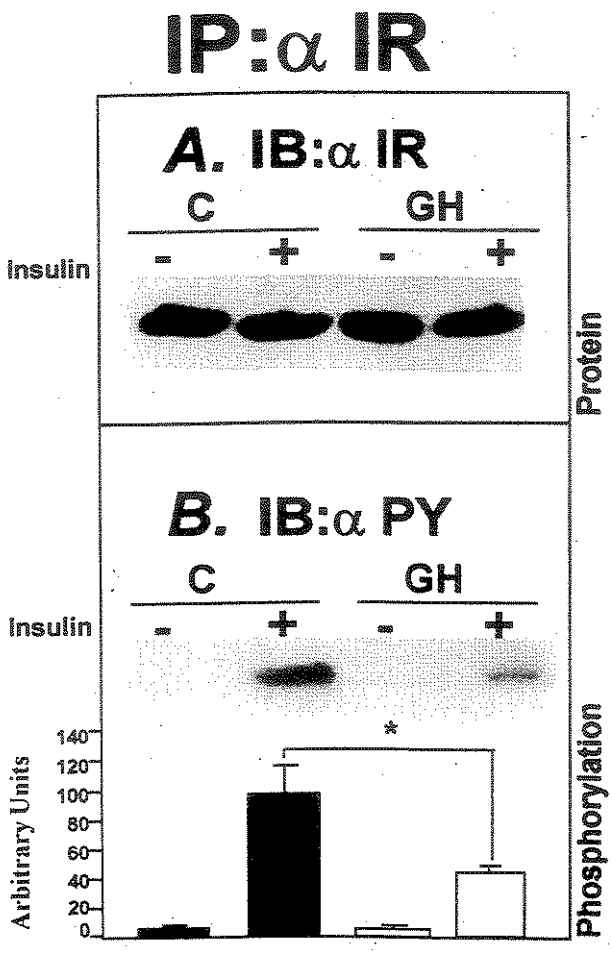

Fig. 3. Insulin-stimulated tyrosine phosphorylation of insulin receptor in intact muscle from epinephrine-treated rats. Saline (0.9%, lanes 1 and 3 or insulin 6 μg, lanes 2 and 4) was administered into the portal vein as a bolus injection and 90 s later the muscle was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-insulin receptor antibody and immunoblotted with the same antibody (A). The same samples were immunoprecipitated with anti-insulin receptor antibody and immunblotted with anti-phosphotyrosine antibody (B). Scanning densitometry of autoradiograms was performed in six experiments. The black bars represent the control group and the white bars represent the epinephrine-treated group. The data are expressed as the mean ± S.E.M. $^*P < 0.05$.

level of IRS-1 phosphorylation, we performed a Western blot analysis of the tyrosyl-phosphorylated proteins in anti-IRS-1 immunoprecipitates before and after stimulation with insulin in both groups of animals. Fig. 4B shows that there was a marked reduction to 53 ± 10% ($P < 0.001$, $n = 9$) in insulin-stimulated IRS-1 phosphorylation in the muscle of animals pretreated with epinephrine. To examine the association of the 85 kDa subunit of PI 3-kinase with IRS-1, blots of samples which had been previously immunoprecipitated with anti-IRS-1 antibodies were incubated with anti-PI 3-kinase antibody. As expected, in both groups an 85 kDa band was present in the IRS-1 immunoprecipitates after exposure to insulin and there was a decrease to 41 ± 8% ($P < 0.001$, $n = 4$) in the intensity of this band in epinephrine-treated rats (Fig. 4C).

Acute epinephrine treatment had no effect on the Shc protein level in muscle as determined by immunoblotting of cell lysates with anti-Shc antibody (Fig. 4D). In samples from muscle previously immunoprecipitated with anti-Shc antibody and immunoblotted with anti-phosphotyrosine antibody, there was no change in insulin-stimulated Shc tyrosine phosphorylation in epinephrine-treated rats when compared to the controls (Fig. 4E). Similarly, no change was observed in the association between Shc/Grb2 when rats treated with epinephrine were compared with controls (Fig. 4F).

4. Discussion

Following insulin stimulation, several proteins undergo tyrosine phosphorylation, including the β chain of the insulin receptor, IRS-1, IRS-2 and Shc proteins [25]. We analyzed the insulin-induced phosphorylation of these signaling intermediates in the muscle of rats treated chronically with GH and in rats receiving an acute infusion of epinephrine. Insulin resistance in these animals was demonstrated by a decrease in the glucose disappearance rate after insulin infusion [13]. We observed a reduction in insulin receptor and IRS-1 tyrosine phosphorylation and also in the PI 3-kinase association with IRS-1 in the muscle of both treated groups. These alterations in the three early steps of insulin action may explain some aspects of the insulin resistance observed in both of these models since these steps seems to have an important role in glucose homeostasis. Previous studies demonstrated that mice homozygous for target disruption of the IRS-1 gene were resistant to the glucose-lowering effects of insulin [5,6], and this correlated with a marked reduction in insulin-stimulated glucose transport in isolated adipocytes [6]. Distinct experimental approaches have also demonstrated a correlation between PI 3-kinase activity and glucose transport [26–28] and PI 3-kinase activity and glycogen metabolism [28]. Thus, it seems reasonable to speculate that the IRS-1/PI 3-kinase pathway may be linked to the activation of glucose transport and to glycogen synthesis in the muscle, and that a reduction in these associations in both models may have a role in the resulting insulin resistance.

Despite the reduction in insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1, no change was observed in the tyrosine phosphorylation of Shc. After insulin-induced Shc phosphorylation, Shc associates with the SH2 domain of the adapter protein Grb2 [29]. This association induces Grb2 to bind to the nucleotide exchange factor SOS, which in turn associates with and activates the GTP binding protein p21Ras. This pathway has been implicated in the mitogenic effects of insulin [12,30]. Although Ras is an upstream activator of the mitogen-associated protein kinase (MAPK) cascade, recent evidence has shown that stimulation of the MAPK pathway by insulin is not required for many of the metabolic activities of the hormone in cultured fat and muscle cells such as glucose uptake and glycogen synthesis [31]. These results are in accordance with our findings since in both animal models of insulin resistance we observed a marked decrease in the glucose disappearance rate that paralleled a decrease in IRS-1 phosphorylation and association with PI 3-kinase, but not Shc tyrosine phosphorylation. It is interesting that the 60% reduction in IRS-1/PI 3-kinase association in both models was quite similar to the 60% reduction in glucose disappearance rate observed in the two cases.

The dissociation of insulin-induced IRS-1 and Shc tyrosine phosphorylation has been described in cell lines with mutations at two tyrosine phosphorylation sites (Y1158, Y1162F,

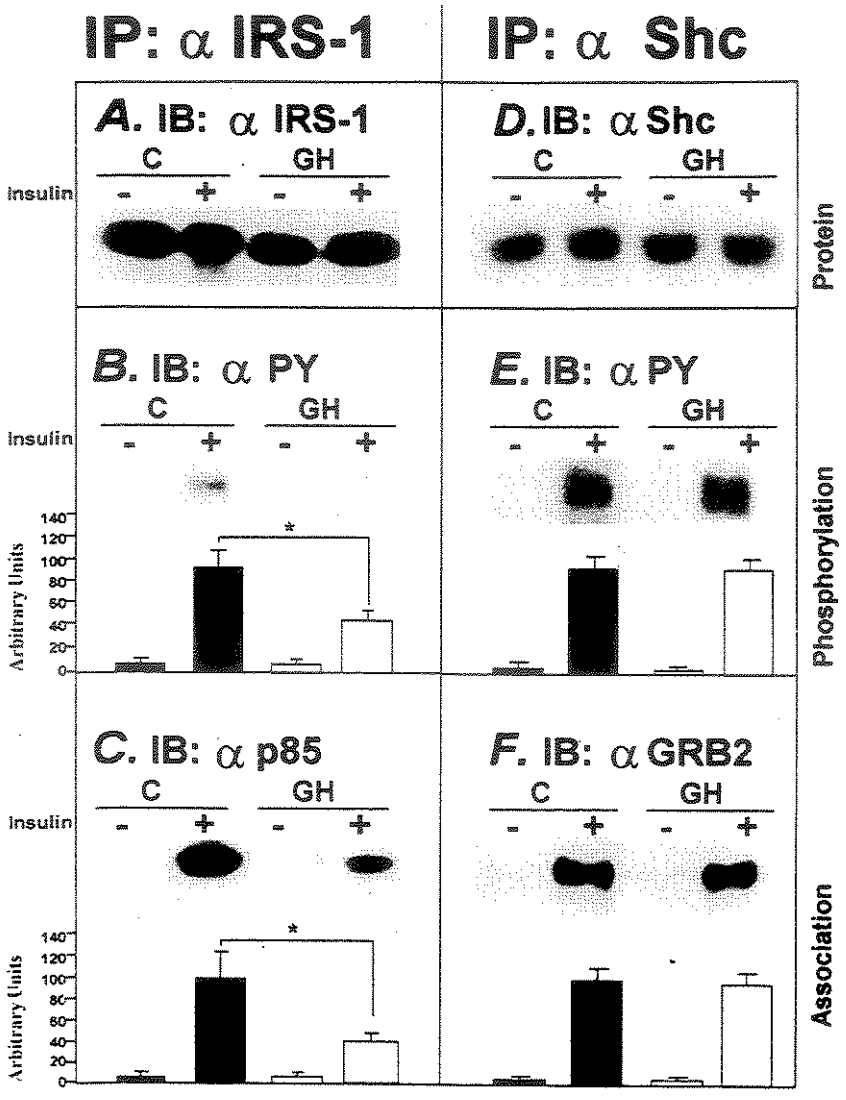

Fig. 4. Insulin-stimulated tyrosine phosphorylation of insulin receptor and Shc in intact muscle from epinephrine-treated rats. Saline (0.9%, lanes 1 and 3 or insulin 6 μg, lanes 2 and 4) was administered into the portal vein as a bolus injection and 90 s later the muscle was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-insulin IRS-1 and immunoblotted with the same antibody (A). The same samples were immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antiphosphotyrosine antibody (B). C: The immunoblotting results of PI 3-kinase in anti-IRS-1 immunoprecipitates from the muscle of control and GH-treated rats. Five minutes after the bolus injection of saline or insulin (6 μg), the muscle was excised and aliquots were immunoprecipitated with anti-Shc antibody and blotted with the same antibody (D). The same samples were immunoprecipitated with anti-Shc antibody and blotted with antiphosphotyrosine antibody (E). F: The immunoblotting results of GRB2 in anti-Shc immunoprecipitates from the muscle of control and epinephrine-treated rats. Scanning densitometry of autoradiograms was performed in six experiments. The black bars represent the control group and the white bars represent the GH-treated group. The data are expressed as the mean ± S.E.M. $^*P < 0.05$.

Y1163F-YFF) on the insulin receptor [32]. Such cell lines showed insulin-induced tyrosine phosphorylation of Shc, Shc-Grb2 complex formation, and p21Ras-GTP formation, but had a reduced tyrosine phosphorylation of IRS-1 as well as a reduced IRS-1 association with Grb2 and PI 3-kinase [32]. These results, together with our own, suggest that a reduction in insulin receptor phosphorylation may differentially induce post-receptor processes by preserving the phosphorylation of some substrates and pathways, but not that of others. A dissociation between IRS-1 and Shc tyrosine phosphorylation without any change in insulin receptor level has also been observed after treatment with dexamethasone [33] or wortmannin [34].

The mechanism by which a reduction in insulin receptor phosphorylation differentially regulates its own substrates is unknown. Shc can directly associate with the insulin receptor, by a binding site to phosphorylated Tyr-960 in the receptor's juxtamembrane region [35]. This is the same binding site for IRS-1 [36], and is consistent with the suggestion that both Shc and IRS-1 compete for this tyrosine residue in their association with the phosphorylated receptor [37]. Although IRS-1 is best known as a substrate for tyrosine phosphorylation, it is mainly a phosphoserine-containing protein. IRS-1 contains over 30 potential serine/threonine phosphorylation sites in motifs recognized by various kinases such as PKC, MAP kinases and cyclic AMP- and cyclic GMP-dependent protein kinases [25,38,39]. Serine phosphorylation also plays a role in the regulation of IRS-1 signalling. Okadaic acid, a serine phosphatase inhibitor, increases serine phosphorylation of IRS-1 in 3T3-L1 adipocytes and this appears to inhibit insulin-

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stimulated tyrosine phosphorylation [40]. Thus, it is possible that serine phosphorylation of IRS-1 (induced by chronic GH treatment and/or hyperinsulinemia in one model and by an increase in intracellular cAMP levels following exposure to epinephrine in another) may reduce this substrate's affinity for the insulin receptor, allowing Shc to be more competitive. In support of this hypothesis, Li and Goldstein [34] have recently shown that reducing IRS-1 serine phosphorylation results in increased insulin-induced IRS-1 tyrosine phosphorylation and decreased Shc tyrosine phosphorylation.

Accelerated dephosphorylation of the insulin receptor and IRS-1 by tyrosine phosphatases cannot be excluded in these models of insulin resistance. In this regard, Wilson and Kaczmarek [41] have demonstrated that an increase in cellular cAMP through the activation of PKA increases the activity of endogenous PTPase, thereby leading to a sequence of dephosphorylation.

A further interesting finding in GH-treated rats was the increase in basal Shc tyrosine phosphorylation without any change in basal IRS-1 tyrosine phosphorylation. This increase in phosphorylation may be related to the hormonal milieu of these animals. There is evidence that GH can induce Shc tyrosine phosphorylation in cultured cells [42] and in animal tissues [43]. It is therefore possible that GH, hyperinsulinemia and/or high serum levels of IGF-1 in the animals we studied could preferentially induce Shc tyrosine phosphorylation to the detriment of IRS-1 tyrosine phosphorylation for the same reasons as those discussed above.

We have shown that chronic GH or acute epinephrine treatment can specifically decrease insulin-induced IRS-1 tyrosine phosphorylation and association with PI 3-kinase. This may help to explain the insulin resistance of these animals. In muscle, both chronic GH treatment and the acute administration of epinephrine preserve insulin-induced Shc tyrosine phosphorylation. Further investigation of the hormonal regulation of the distinct signaling pathways activated by insulin receptors will contribute to our understanding of the mechanisms involved in this phenomenon.

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Publicação 5

REGULATION OF INSULIN-STIMULATED TYROSINE PHOSPHORYLATION OF SHC AND SHC/GRB2 ASSOCIATION IN LIVER, MUSCLE AND ADIPOSE TISSUE OF EPINEPHRINE- AND STREPTOZOTOCIN- TREATED RATS

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Running Title: Regulation of Shc and Grb2 by insulin

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Abstract

Shc protein phosphorylation has been extensively characterized as the initial step that activates a complex mitogenic pathway through its association with Grb2. In the present study, we investigate the adrenergic control of insulin-induced Shc phosphorylation and Shc-Grb2 association, and the modulating effect of streptozotocin-induced diabetes mellitus on Shc phosphorylation and Shc/Grb2 association. Acute epinephrine treatment, which leads to a normoglycemic insulin resistant state, do not affect insulin-induced Shc tyrosine phosphorylation or Shc-Grb2 association in liver, muscle or fat. In contrast, a significant increase in insulin-induced Shc phosphorylation is observed in liver and muscle of rats treated chronically with streptozotocin. The association Shc/Grb2 is also increased in both tissues following insulin treatment. These data suggest that while epinephrine preserves the insulin-induced phosphorylation of Shc and the mitogenic pathway stimulated by Shc-Grb2 association, STZ treatment leads to a tissue-specific increase in the activity of the initial step that ultimately results in the activation of the Shc/Grb2 mitogenic pathway.

Keywords: *epinephrine, diabetes mellitus, streptozotocin, IRS-1, Shc, Shc-Grb2 association.*

Introduction

The insulin receptor (IR) is a protein with endogenous tyrosine kinase activity which following the activation by insulin, undergoes rapid autophosphorylation and phosphorylates intracellular protein substrates, including insulin receptor substrates 1-4, and Shc (1-5) Tyrosine phosphorylated IR couples to downstream SH2- or SH3-containing signaling molecules, through the phosphotyrosil residues of activated insulin receptor substrates (IRSs). The p85 regulatory subunit of PI 3-kinase (6), Grb2 (7), SHP2 (Syp) (8) and Nck (9), are some of the proteins regulated by this pathway. Like IRSs, Shc protein is also an IR substrate (5,10) that acts as a docking molecule for the SH2/SH3 adapter protein Grb2 (11,12). Shc/Grb2 association is upstream to the activation of an important signaling pathway leading to the stimulation of mitogen-activated protein kinase (MAPK) (13-15). We have reported recently that insulin can induce time- and dose-dependent Shc tyrosine phosphorylation and association with Grb2 in rat tissues (16), and that in some situations of insulin resistance there is an increased correlation between insulin levels and Shc tyrosine phosphorylation (17). Thus, IRSs and Shc constitute downstream coupling molecules which connect the activated receptor to distal events.

Insulin resistance is defined as a subnormal biological response to a given concentration of insulin. Insulin resistance is characteristic of many disease states such as type 2 diabetes, uncontrolled type 1 diabetes, obesity and hypertension. Supranormal concentrations of epinephrine are also known to cause insulin resistance (18-20). Previous studies have demonstrated that in streptozotocin-induced diabetes (an animal model of type 1 diabetes) there is increased IRS-1 tyrosine phosphorylation and association with PI 3-kinase (18,21). In contrast, in

rats treated with an excess of epinephrine, there is a reduction in the insulin-induced IRS-1 phosphorylation level and in the association with PI 3-kinase (20). These data demonstrate that IRS-1 is specifically regulated in animal models of insulin resistance, and may play a critical role in the altered insulin action observed in such models. In additiont fatty acid-induced insulin resistance was recently associated with a decrease in insulin-stimulated MAP kinase (22). TNF-α and hyperglycemia also regulate Shc phosphorylation in cultured cells (23), suggesting that Shc may be involved in the molecular mechanisms of insulin resistance. However, the regulation of Shc in tissues from stz- and epinephrine- treated rats has not yet been investigated.

In this study, we examined the insulin-induced Shc tyrosine phosphorylation and association with Grb2 in liver, muscle and fat of streptozotocin- and epinephrine-treated rats

Materials and Methods

Materials

The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA). Tris[hydroxymethyl]amino-methane (Tris), phenylmethylsulfonylfluoride (PMSF), aprotinin, silicone, dithiothreitol and streptozotocin (N-[methylnitrosocarbamoyl]-D-glucosamine) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium amobarbital were purchased from Eli Lilly Co. (Indianapolis, IN), and regular highly purified insulin (Iolin R) was from Biobrás. Protein A-Sepharose 6 MB was purchased from Pharmacia (Uppsala, Sweden). [^{125}I]Protein A was from Amersham (Aylesbury, UK), and nitrocellulose

(BA85; 0.2 μm) was obtained from Schleicher and Schuell (Keene, NH). Male Wistar rats were from the University's Animal Breeding Center. Monoclonal antiphosphotyrosine antibodies (1 μg/ml) were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-Shc and anti-GRB2 rabbit polyclonal antibodies (100 μg/ml) were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

Wistar rats 6 weeks old (mean body weight of 148 ± 3 g) were fed standard rodent chow and water *ad libitum*. Food was withdrawn 12-14 h before the experiments. Diabetes was induced with streptozotocin (stz) dissolved in citrate buffer (pH 4.5) and administered intravenously in a single dose of 60 mg/kg body weight (Bw) to rats fasted overnight. Acute epinephrine treatment involved a single injection of 25 μg of 5% (w/v) epinephrine/100g Bw given 10 min before the experimental procedure. All experiments with animals were approved by the Ethics Committee of the State University of Campinas (UNICAMP).

Methods

The rats were anesthetized with sodium thiopental (100 μg/kg Bw, ip) and used 10-15 min later, as soon as anesthesia was assured by the loss of foot and corneal reflexes. The abdominal cavity was opened, the portal vein exposed and 60 μg of insulin was injected. Samples of liver, skeletal muscle and adipose tissue were collected 5 min after the insulin infusion, minced coarsely, and homogenized immediately in aproximately 10 volumes of solubilization buffer A at 4 ^{0}C, using a Polytron PTA 20S homogenizer (Brinkmann Instruments, model PT 10/35), operated at maximum speed for 30 s. Buffer A (for solubilization) consisted of 1% Triton X-100, 50 mM Hepes (pH 7.4), 100 mM sodium pyrophosphate, 100 mM

sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonylfluoride, and 0.1 mg of aprotinin/ml.

For both experimental models studied, the tissues were extracted in an identical manner. The extracts were centrifuged at 30,000 x g in a Beckman 70.1 Ti rotor at 4 °C for 20 min to remove insoluble material, and the resulting supernatant was used for immunoprecipitation with 10 µl of polyclonal anti-Shc antibody. The immune complexes were precipitated with protein A-Sepharose 6MB and were washed three times with 50 mM Tris (pH 7.4), containing 2 mM sodium vanadate, and 0.1% Triton X-100.

Protein analysis by immunoblotting

After washing, the pellet was resuspended in Laemmli sample buffer (24) with 100 mM dithiothreitol and heated in a boiling water bath for 5 min. The samples were subjected to SDS-PAGE (10% polyacrylamide gels) in a Bio-Rad miniature slab gel apparatus. Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin *et al* (25). Nonspecific protein binding to the nitrocellulose was reduced by pre-incubating the filter overnight at 4°C in blocking buffer (5% non-fat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The prestained molecular mass standards used were phosphorylase B (105 kDa), bovine serum albumin (82 kDa), ovalbumin (49 kDa), carbonic anhydrase (33.3 kDa), soybean trypsin inhibitor (28.6 kDa) and lysozyme (19.4 kDa). The nitrocellulose filter was then incubated for 4 h at 22 °C with anti-phosphotyrosine antibody (1 µg/ml), anti-Shc antibody (1:100) or GRB2 antibody (1:100) diluted in blocking buffer (3% non-fat dry milk) and then washed for 30 min in blocking buffer without non-fat dry milk. The blots were then 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. [^{125}I]-Protein A bound to the antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at -70 °C for 12-48 h. Band intensities were quantified by optical densitometry (Hoefer Scientific Instruments, San Francisco; model GS 300) of the developed autoradiographs.

General procedures

Protein quantification was performed using the Bradford dye method (26), and the Bio-Rad reagent with BSA as a standard. Plasma glucose levels were determined with a Beckman glucose analyser (Beckman Instruments, Inc., Palo Alto, CA). Acute epinephrine-treated rats and chronic stz-treated rats and their respective controls underwent an intravenous insulin tolerance test (60 μg of insulin, injected into the portal vein). Samples were collected from the tail vein at 0 (basal), 4, 8, 12 and 16 min after hormone injection. Plasma glucose disappearance rate (K_{itt}) was obtained from the formula $0.693/t_{1/2}$. The plasma glucose $t_{1/2}$ was calculated from the slope of the least square analysis of the plasma glucose concentrations during the linear phase of decline

Statistical analysis

The experiments were always performed by studying the treated rats of animals in parallel with a control group. Comparisons of the Shc levels between controls vs. diabetics and controls vs. acute epinephrine-treated rats, were analyzed using the unpaired *t* test. A p value ≤ 0.05 was considered to indicate significance.

Results

1. Characteristics of epinephrine- and stz-treated rats

To establish the degree of insulin resistance in epinephrine-treated rats, ten rats from each experimental group and their respective matching controls underwent an insulin tolerance test. The plasma glucose disappearance rate (k_{itt}) of the control group was 4.40 ± 0.39%/min. Acute epinephrine treatment induced insulin resistance, as reflected in the glucose disappearance rate of 1.89 ± 0.63%/min ($p<0.05$ related to the control group). In stz-treated rats, plasma glucose and serum insulin levels were measured, and considered diabetics when glucose was over 300 mg/dl. The glucose disappearance rate of STZ diabetic rats was 1.94 ± 0.81%/min ($p<0.01$ related to control group). Table I sumarizes the body weight, plasma glucose and serum insulin levels of control, epinephrine- and stz-treated rats.

2. Shc protein expression in tissues of diabetic or epinephrine-treated rats

Shc protein expression in liver, muscle and adipose tissue of stz-diabetic rats and epinephrine-treated rats was not significantly different from the respective controls before and after acute stimulation with insulin (data not shown). The predominant isoform phosphorylated in muscle was $p52^{Shc}$, whereas in liver and fat $p52^{Shc}$ was the only phosphorylated isoform detected.

3. Effect of epinephrine on Shc phosphorylation and Shc/Grb2 association

Insulin-induced tyrosine phosphorylation of Shc in liver of epinephrine-treated rats did not differ from that of control rats (control 100 ± 9% vs epinephrine-treated 85 ± 10%; $p=0.2$) (Fig. 1A, left panel). The extent of insulin-induced Shc/Grb2 association in this tissue was highly correlated with the level of insulin-induced Shc phosphorylation (control 100 ± 23% vs epinephrine 74 ± 2%, $p=0.9$) (Fig. 1A, right panel). Basal levels of Shc/Grb2 association in liver of epinephrine-treated rats increased by 50% ($p<0.003$) (Fig. 1A, right panel).

Insulin-induced tyrosil phosphorylation of Shc in the muscle of epinephrine-treated rats was similar to the controls (control 100% ± 8% vs epinephrine 103% ± 8%, p=0.4) (Fig. 1B, left panel). Insulin-induced Shc/Grb2 association levels in muscle of epinephrine-treated rats, did not differ from the Shc/Grb2 association levels displayed by the controls (control 100 ± 5% vs epinephrine 97 ± 1%, p= 0.21) (Fig. 1B, right panel). In muscle of epinephrine-treated rats there was a significant increase in the basal levels of Shc/Grb2 association (control 28 ± 2% vs epinephrine 65 ± 9%, $p<0.05$)

In the adipose tissue of epinephrine-treated rats, the levels of insulin-induced Shc protein phosphorylation were similar to the levels observed in their controls (control 100 ± 6% vs epinephrine 91 ± 15%; p=0.8) (Fig. 1C, left panel). In these animals, the insulin-induced Shc/Grb2 association in adipose tissue was strongly correlated with the level of phosphorylation of Shc (control 100% ± 6%, epinephrine 96% ± 4%, p=0.2) (Fig 1C, right panel). Basal level of Shc/Grb2 association in fat of epinephrine-treated rats was significantly increased (control 37 ± 17%, epinephrine 86 ± 4%, p=0.05) (Fig. 1C, right panel)

4. Effect of stz on Shc phosphorylation and Shc/Grb2 association

Insulin-induced tyrosine phosphorylation levels of Shc in liver of stz-treated rats was significantly higher compared to controls (control 100 ± 7% vs stz 175 ± 5%, $p<0.001$) (Fig. 2A, left panel). The extent of insulin-induced Shc/Grb2 association was strongly correlated with the level of phosphorylation of Shc (control 100 ± 13% vs STZ 194 ± 8%, $p<0.001$) (Fig. 2A, right panel).

In muscle of stz-treated rats there was an increase in the basal phosphorylation of Shc (47 ± 16% above the control, $p<0.05$) (Fig. 2B, left panel), which was not accompanied by an increase in Shc/Grb2 association before the

injection of insulin. Insulin-induced Shc tyrosine phosphorylation was higher in muscle of stz diabetic rats (control 100 ± 11% vs stz 180 ± 12%, $p<0.01$) (Fig. 2B, left panel), with a corresponding increase in the association between Shc and Grb2 (control 100 ± 18% vs stz 162 ± 8%, $p<0.01$) (Fig 2B, right panel).

In adipose tissue from stz-treated rats there was a non-significant increase in insulin-induced Shc phosphorylation levels compared to controls (control 100 ± 4% vs stz 133 ± 9%; $p=0.2$) (Fig. 2C). The insulin-induced Shc/Grb2 association was consistent with the Shc tyrosil phosphorylation levels, and did not differ from the controls (control 100 ± 6% vs stz 125 ± 8%, $p=0.1$) (Fig. 6C).

Discussion

Following insulin stimulation, several proteins undergo tyrosine phosphorylation, including the β chain of the insulin receptor, IRS-1, IRS-2 and Shc (27). Shc belongs to a family of signal-transduction proteins implicated in several intracellular interactions between different signaling systems (15). Once phosphorylated, Shc binds to the adapter protein Grb2, activating a complex mitogenic pathway, acting as a key docking that enhances or counter-regulates the tyrosine kinase receptors actions over specifically mitogenic processes (11-15).

In the present study, we analyzed the insulin-induced phosphorylation of $p52^{Shc}$ and the relationship between insulin-induced Shc phosphorylation and Shc/Grb2 association in liver, muscle and fat of rats treated acutely with epinephrine or chronically with stz. Insulin resistance in both groups was demonstrated by a decrease in the glucose disappearance rate after insulin infusion (28). The insulin-induced tyrosine phosphorylation levels of Shc, and the Shc/Grb2 association were similar in liver, muscle and fat of acute epinephrine-treated rats compared to the controls. However, there was a significant increase in basal Shc/Grb2 association

levels in the three tissues studied. Comparison of these results with previous studies done under similar conditions (20, 29, 30), revealed a clear dissociation between insulin-induced Shc and IRS-1 tyrosine phosphorylation in epinephrine-treated rats in the tissues examined. Indeed, while the insulin-induced tyrosine phosphorylation levels of IRS-1 in liver and muscle of epinephrine-treated rats were lower compared to controls (20), the insulin-induced Shc phosphorylation in the same tissues were similar to controls. The decrease in IRS-1 tyrosine phosphorylation in epinephrine-treated rats correlated with that of the IR (20). The mechanism by which a reduction in insulin receptor phosphorylation differentially regulates its own substrates is unknown. Shc can directly associate with the insulin receptor by binding to phosphorylated Tyr-960 in the receptor juxtamembrane region (10), which is the same binding site for IRS-1 (31). These suggested that IRS-1 competes for this tyrosine residue during association with the phosphorylated receptor (32). It is possible that the serine phosphorylation of IRS-1 induced by an increase in intracellular cAMP levels following epinephrine treatment may reduce its binding affinity for the insulin receptor, allowing Shc to be more competitive. In support of this hypothesis, Li and Goldstein (33) have shown that reducing IRS-1 serine phosphorylation results in increased insulin-induced IRS-1 tyrosine phosphorylation and decreased Shc tyrosine phosphorylation.

The dissociation of insulin-induced IRS-1 and Shc tyrosine phosphorylation observed after acute epinephrine treatment has been described in cell lines with mutations at two tyrosine phosphorylation sites on the insulin receptor (34). Such cell lines showed insulin-induced tyrosine phosphorylation of Shc, Shc/Grb2 complex formation, and p21ras-GTP formation, but had reduced tyrosine phosphorylation of IRS-1 and reduced IRS-1 association with Grb2 and PI 3-kinase. These results, together with our own, suggest that a reduction in insulin receptor

phosphorylation may differentially induce post-receptor processes by preserving the phosphorylation of some, but not all, substrates and pathways.

The increased Shc-Grb2 association during the basal state in liver, muscle and adipose tissue of epinephrine-treated rats, suggests that epinephrine-induced tyrosine phosphorylation of p52 Shc, that leads to its association with Grb2 and MAPK activation. Studies showing that norepinephrine, through the alpha-1 adrenergic receptor, directly stimulates p52Shc and MAPK in human vascular smooth muscle cells, support this hypothesis (35). High levels of TNFα due to epinephrine treatment may also contribute to the increase of basal Shc tyrosine phosphorylation levels and Shc-Grb2 association (23).

We also studied the effect of stz-induced diabetes mellitus, which is an insulin-resistant state characterized by deficient insulin production and action, on the regulation of Shc. In this state, high glucose levels were detected, and Shc phosphorylation was increased, so that the Shc/Grb2 association after stimulation with insulin was considerably augmented in liver and muscle, and to lesser extent in rat adipose tissue. In stz diabetic animals under similar conditions, there was also an increase in insulin-induced IRS-1 tyrosine phosphorylation, a well as IRS-1 associated PI-3 kinase activity.

The mechanism responsible for the increase in IRS-1 and Shc tyrosine phosphorylation in liver and muscle of stz diabetic animals is not known, but may be related to the increase in insulin receptor number and phosphorylation (18). However in other animal model of insulin resistance with hypoinsulinemia (72 h fasted rats), in which there is an increase in insulin receptor number and phosphorylation, the increase in IRS-1 tyrosine phosphorylation is not accompained by an increase in Shc phosphorylation. Since the difference between these two models is related mainly to glucose levels, it is possible that high glucose levels, perhaps by increasing the osmolarity, may contribute to the increased Shc

phosphorylation level and association with Grb2 in liver and muscle (36). The upregulation of substrate tyrosine phosphorylation in the presence of hypoinsulinemia could also potentially result from the reduced activity of a phosphotyrosine-protein (PTP) phosphatase, which is known to be decreased in the skeletal muscle of stz-diabetic rats (40-42).

The increase in IRS-1 and Shc tyrosine phosphorylation in stz diabetic animals occurs despite the state of decreased insulin responsiveness in both liver and peripheral tissues of these animals (37-39). The fact that the glucose disappearance rate was lower in stz diabetic rats suggests that tissue or circulating factor such as fatty acids, ketones, counterregulatory hormones, and acidosis may antagonize the stimulatory action of insulin on glucose uptake and metabolism as previously discussed. It is possible that the increase in IRS-1 and Shc phosphorylation in liver and muscle of stz-diabetes could be a response to the impairment of insulin action.

The results of our study suggest that while epinephrine preserves the insulin-induced phosphorylation of Shc and the mitogenic pathway stimulated by Shc/Grb2 association, stz treatment leads to a tissue-specific increase in the activity of the initial step that ultimately results in the activation of the Shc/Grb2 mitogenic pathway.

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Figure Legends

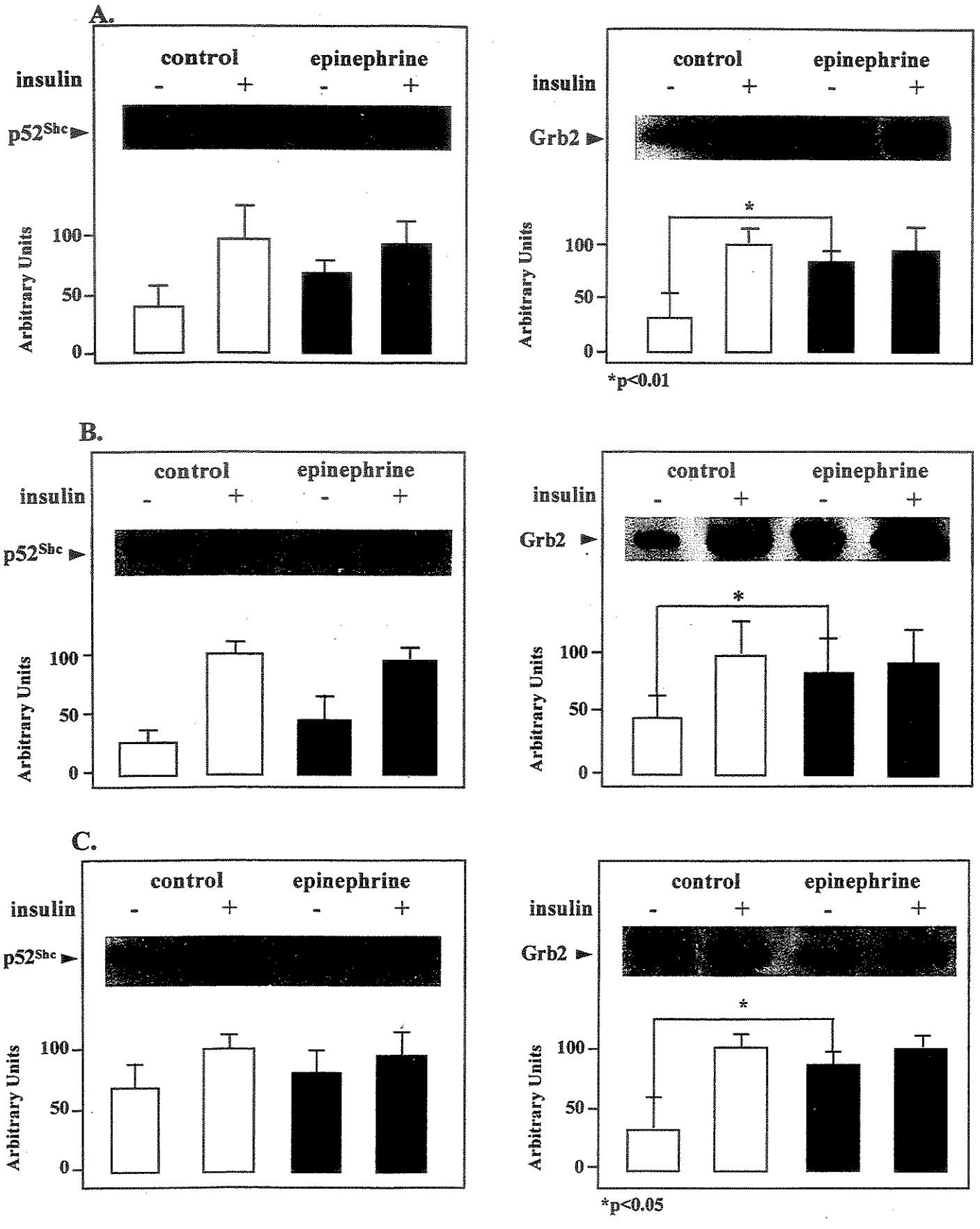
Fig 1. Insulin stimulates tyrosine phosphorylation of Shc and Shc/Grb2 association in tissues from control and epinephrine-treated rats: The proteins were isolated and processed as described in Methods and were detected with anti-Shc/antiphosphotyrosine antibodes (left panels) and anti-Shc/anti-Grb2 antibodies (right panels) in *(A)* liver, *(B)* muscle and *(C)* adipose tissue extracts. The levels of Shc tyrosil phosphorylation and Shc-Grb2 association were determined by scanning densitometry of six separate experiments for each tissue and were expressed as a percentage of the insulin-stimulated control (100%). The columns and bars and represent the mean ± SEM. $p<0.05$.

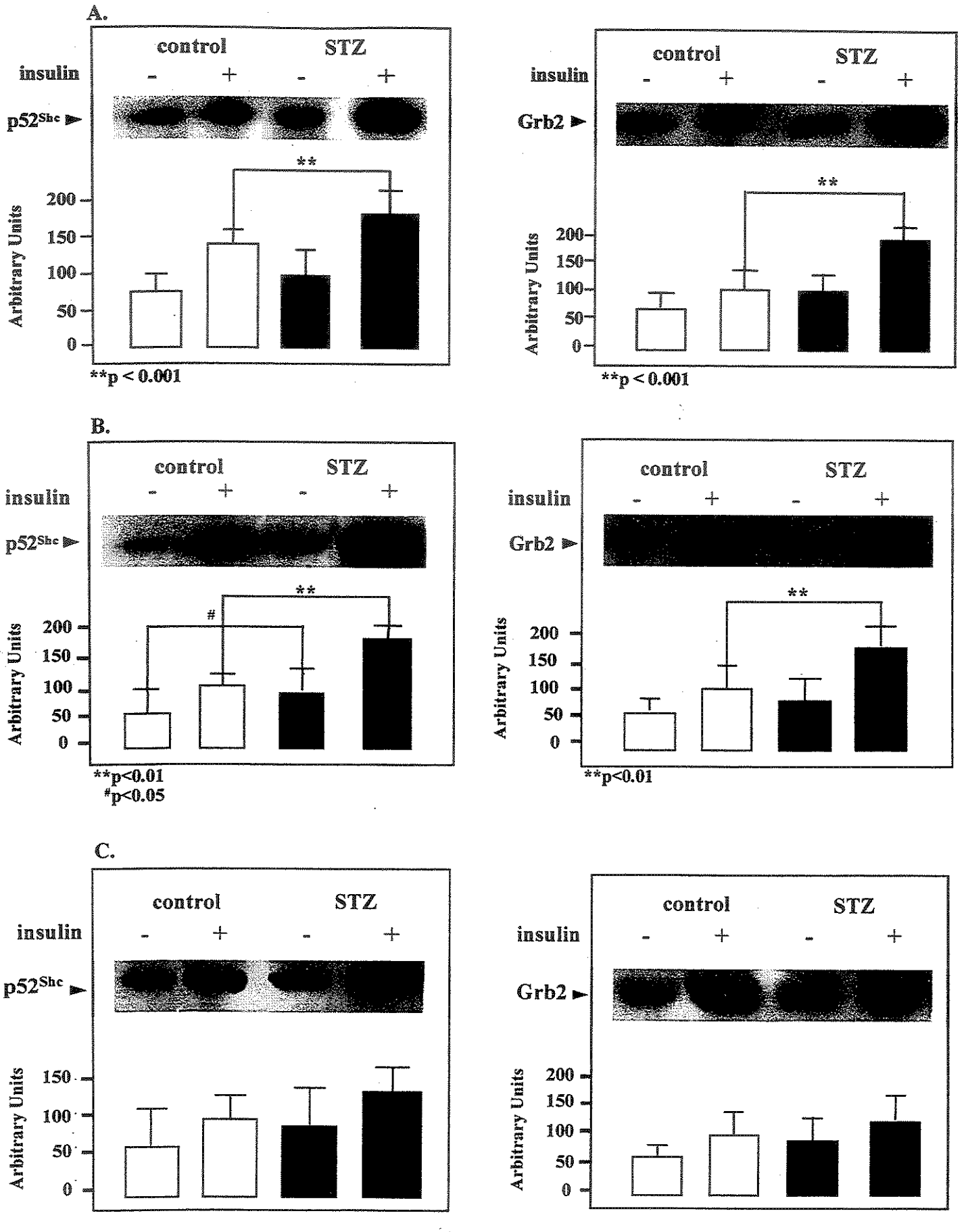
Fig 2. Insulin stimulates tyrosine phosphorylation of Shc and Shc/Grb2 association in tissues from control and stz-treated rats: The proteins were isolated and processed as described in Methods and were detected with with anti-Shc/antiphosphotyrosine antibodes (left panels) and anti-Shc/anti-Grb2 antibodies (rigth panels) in *(A)* liver, *(B)* muscle and *(C)* adipose tissue extracts. The levels of Shc tyrosil phosphorylation and Shc-Grb2 association were determined by scanning densitometry of seven, six and seven separate experiments for liver, muscle and fat respectively, and were expressed as a percentage of the insulin-stimulated control (100%). The columns and bars represent the mean ± SEM. $p<0.05$.

Table I. Characteristics of the rats studied

The data are expressed as the mean ± SEM. *p≤0.05 compared to the corresponding control.

Groups	n	Body weight (Bw)	Basal Plasma glucose	Basal Serum insulin
		(g)	(mg/dl)	(μU/ml)
Control	20	148±3	115±2	29±2
Epinephrine	12	144±3	117±3	41±4
STZ diabetes	16	102±3*	452±2*	6±6*


A.
control
epinephrine
insulin
- + - +
p52Shc
Arbitrary Units
100
50
0
Grb2
*
*p<0.01
B.
*p<0.05
C.


A.
control
STZ
insulin
-
+
-
+
p52Shc
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Arbitrary Units
200
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control
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Grb2
Arbitrary Units
200
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Publicação 6

RAT PANCREATIC β-CELLS EXPRESS THE INSULIN RECEPTOR SUBSTRATE - 3 ($pp60^{IRS-3}$)

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Running Title: IRS-3 expression in pancreatic β-cells

Summary

Adipocytes express a 60 kDa protein which undergoes rapid tyrosine phosphorylation in response to insulin and then binds to PI-3 kinase. This protein has been considered a new member of the insulin receptor substrate (IRS) family, and designated IRS-3. In the present study we show for the first time that purified rat β-cells express $pp60^{IRS-3}$. The protein is immunoreactive with anti-IRS-2, and it is highly expressed in β-cells. Antibodies against IRS-2 and phosphotyrosine also detected the presence of another protein of 125 kDa in β-cells, but not in liver or adipose tissue. IRS-3 mRNA is expressed in β-cells, but the content of this mRNA is not affected by a 48 h culture at different glucose concentrations or in the presence of interleukin-1β. Insulin may exert a feedback loop which contributes to regulate pancreatic β-cell function, and further studies are now required to clarify

the role for IRS-3 and the 125 kDa protein in the insulin signal transduction at the β-cell level.

Key words: IRS-2, IRS-3, pancreatic β-cells, insulin.

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1. INTRODUCTION

Insulin secretion tightly regulates glucose homeostasis by stimulating the peripheral use of glucose and inhibiting hepatic glucose production [1]. Insulin action is initiated by its binding to the extracellular domain of the insulin receptor (IR) and subsequent activation of the tyrosine kinase activity of the receptor cytoplasmic domain [2]. Once activated, the IR phosphorylates proteins which will complex with a variety of SH2 domain-containing proteins and thus trigger signaling pathways that lead to the diverse actions of insulin [3].

In contrast to the extensive studies performed in liver, muscle and adipose tissue [4-5], relatively little is known on the effects of insulin in pancreatic β-cells. The recent detection of insulin receptor (IR) mRNA and the insulin receptor 1 (IRS-1) protein in βTC3 cells, an insulin-producing β-cell line [6], raises the question whether a feedback loop operates through the IR in β-cells. Moreover,

IRS-1 and IRS-2 are also implicated in the signalling of insulin growth factor-1 (IGF-1), for which rat β-cells have specific receptors [7]. Thus, the finding of IRS-1 and IRS-2 in insulin-producing cells would be consistent with the possibility that islet β-cells respond to insulin and/or insulin growth factor (IGF) via intracellular transducion pathways that are similar to those in peripheral tissues [8-10]. Glucose and insulin have been found to induce phosphorylation of the insulin receptor and its primary substrates insulin receptor 1 and 2 (IRS-1 and IRS-2) in whole rat islets [11]. What is still unclear is whether the proteins involved in insulin signal transduction are indeed present in primary non-tumoral β-cells.

To further examine signal transduction of insulin at the β-cell level, we studied the expression of the insulin receptor substrate-2 (IRS-2), one of the early substrates of the activated IR, in purified rat β- and non β-cells. IRS-2 has been recently proposed as an important regulatory protein for β-cell development and survival [12]. During this study, we noticed that IRS-2 was associated with a 60 kDa band which showed similarities with the 60 kDa insulin receptor substrate-3 ($pp60^{IRS-3}$) recently described in adipocytes [13]. To identify the expression of IRS-3 mRNA in β-cells, we developed a reverse transcribed-polymerase chain reaction (RT-PCR) assay for IRS-3 mRNA in purified islet cells.

2. MATERIALS AND METHODS

Preparation of purified β-cells

Pancreatic islets were isolated from adult male Wistar rats by collagenase digestion and dissociated into single cells in calcium-free medium containing trypsin and deoxyribonuclease (Dnase) [14]. Single β-cells were purified by autofluorescence-activated cell sorting, using cellular light-scatter and flavin adenine dinucleotide (FAD)-autofluorescence as discriminating parameters [14]. The β-cell preparations contained >90% β-cells, while the non-β cells preparations contained >60% α–cells and <20% β-cells [14].

Cell culture

The two cell preparations (300K β-cells and 900K non-β cells) were suspended in Lux dishes (Miles, Maperville, IL) containing 3 ml culture medium, reaggregated for 2 h in a rotatory shaking incubator (Braun, Melsugen, Germany), and then further cultured under static serum-free conditions. Culture medium was Ham's F10 containing 10 mmol/liter glucose supplemented with 0.075 mg/ml penicillin, 0.1 mg/ml streptomycin, 2 mmol/liter L-glutamine, 1% (wt/vol) BSA pretreated with charcoal (BSA, fraction V, RIA grade, Sigma, St Louis, MO), and 50 μmol/liter 3-isobutyl-1-methylxantine (IBMX, Janssen Chimica, Beerse, Belgium) [15]. The presence of IBMX preserves β–cell viability in culture [15]. In the first series of experiments, β- and non-β-cell subpopulations were cultured for 24 h at 2.8 mM glucose and then exposed for 90 sec to 22.2 mM glucose, 10^{-7} M human insulin (a kind gift from Eli Lilly, Indianapolis, USA) or 10^{-8} M IGF-1 (a

kind gift from Eli Lilly, Indianapolis, USA). These cells were collected directly for Western blot analysis (see below). In a second series of experiments, 100K β- or non β-cells were incubated for 48 h in 1 ml culture medium at 2.8, 10 or 22.2 mM glucose or 2.8 mM glucose + 10^{-7} M insulin before collection for RT-PCR (see below).

Preparation of rat tissue samples for control tests

Wistar rats (200-250 g body weight) were starved for 16 h before anesthesia with sodium thiopental (100 μg/kg body weight, ip) and used 10-15 min later. The abdominal cavity was opened, the portal vein exposed and 2.8 mM or 22.2 mM glucose, or 10^{-7} M of insulin or 10^{-8} M of IGF-1was injected. Samples of liver and adipose tissue were collected 90 sec. after infusion (5). The tissues were minced and homogenized immediately in approximately 10 volumes of lysis buffer (see below) at 4^{0}C, using a Polytron PTA 20S homogenizer (Brinkmann Instruments, model PT 10/35), operated at maximum speed for 30 s. The extracts were centrifuged at 30,000 x g in an Eppendorf centrifugue at 4 °C for 20 min to remove insoluble material. Protein quantification was performed using the micro BCA Protein Assay (Pierce, Chemical Co., Rockford, IL) and similar amounts of protein (100 μg/lane) were ran in the same gel and following the same steps used for β-cells and non β-cells preparations.

Immunoblot analysis of IRS-2

Groups of $3x10^5$ β-cells and $9x10^5$ non-β cells were sonicated in 30 µl of modified radioimmunoprecipitation (RIPA) lysis buffer (1x PBS, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM PMSF in isopropanol, aprotinin 1 µg/ml, 1 mM sodium orthovanadate, 1 mM NaF). The extracts were centrifuged at 30.000 x g in an Eppendorf centrifuge at 4°C for 20 min to remove insoluble material. Protein quantification was performed using the micro BCA Protein Assay (Pierce, Chemical Co., Rockford, IL), and equal amounts of protein (25 µg/lane) were ran on a 10% SDS-polyacrilamide gel. After electrophoresis, proteins were electrically transferred to nitrocellulose filters and then incubated with a mouse monoclonal anti-phosphotyrosine antibody (1:1000; Transduction Laboratories, Lexington, UK), or rabbit polyclonal anti-IRS-2 antibody (non-IRS-1 cross reacting, 1:1000; Upstate Biotechnology, Lake Place, NY-USA). Horseradish peroxidase-linked whole anti-mouse or anti-rabbit were used as second antibodies. Peroxidase activity was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

mRNA isolation and RT-PCR

Poly(A)+ mRNA was isolated from cell aggregates (10^5 β or non-β-cells) using oligo(dT)25-coated polystyrene Dynabeads (Dynal, Oslo, Norway). The yield of mRNA in each sample was evaluated by RT-PCR amplification of the

housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The reverse transcription reaction was performed at 42°C for 1h, and contained (per 10 µl): mRNA equivalent to $6x10^3$ cells, 1x reverse transcription buffer, 5 mM $MgCl_2$, 1 mM of each dNTP, 2.5 µM random hexamer primers and 100 units of Moloney murine leukemia virus reverse transcriptase (Perkin Elmer, Norwalk, CT, USA) [16]. The subsequent PCR reaction contained (in 25 µl reaction solution): 5 µl cDNA, 0.4 µM of forward and reverse primers, 200 µM of each dNTP, 1x PCR buffer, 2 mM $MgCl_2$, and 0.625 U AmpliTaq Gold DNA polymerase (Perkin Elmer). PCR specificity and efficiency was improved by using hot start PCR with 12 min predenaturation at 95°C and then 27 (GAPDH) or 32 (IRS-3) cycles of 94°C for 45s, 58°C for 45s and 72°C for 80s. The number of cycles selected allowed linear amplification of the amount of cDNA under study (data not shown). The primer sequences used were: IRS-3F 5'-GGTGCCTGCACTATTAGCAA-3',

IRS-3R 5'-AGCAAGGACAAGCGCAGGAC -3',

GAPDH-F 5'-TCACTCAAGATTGTCAGCAA-3',

GAPDH-R 5'-AGATCCACGACGGACACATT-3'

The ethidium bromide-stained agarose gels were photographed under UV-transillumination using Kodak Digital Science DC40 camera (Kodak, Rochester, NY, USA) and the PCR band intensities on the image were quantified by Biomax

1D Image analysis software (Kodak) and expressed in pixel intensities. The target cDNAs present in each sample was corrected for the respective GAPDH value.

Data analysis

Data are presented as means ± SEM. Statistical significance of differences were calculated by ANOVA, as indicated.

3. RESULTS

Expression of Insulin Receptor Substrate-2 in β- and non-β-cells

To determine the expression of IRS-2 in purified pancreatic β- and non-β-cells, both cell populations and the control tissues (liver and fat) under treatment with glucose (22.2 mM), were subjected to Western blotting using anti-PY antibody. Rat liver samples showed, as expected, a 180 kDa band which corresponds to the IRS-2 molecular weight (Fig. 1, lane 1). Adipose tissue samples showed only a faint band at the IRS-2 level, but contained a clear 60 kDa band (Fig. 1, lane 5). IRS-2 was strongly positive in islet β-cells (Fig. 1, lane 3), whith a protein load that was four-fold smaller than for liver (Fig. 1, compare lanes 1 and 3), but was absent on non-β-cells. Interestingly, the β-cell blots showed a 60 kDa band, comparable to the band observed in the rat adipose tissue (Fig. 1, compare lanes 3 and 5). Liver and islet non-β-cells did not express this 60 kDa band (Fig. 1, lanes 1-2 (liver) and 7-8 (fat)).

The β-cells also showed a clearly defined band at aproximately 125 kDa, which was not present in the other preparations (lanes 3-4, Fig. 1). A band of similar MW has been previously described in insulin-producing βTC3 cells and in NEDH rat insulinoma tissue (RITz cells) [17-18].

With the use of an anti-phosphotyrosine antibody, liver samples previously exposed to insulin *in vivo* showed the expected 180 kDa, 160 kDa and 95 kDa bands for respectively IRS-2, IRS-1 and IR (Fig. 1, lane 2). Pancreatic β-cells exposed to insulin 10^{-7} M evidenced phosphorylation of the above described 125 kDa protein, but not of the 180 kDa band corresponding to IRS-2 (Fig. 1, lane 4). Interestingly β-cells, but not non-β-cells, presented a phosphorylated band migrating at 60 kDa, which strongly increased in intensity following exposure to insulin (Fig. 1, lane 4) or IGF-1 (Data not shown). This band was comparable to that observed in the rat epididymal fat pad samples after exposure to 10^{-7} M insulin (Fig. 1, lane 6). Non-β-cells did not present insulin-phosphorylated proteins at these two molecular weigths (Fig. 2 lane 8).

Presence of $pp60^{IRS-3}$ mRNA in FACS purified β- and non-β-cells

Since we did not have access to antibodies specifically directed against IRS-3, we developed a RT-PCR to detect the mRNA encoding for this protein. The primers used were able to amplify the IRS-3 gene spanning an intron of 170 bp, in a 401 kb cDNA band (Fig. 3). Sequencing of this cDNA product by

automated DNA sequencing (ABI PRISM 310 Genetic Analyzer), showed 99% identity with the described sequence of rat IRS-3 (Accession No U93880) (data not shown). Using this RT-PCR we observed that β-cells, and to a less degree non-β cells, expressed IRS-3 mRNA (Fig. 3). When corrected for GAPDH expression, we still noticed a lower IRS-3 expression in non-β-cells (data not shown). Next we evaluated whether culture at different glucose concentrations would modify IRS-3 mRNA expression. Higher glucose concentrations induced an increase in medium insulin accumulation (Table 1). Medium insulin accumulation in non β-cells was always below 1% of the values observed in β-cells (data not show). However, these cells presented a 4-fold increase in insulin secretion when comparing cells cultured at 2.8 mM or 20 mM glucose (data not shown), consistent with a minor contamination by β-cells in these preparations. None of those different treatments modified IRS-3 mRNA expression (Table 1). In a separate series of experiments, we exposed β-cells for 24 h to the cytokine IL-1β (50 U/ml), a treatment previously shown to profoundly suppress β-cell function [16]. However, the cytokine failed to affect IRS-3 expression, which in IL-1β treated cells was 97 ± 9 % of that observed in control islets (n = 4).

4. DISCUSSION

It has been recently demonstrated that pancreatic β-cell lines express insulin receptor (IR) mRNA [6]. Insulin induces IR tyrosine kinase activity and leads to

activation of its intracellular signaling pathway in β-cell lines such as βTC3 [19]. Moreover, overexpression of the wild type IR in insulin-producing cells stimulates tyrosine kinase activity and increases insulin mRNA expression [10]. This, and the observations that mice with defective IRS-2 expression (IRS-2-/- mice) present β-cell failure and diabetes mellitus [12] while mice with β-cell-specific knockout of the insulin receptor have insulin secretory defects and glucose intolerance [20], suggest that insulin signaling is required for adequate β-cell function. In the present study we investigated the expression of IRS-2 in pancreatic β-cells purified from normal adult rats, and compared it with that of non-β cells. Immunoblot analysis indicated the presence of IRS-2 in β-cells, with much lower expression in non-β cells. We also detected a 60 kDa band in β-cells and in adipose tissue, but not in liver or in islet non-β–cells. The co-expression of a 60 kDa protein with IRS-2 in rat pancreatic β-cells raised the posibility of a cross-reacting phenomenon with the insulin receptor substrate 3 (IRS-3), which has been previously described in rat periepididimal adipose tissue [21-22]. The presence of 60-70 kDa phosphotyrosine proteins (p60-70), which are able to interact with IR, IRSs and p85 (the regulatory subunit of PI-3 kinase) after insulin stimulus, has also been described in rat hepatoma cells overexpressing normal insulin receptors (HTC-IR) [23-24] and in NIH 3T3 cells [25]. The adipocyte protein that binds PI-3 kinase was reported first [26], and this is probably the same protein recently characterized in rat adipocytes [21-22] and named insulin receptor substrate-3

($pp60^{IRS-3}$). Rat β-cells also contained a third proeminent band at 125 kDa, which was previously described in β-cells lines, and assumed to represent an early step in the insulin signal transduction pathway [17-18]. It is noteworthy that this 125 kDa protein is immunoreactive with anti-IRS-2 antibody in Western blots (Fig. 2) and is phosphorylated following exposure to insulin, suggesting an autocrine effect upon the β-cell phosphorylation state. Further studies are required to characterize the structure and function of this new protein.

Additional evidence for the presence of IRS-3 in β-cells comes from RT-PCR analysis indicating IRS-3 mRNA expression in these cells. Culture at different glucose concentrations, or in the presence of insulin or IL-1β, did not modify IRS-3 mRNA expression in β-cells.

IRS-3 protein generally resemble IRS-1 and IRS-2. It is composed of a plekstrin homology (PH) domain, a phosphotyrosine binding domain (PTB) and a short tail of tyrosine phosphorylation sites which engage and activate proteins with SH2 domains [13]. Moreover, insulin-exposed adipocytes present an early binding of IRS-3 to p85, which precedes IRS-1 or IRS-2 binding [27]. This suggests that IRS-3 may be the initial substrate of the activated insulin receptor and a strong activator of PI-3 kinase in the adipose tissue [28].

The present observation that IRS-3 is expressed in β-cells provides a new insight into the complex pathways regulating insulin signaling in these cells where, as in insulin target tissues, the activation of PI-3 kinase might represent a

crucial step [9]. In insulin responsive tissues PI-3 kinase activity is implicated in the stimulation of both general and growth-regulated protein synthesis [29], the stimulation of DNA synthesis [30], as well as the regulation of vesicle trafficking and glucose transport [31]. Studies performed in the insulin producing cell lines HIT-T15 [9] and βTC6-F7 [10], showed that PI-3 kinase may be involved in insulin-mediated up-regulation of insulin gene transcription, through the IRS-2/PI-3 kinase/p70 ribosomal s6 protein kinase and CaM kinase pathways [9] [32].

At present, the physiological significance for the presence of different isoforms of IRS in the same tissue is not understood, but it may reflect a "safe mechanism" to ensure adequate insulin signal transduction. In line with this possibility, IRS-2 apparently compensates for the absence of IRS-1 in hepatocytes from IRS-1-/- mice, but fails to do so in skeletal muscle or adipocytes [33-35]. Alternatively, these isoforms may have evolved to mediate specific and different functions associated to the insulin stimulus [36]. This may explain the differential time course and level of association between IRS proteins and PI-3 kinase in adipocytes [27]. Further studies are now required to clarify the putative contributions of IRS proteins, as well as the role for IRS-3 and PI-3 kinases, for insulin-and IGF-1 mediated signal transduction in pancreatic β-cells.

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LEGENDS TO THE FIGURES

Figure 1. **IRS-2 and p60 expression in islet β- and non-β-cells.** Western Blot of β-cells and non-β-cells (25 μg protein each) were performed as described under "Methods". The bands were detected with anti-phosphotyrosine antibody. Rat liver and adipose tissue were used as positive controls (100 μg protein each). *Lane 1.* Liver exposed to 22.2 mM glucose for 90 sec; *Lane 2.* Liver exposed to 10^{-7} M insulin for 90 sec; *Lane 3.* β-cells exposed to 22.2 mM glucose for 90 sec; *Lane 4.* β-cells exposed to 10^{-7} M insulin for 90 sec; *Lane 5.* periepididymal adipose tissue exposed to 22.2 mM glucose for 90 sec; *Lane 6.* periepididymal adipose tissue exposed to 10^{-7} M insulin for 90 sec; *Lane 7.* non-β-cells exposed to 22.2 mM glucose for 90 sec; *Lane 8.* non-β-cells exposed to 10^{-7} M insulin for 90. The blot is representative of four independent experiments.

Figure 3. **Expression of IRS-3 mRNA in rat β and non-β cells.** Poly(A)$^+$ mRNA was extracted from 10^5 rat β and non-β cells, reverse transcribed to cDNA and amplified by PCR. Each PCR reaction contained cDNA equivalent to 1.5 x 10^3 cells. Lane 1, β-cells; lane 2, non β-cells; lane 3, rat hepatocyte genomic DNA; M, pBR322/*Hae* III DNA marker. The sizes of PCR fragments of cDNA (401 bp) and genomic DNA (571 bp) are indicated. The figure is representative for four independent experiments.

Table 1. Medium insulin accumulation and IRS-3 mRNA expression in β–cells cultured under different experimental conditions

	G 2.8mM	G 10mM	G 22.2mM	G 2.8mM + 10^{-7} M Insulin
Medium insulin (**ng/10^3 cells**)	10±1	46±2[a]	62±4[a,b]	n.d.
IRS-3 mRNA (**O.D./GAPDH**)	1±0.1	0.9±0.1	0.6±0.1	0.9±0.1

Rat β-cells were cultured for 48 h (3 x 10^5 cells/condition) at 2.8mM glucose, 10mM glucose, 20mM glucose or 2.8 mM glucose + 10^{-7} M Insulin. After culture, insulin content of the medium was measured by radioimmunoassay (RIA). IRS-3 mRNA expression was determined by RT-PCR, quantified by densitometry and corrected for GAPDH expression. Data represents means ± SEM of 4 separate experiments. Statistical significance of differences was calculated by ANOVA. [a]$p<0.001$ vs G 2.8 mM; [b]$p<0.01$ vs G10 mM. n.d.: not determined.

IB: α PY