LILIA APARECIDA COLETTO VIEIRA DA SILVA

TRANSMISSÃO DO SINAL INSULÍNICO EM CORAÇÃO E AORTA: REGULAÇÃO EM MODELOS ANIMAIS DE RESISTÊNCIA À INSULINA.

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Tese apresentada no Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Doutor em Ciências Biológicas. Área de concentração: Fisiologia.

ORIENTADOR: Prof. Dr. Mário José Abdalla Saad

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Dedicatória

Dedico a minha querida mãe ,

Ao meu marido e aos meus filhos Mateus e Pedro Augusto

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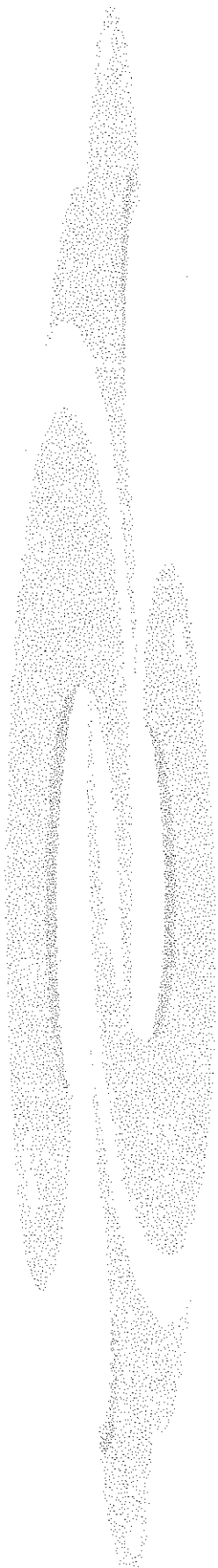
ATP	trifosfato de adenosina
AII	angiotensina II
AMPc	monofosfato de adenosina cíclico
BSA	albumina sérica bovina
DNA	ácido desoxirribonucléico
DTT	ditiotreitol
DEXA	dexametasona
EDTA	ácido etilendinitrilotetracético sal dissódico
EGF	fator de crescimento epidermal
FDG	fluorodeoxiglicose
Fyn	proteína quinase citoplasmática
GRB2	proteína ligadora do receptor para fator de crescimento
GTP	trifosfato de guanosina
GLUT	transportador de glicose
IR	receptor de insulina
IGF-1	insulin-like growth factor-1
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
^{125} I	isótopo radioativo do iodo
JAK2	proteína citoplasmática da família Janus

kDa	quilo Daltons
Mr	peso molecular
Nck	molécula adaptadora
pp185	proteína fosforilada de 185 kDa
PDGF	fator de crescimento derivado de plaqueta
PI 3-quinase	fosfatidilinositol 3-quinase
PKA	proteína kinase A
PKC	proteína kinase C
PSMF	fenil-metil-sulfonil-fluoreto
SDS-PAGE	gel de poliacrilamida e sódio dodecil-sulfato para eletroforese
Src	oncogene originalmente definido como produto do sarcoma
SH2	segunda homologia ao Src
SHPTP2	fosfatase tirosina específica
Shc	molécula adaptadora e substrato do receptor de insulina
Syp	fosfatase tirosina-específica
STZ	estreptozotocina
Tris	Tri(hidroximetil)-aminometano
Tyr	tirosina
μCi	microCuri

SUMÁRIO



RESUMO

A insulina estimula a atividade tirosina quinase de seu receptor, resultando na fosforilação de seu substrato citosólico, o IRS-1, que se associa à enzima fosfatidilinositol 3-quinase ativando-a. Estudos anteriores em modelos animais demonstraram que o IRS-1 apresenta uma regulação tecido-específica. No presente estudo, investigamos os níveis protéicos e os graus de fosforilação do receptor de insulina e do IRS-1, bem como a associação entre IRS-1 com a PI 3-quinase, após estímulo insulínico em coração de ratos, *in vivo,* em cinco modelos de resistência à insulina: ratos diabéticos pela estreptozotocina, tratamento crônico com dexametasona, tratamento agudo com adrenalina, envelhecimento e jejum prolongado. Caracterizamos também as etapas iniciais da ação insulínica em aorta de ratos e sua modulação no jejum prolongado.

Nossos resultados mostraram que não houve mudanças nos níveis e grau de fosforilação do receptor de insulina, determinado pelo immunoblotting com anticorpo antifosfotirosina em coração de animais diabéticos, ou tratados com dexametasona ou no envelhecimento. O tratamento com adrenalina induziu uma redução de 35% no grau de fosforilação em tirosina do receptor de insulina. Em amostras préviamente immunoprecipitadas com anticorpo anti-IRS-1 e incubadas com anticorpo antifosfotirosina, observamos uma redução de 25% e 75% nos níveis de fosforilação desta proteína em coração de ratos tratados com dexametasona ou adrenalina, respectivamente, mas não encontramos alterações significativas no coração de ratos diabéticos e velhos. Após estímulo insulínico, detectamos uma diminuição na associação IRS-1/PI 3-quinase em coração de ratos tratados com dexametasona, com adrenalina e nos animais diabéticos. Esta redução da interação IRS-1/PI 3-quinase pode contribuir para explicar a diminuição de captação de glicose observada no coração desses modelos animais de resistência à insulina. No envelhecimento, a ausência de alterações nas etapas iniciais de ação insulínica em coração contrasta com os resultados observados em fígado e músculo, e contribuem para justificar a utilização normal de glicose exclusivamente em coração de animais envelhecidos. Nos animais submetidos ao jejum prolongado, houve um aumento nos níveis de fosforilação do receptor de insulina e do IRS-1, quando estimulados pela insulina, acompanhado por um aumento da associação IRS-1/PI 3-quinase tanto em coração como em aorta. Essa modulação das etapas iniciais da ação insulínica descrita em coração e aorta no jejum é similar ao observado em fígado, músculo e tecido adiposo.

Nossos resultados demonstram que as proteínas envolvidas nas etapas iniciais da transmissão do sinal insulínico estão presentes em coração e aorta de ratos, e que apresentam uma regulação diferenciada dependendo do modelo analisado, contribuindo para explicar a modulação específica da utilização de glicose em coração nesses modelos animais.

1. INTRODUÇÃO

A insulina é o principal hormônio regulador do metabolismo de nutrientes. Em resposta ao aumento da concentração da glicose sangüínea, as células β das ilhotas pancreáticas liberam a insulina na corrente sangüínea, que é conduzida para os tecidos alvos. Nesses tecidos, promove o influxo de nutrientes e bloqueia a liberação de outras formas de energia estocadas (BIRNBAUM, 1993). Em músculos estriado e cardíaco estimula a síntese de proteínas e a captação de glicose, e no tecido adiposo ativa a lipogênese bloqueando paralelamente a lipólise. A insulina, também promove diversos efeitos em uma ampla variedade de células e tecidos (MYERS *et al.*, 1996).

As ações da insulina, na célula, iniciam-se pela ligação do hormônio a seu receptor na membrana plasmática (FREYCHET *et al.*, 1971; CUATRECASAS *et al.*,1972). Este receptor está virtualmente presente em todos os tecidos de mamíferos, variando em número (KAHN 1985). O receptor de insulina é uma glicoproteína heterotetramérica constituído por duas subunidades α, cada uma com peso molecular de 135.000, e duas subunidades β, cada uma com peso molecular de 95.000, ligadas por duas pontes de dissulfeto para dar a estrutura β-α-α-β (MISSAGUE *et al.*, 1991, KASUGA *et al.*, 1982). A subunidade α é inteiramente extracelular e contém o sítio de ligação da insulina. A subunidade β é uma proteína transmembrana responsável pela transmissão do sinal.

A subunidade α, que é extracelular, confere alta afinidade de ligação da insulina ao receptor. Os aminoácidos cisteína da subunidade α e da porção extracelular da subunidade β participam das pontes de dissulfeto entre as subunidade α e β. O componente transmembrana da subunidade β é responsável pela transmissão do sinal insulínico, para a porção citoplasmática.

A insulina liga-se à subunidade α e estimula a capacidade quinase da subunidade β de seu receptor, que se autofosforila. O ATP atua como doador de fosfato e a fosforilação ocorre exclusivamente em tirosina (WHITE 1984).

A aufosforilação do receptor de insulina ocorre através de uma cascata intramolecular de reações de fosforilações (WHITE *et al.*, 1988). Como resultado, pelo

menos cinco tirosinas na porção intracelular da cadeia β são fosforiladas, estando três delas nas posições 1158, 1162, 1163. Quando estão fosforiladas, a atividade quinase é implementada em direção a outros substratos exógenos (YU & CZECH 1984).

Em células intactas, o receptor é também fosforilado em aminoácidos serina e treonina (KASUGA *et al.*,1982). Isto pode ser estimulado por tratamento prolongado com insulina, ésteres de forbol e análogos do AMP cíclico, e presumivelmente é o resultado da fosforilação do receptor por 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 (TAKAYAMA *et al.*, 1988). Esta regulação da atividade quinase por fosforilações em aminoácidos distintos apresenta-se como um importante mecanismo potencial para regular o receptor de insulina em situações fisiológicas e patológicas (SAAD 1996).

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

Para propagar o sinal, a maioria dos receptores tirosina-quinase, uma vez autofosforilados, ligam-se diretamente às proteínas com domínio SH2. Ao contrário, o receptor de insulina apresenta uma variação desse modelo pois, uma vez autofosforilado, estimula sua atividade tirosina-quinase e fosforila proteínas intermediárias ou substratos do receptor responsáveis pela associação à proteínas com domínio SH2 (CHEATAM & KAHN,1995).

O primeiro e mais estudado substrato do receptor de insulina é uma proteína que migra numa banda de aproximadamente 185kDa, denominada pp 185, pela sua mobilidade eletroforética (WHITE *et al.*,1985).

A pp 185 foi clonada e denominada substrato 1 do receptor de insulina ou IRS-1 (SUN *et al.*,1991), e nos últimos anos, demonstrou-se que outra proteína também migra nesta banda, que recebeu o nome de IRS-2 (TAMEMOTO *et al.*,1994, ARAKI *et al.*,1994, SUN et al.,1995). O DNA complementar (cDNA) do IRS-1 prevê uma proteína de 1235 aminoácidos com diversas características interessantes. Primeiro, não há porção transmembrana, é uma proteína de localização citoplasmática. Há uma seqüência de aminoácidos compatível com a seqüência de ligação para nucleotídeos (ATP ou GTP), embora não haja nenhuma homologia com proteínas quinases conhecidas. Ainda em relação a homologia de seqüências de aminoácidos, há múltiplos sítios de fosforilação em serina, treonina e tirosina. Mais importante, o IRS-1 é um excelente substrato para o receptor de insulina e é fosforilado em múltiplos sítios tirosina após estímulo insulínico.

A capacidade do IRS-1 de ser fosforilado em tirosina após estimulação insulínica, e de participar na transmissão do sinal, depende de uma característica estrutural, que é a presença de múltiplos sítios tirosina em localizações estratégicas. Baseado em análise seqüencial, existem 22 sítios potenciais para fosforilação em tirosina presentes no IRS-1. Nove destes possuem uma de duas seqüências repetitivas: YMXM ou YXXM, onde Y é tirosina, M é metionina é X e qualquer outro aminoácido. Seguindo-se a estimulação insulínica, essas tirosinas, bem como outras tirosinas potenciais na molécula de IRS-1, são rapidamente fosforiladas. Essa fosforilação em vários sítios resulta em uma ligação não covalente do IRS-1 com proteínas intracelulares específicas (SHOELSON *et al.*,1992).

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

3-fosfato, sugerindo que a PI 3-quinase é realmente ativada durante a estimulação com esse hormônio.

Estudos anteriores demonstram que a associação do IRS-1 com a PI 3-quinase é essencial para o transporte de glicose em tecido muscular esquelético e cardíaco (TSAKIRIDIS *et al* ,1995). Além da PI 3-quinase, pelo menos três outras proteínas com porção SH2 podem se associar ao IRS-1 como: SH-PTP2, GRB2 e Nck. Essas associações, podem ter um importante papel no crescimento celular. (CHEATHAN & KAHN,1995).

O IRS-1 é uma proteína crucial no processo de transmissão do sinal insulínico, localizada estratégicamente na fase inicial desta sinalização. Atua como proteína "ancoradouro" que ativa diversas enzimas, sendo provavelmente um ponto de ramificação dos efeitos pleiotrópicos da insulina. Portanto, o estudo da regulação do IRS-1 em tecidos sensíveis à ação insulínica, pode contribuir para a compreensão de mecanismos moleculares pós-receptores de alteração da sensibilidade a este hormônio, em modelos animais de resistência à insulina.

Algumas dessas vias metabólicas de transmissão do sinal insulinico foram caracterizadas em cultura de células, ou em tecidos onde a insulina tem papel determinante na homeostase da glicose (SAAD *et al.*,1994). Vários estudos demonstram uma regulação tecido-específica dos níveis e grau de fosforilação do IRS-1 quando analisado em fígado, músculo ou tecido adiposo (SAAD, *et al.*,1995a; SAAD *et al.*,1995c; CARVALHO *et al.*,1996, VELLOSO *et al.*, 1995, CARVALHO 1997). Entretanto, a regulação do IRS-1 em tecido cardíaco não foi ainda investigada.

Nos últimos dez anos, diversos estudos demonstraram associações entre resistência à insulina ou hiperinsulinemia, hipertensão arterial, aterosclerose ou hipertrofia do miocárdio. (BARON *et al.*, 1994; SOUZA *et al*, 1995; KATZ, *et al.* 1995; CARVALHO *et al.* 1997). Estudos recentes demonstram que animais transgênicos, que não expressam o GLUT 4 (proteína transportadora de glicose sensível à insulina) apresentam resistência à insulina e hipertrofia marcante do miocárdio. Nesse sentido, a investigação das etapas iniciais da ação insulínica e sua regulação em coração e aorta

adquire importância, para uma melhor compreenção dos mecanismos moleculares que podem estar envolvidos nessas associações.

O objetivo do presente estudo foi investigar os níveis e grau de fosforilação do receptor de insulina e do IRS-1, bem como a interação deste com a PI 3-quinase em coração de ratos em cinco situações de resistência à insulina: diabetes induzido por estreptozotocina, tratamento crônico com dexametasona, tratamento agudo com adrenalina, envelhecimento e jejum prolongado. Constou também dos objetivos investigar as etapas iniciais da ação insulínica em aorta de ratos e sua regulação no jejum prolongado.

2. PUBLICAÇÕES

REGULATION OF IRS-1 IN THE HEART OF INTACT RATS: EFFECTS OF STZ-DIABETES, DEXAMETHASONE AND AGING.

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ABSTRACT

Insulin receptor, IRS-1 and PI 3-kinase represent three of the earliest steps in insulin action. It was previously demonstrated that IRS-1 and PI 3-kinase have a tissue-specific regulation.

In the present study we have examined the levels and phosphorylation state of the insulin receptor and insulin substrate 1 (IRS-1) as well as the association between IRS-1 and phosphatidylinositol 3-kinase (PI 3-kinase) in the heart of three animal models of insulin resistance: STZ diabetes, dexamethasone treated rats and aging. We infused insulin in vivo, extrated the heart and then performed by immunoprecipitation and immunoblotting with anti-insulin receptor, anti-IRS-1, anti-PI 3-kinase and antiphosphotyrosine antibodies. There were no changes in the insulin receptor concentration and the insulin-stimulation of receptor autophosphorylation, as determined by immunoblotting with antiphosphotyrosine antibody in heart of all experimental models. IRS-1 protein levels did not changes in heart of the three groups of animals. In samples previously immunoprecipitated with anti-IRS-1 antibody and blotted with antiphosphotyrosine, the insulin-stimulated IRS-1 phosphorylation levels in the heart of dexamethasone-treated rats decreased 74 ± 4% ($p<0,05$), but no changes was observed in the other models. The insulin-stimulated IRS-1/PI 3-kinase association decreased to 39 ± 13% ($p<0,05$) in heart of desamethasone-treated rats and also decreased to 47 ± 14% in STZ-diabetc rats.

These results suggest that the reduction in IRS-1 tyrosine phosphorylation and/or association with PI 3-kinase may have a role in the reduced glucose utilization describe in heart in STZ diabetes and dexamethasone treated rats. In parallel, the absence of changes in the early steps of insulin action is in accordance with normal insulin-induced glucose uptake in heart of aging rats.

INTRODUCTION

Insulin stimulates a diverse array of cellular responses, including cell growth and metabolic reponses such as glucose uptake and glycogen synthesis. Ligand activation of the tyrosine kinase domain of the insulin receptor is probably the central event in mediating a cellular insulin response (39). One function of the tyrosine kinase is to autophosphorylate the insulin receptor itself, which leads to activation of the tyrosine kinase catalytic domain (6,15). A second important function of the tyrosine kinase is to stimulate tyrosine phosphorylation of several intermediated proteins, including the insulin receptor substrates (IRSs), as IRS-1 (26), IRS-2 (34), IRS-3 (21), IRS-4 (22). These molecules, rather than the insulin receptor itself, then couple to a downstream signaling pathway by serving as binding sites for src homology (SH2) domain containing signaling molecules (39). IRS-1 binds to the 85 kDa subunit of phosphatydilinositol 3-kinase (PI 3-kinase) thus activating this enzyme (2). Previous studies demonstrated that IRS-1 and PI 3-kinase have a tissue specific regulation, and this can contribuite to explain the differential effects of insulin in situations of insulin resistance (27).

Insulin resistance is defind as a subnormal biologic response to a given concentration of insulin. It is well known that the effects of insulin are pleiotropic, but the term "insulin resistance" usually refers to the action of insulin on glucose homeostasis (30). Many physiologic states and circulating factors can adversely affect the action of insulin. Glucocorticoids, glucagon, catecholamines, and growth hormone induce insulin resistance in cases of excess secretation of individual hormones which occurs in specific endocrinopathies. Several complex physiologic and pathologic states, incluing obesity, NIDDM, diabetc ketoacidosis, uremia, cirrhosis, fasting, pregnancy, hypertension, puberty, and aging are associated with resistance to insulin (30).

Heart is a insulin sensitive tissue (37), but the regulation and phosphorylation of insulin receptor and IRS-1 in rat heart *in vivo*, have not been examined. In the present study we have investigated the phosphorylation state of the insulin receptor and of IRS-1 as well as IRS-1/PI 3-kinase in the heart of three models of insulin resistance: STZ-diabetic, dexamethasone-treated and aging rats.

MATERIALS AND METHODS

Materials: The reagent for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonyl fluoride (PMSF), aprotinin, dithiothreitol, Triton X-100, Tween 20, Streptozoticin, glycerol were obtained from Sigma Chemical Co.(St Louis, MO). Protein A Sepharose 6MB was obtained from Pharmacia (Uppsala, Sweden), [I^{125}] protein A from Amersham (Avlesbury,U.K.), ande nitrocellulose paper (BA85, 0.2μm) from Schleicher& Schuell (Keene, NH). Sodium thiopental and human recombinant insulin (Humulin R) were obtained from Eli Lilly (Indianapolis, IN). Male rats were obtained from the UNICAMP Central Breeding Center .Monoclonal antiphosphotyrosine antibodies and anti PI 3-kinase (p85) antibody were obtained from Upstate Biochnology (Lake Placid, NY), and IRS-1 and anti-insulin receptor antibody, from Santa Cruz Technology (Santa Cruz, CA).

Animals: Male Wistar rats age 3-month-old were provided with standard rodent diet and water ad libitum. Food was withdrawn 12-14 h before experiments. Diabetes was induced with streptozotocin (STZ) in citrate buffer, pH 4.5, administered e.v. in single dose of 75 mg\kg body wt to overnight fasted rats. Diabetes rats were studied with 300mg % of glicemia The others rats were injected daily at ~ 10:00 am. with 1 mg/100g i.p. dexamethasone or an equal volume of saline (controls) for 5 days. And finally we used old rats with 20 months or young rats (3 months). The studies were performed in parallel for the controls and experimentals rats in each pair.

Methods: Rats were anaesthesed with sodium amobarbital (15 mg/kg bw, i.p.) and used experiments 10 - 15 minutes later i.e. as soon as anaesthesia was assured by loss of pedal and corneal reflexes. The abdominal cavity was opened, the cava vein was exposed, and 0.5 ml normal saline (0.95 NaCl) with or without 10^{-5} M insulin was injected. After 90 sec., the piece heart was removed, minced coarsely, and homogenized immeditely in aproximately 6 volumes of solubilization extration Buffer B using a polytron PTA 20S generator (Brinkmann Instruments model pT 10\35, Westbury, NY) operating at maximum speed for 30 sec. The buffer B contained 1% triton X-100, 10 mM Tris (pH 7.4), 100 mM sodium pirophosphatase, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium

vanadate, 2 mM PSMF and 0.1 mg\ml aprotinin at 4°C. After centrifugation, the supernatant solution of the tissue was than used for protein determination the Bradford (4) dye binding method using the Bio-Rad reagent and bovine serum albumin (BSA) as the standard. The supernatants used for immunoprecipitation with α IR, αIRS-1 and α PI 3-kinase antibodies and protein A-Sepharose 6 MB.

Protein analysis by immunoblotting: The precipitated proteins were treated with Laemmli sample buffer (20) containing 100 mmol/1dithiothreitol and heated in a boiling water bath for 4 min., after with they subjected to SDS-PAGE (6% bis-acrylamide) in a Bio-Rad miniture apparatus (Mini-Protean, Bio-Rad Laboratories, Richmond, CA).

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 describe by Towbin et al.(36), except for addition of 0.02% SDS to the transfer buffer to enhance the elution of high mW, proteins. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4°C in blocking buffer (5% nonfat dry milk,10 mmol/1 Tris, 150 mmol/1 NaCl, and 0.02% Tween 20). The nitrocellulose blot was incubated with antiphosphotyrosine antibodies (1μg/ml) or with PI 3-kinase (1:500) diluted in blocking buffer (0.3% BSA insted of non fat dry milk) overnight at 4°C and then washed for 60 min. with the blocking buffer without milk. The blots were subsequently incubated with 2 μCi of [^{125} I]protein A (30μCi/μg) in 10 ml of blocking buffer for 2 h at room temperature, and then washed again for 30 min.as describe above. [^{125} I]Protein A bound to the antiphosphotyrosine and antipeptide antibodies was detect by autoradiography using preflashed Kodak XAR film (Eastman Kodak,Rochester, NY) with Cronex lightning Plus intensifying screens (DuPont, Wilmington, DE) at -80°C for 12-48 h. Band intensities were quantitated by optical densitometry (Eagle Eye) of the developed autoradiographs.

Statistical analysis: Experiments were always performed by stuying groups of animals in parallel. For comparisons, Student's unpaired *t* test was used. The levels of significance was set at $P<0.05$.

RESULTS

Effect of STZ diabetes on insulin receptor and IRS-1 phosphorylation levels in the rat heart.

In STZdiabetc rats, there were no changes in the insulin receptor levels in heart as shown by immunoblotting with an antibody to the COOH terminus of the insulin receptor (Fig.1A), insulin induced receptorphosphorylation was similar in control and STZ diabetic rats (Fig.1B). In contrast, he levels of IRS-1 protein in heart were reduced to 67 ± 7% ($p<0,05$) in STZ treates rats (Fig.2A).

In samples from heart previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antiphosphotyrosine antibody there was a small increase in insulin-stimulated IRS-1 phosphorylation to 126 ± 36% of the control value, but not significant (Fig2B).

Previous studies (Kelly e saad) have suggested that there is a relatively stable, high affinity interaction between IRS-1 and 85 kDa subunit of PI 3-kinase, such that both proteins are co-precipitated by antibodies to each protein. After stimulation with insulin, a band with the expected molecular weight of the regulatory subunit of PI 3-kinase (85 kDa) was present in anti-IRS-1 antibody immunoprecipitates of heart from experimental and controls animals. This observation is consistent with the existence of a stable complex between IRS-1 and PI 3-kinase. However, the amount of PI 3-kinase associatd with IRS-1 was reduced by 47 ± 6% ($p<0.05$) in STZ diabetic rats (Fig.2C).

Effect of chronic treatmet with dexamethasone on insulin receptor and IRS-1 phosphorylation in rat heart.

As in the heart, dexamethasone treatment produced no significant change in the insulin receptor protein level (Fig.3A) as well as following stimulation with insulin, phosphorylation of the insulin receptor (Fig.3B).

Similary, no significant change occured in the level of IRS-1 protein in heart of desamethasone treated rats when compared to the controls (Fig.4A). To define better the

level of IRS-1 phosphorylaiton, 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 reduction to 74 ± 4% ($p<0.05$, n=6) in insulin stimulated IRS-1 phosphorylation in the heart of animals treated with desamethasone. To examine the association of 85 kDa subunit PI 3-kinase with IRS-1, blots of samples which had been previously immunoprecipitated with anti-IRS-1 antibody were incubated with anti-PI 3-kinase antibody. As expected, in both groups na 85kDa band was present in the IRS-1 immunoprecipitates after exposure to insulin and there was a decrease to 39 ± 13 % ($p<0.05$, n=4) in the intensity of this band in desamethasone treated rats (Fig.4C).

Effect of aging on insulin receptor and IRS-1 phosphorylation in rat heart.

There was no change in insulin receptor levels in heart of rats 2-20 months old, as determined by immunoblotting with an antibody to the COOH-terminus of the insulin receptor (Fig.5A). Insulin receptor phosphorylation, induced by insulin, was similar in young and old rats (Fig.5B). Using the specific antipeptide antibody against IRS-1, there was no change in IRS-1 protein in heart during aging (Fig.6A).

Heart samples were also immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antiphosphotyrosine antibody. The level of insulin-stimulated IRS-1 phosphorylation was similar to the controls (Fig.6B).

After stimulation with insulin, a band with the expected molecular weight of the regulatory subunit of PI 3-kinase (85 kDa) was present in anti-IRS-1 antibody immunoprecipitates of heart from old rats. This observation is consistent with the existence of a stable complex between IRS-1 and PI 3-kinase. However, there was no change of PI 3-kinase associated with IRS-1 in old when compared with young rats (Fig.6C).

DISCUSSION

Insulin binding to the insulin receptor leads to the tyrosine phosphorylation of several insulin receptor substrates (IRSs), including IRS-1, IRS-2, IRS-3, IRS-4, Shc and Jak2 (21,46,52). These insulin receptor substrates then bind to a number of proteins in the cell via interaction of tyrosine phosphorylation mofis. and specific domains of the target proteins termed SH2 (Src homology 2/domains (34). For IRS-1, the SH2 proteins include PI 3-kinase, the cytoplasmatic tyrosine kinase Fyn, the protein tyrosine phosphatase SHP2, and the adaptor molecules Nck and Grb2 (25). Exactly how IRS-1 binding to SH2 domain proteins results in signal transduction is still not understood, but for PI 3-kinase binding of phosphorylated IRS-1 results in stimulation of enzymatic activity (23).

The regulation of IRS-1 tyrosine phosphorylation and interaction with PI 3-kinase in liver, fat and muscle have been shown to be tissue-specific (34,38). However the modulation of these early steps in insulin action in heart in situations of insulin resistance have not yet been investigated. Futhermore, in some situation of insulin resistance in humans and animals it has been described reduced glucose uptake in skeletal muscle but not in heart. In the present study, we have evaluated the modulation of insulin receptor and IRS-1 phosphorylation and also the association of PI 3-kinase with IRS-1 in heart of three animal models of insulin resistance: STZ-diabetes, dexamethasone-treated rats and aging.

Streptozotocin diabetic rats are characterized by insulin deficiency and insulin resistance(3,18). The insulin resistance is observed in liver, muscle and has also been describe in heart. In heart there is a decrease in insulin-induced glucose uptake and metabolism in 3 day diabetic rats. The molecular mechanism responsible for this loss of insulin sensitivity is not known (42).

The present study show that there is no change in insulin receptor protein level and insulin-induced receptor and IRS-1 tyrosine phosphorylation levels in heart of STZ diabetes, in spite of a reduction of ~ 40% in IRS-1 protein levels. Another possible mechanism that may have a role in this reduced interaction is an increase in serine/threonine phosphorylation of IRS-1. In this line, its is well established that there is an

increase in PKC activity in heart of diabetc rats (1), and PKC can induce serine and/or threonine phosphorylation of IRS-1 without affecting its tyrosine phosphorylation (31). In accordance, recently Kolter et al. demonstrated that in isolated ventricular cardiomyocytes of obese Zucher rats there is an increase in serine phosphorylation level of IRS-1 (without changes in tyrosine phosphorylation levels) which reduces its interaction with PI 3-kinase. These data suggest that in heart of these animals, a decrease in IRS-1 protein level is not sufficient to induce a reduction in the tyrosine phosphorylation level of this protein, which is mainly driven by insulin receptor. On the other side, there was a reduction in insulin induced IRS-1 association with PI 3-kinase. In this regard IRS-1 protein levels may have a role in this interaction, because there are specific tyrosines that are involved in IRS-1/PI 3-kinase association(37) and an increase in stoichiometry of IRS-1 phosphorylation may not be accompained by an increase in its association with PI 3-kinase. These decrease in IRS-1/PI 3-kinase association in heart is different from liver and muscle where we found an increase in insulin receptor and IRS-1 tyrosine phosphorylation levels acompained by an increase in IRS-1/PI 3-kinase association (37), demonstrating a tissue-specific regulation of early steps in insulin action in STZ diabetes.

Dexamethasone treatment induces marked insulin resistance in rats as evidenced by altered glucose metabolism in both peripheral tissue and liver, as well as decreased insulin-stimulated amino acid uptake in muscle, and decreased lipogenesis in adipocytes (2,7,14,38). Previous study using fluorodeoxyglucose (FDG), demonstrated that dexamethasone reduces in a similar pattern both skeletal muscle and myocardium FDG uptake. In both tissues after dexamethasone-treatment there is no change in the amount of GLUT 4 . The results of the present study demonstrated that after 5 days of dexamethasone there is a decrease in IRS-1 protein and phosphorylation and also a decrease in IRS-1/PI 3-kinase association. This decrease in association was also observed in other tissues, suggesting that there is a widespread reduction in insulin signal transduction after treatment with dexamethasone. Whether the changes induced by dexamethasone in early steps in insulin action in heart is secundary to the hyperinsulinemia of these animals remains to be determined. In this regard, we and other have demonstrated that chronic exposure of different cell lines to insulin induced a decrease in IRS-1 protein and phosphorylation levels and also in association with PI 3-kinase. In situation of hyperinsulinemia the

decrease in IRS-1 protein levels may be consequence of an increase in rate of degradation of this protein(2,7).

The reduction in insulin-induced in IRS-1/PI 3-kinase association in STZ-diabetes or in dexamethasone treated rats may have a role in reduced glucose uptake in the heart of these animals. The regulation of GLUT 4 translocation to the plasma membrane is essential for the rapid effect of insulin on glucose uptake in heart. It is sequestered in an intracellular vesicular compartment under basal conditions, and insulin stimulates its accumulation at the plasma membrane which stimulates glucose influx (35). Considerable evidence now indicates that activation of PI 3-kinase during association with IRS-1 provides one of the essential signals for GLUT 4 translocation.

A well studied rodent model of aging is the 20 month old rat. The insulin resistance that develops during aging is associated with an increase glucose intolerance (6). The rate of glycogen synthesis in response to stimulation by insulin is markedly decreased in the soleus muscle of 85-week-old Wistar rats (3), and impaired insulin-stimulated glucose uptake due to a depletion of the pool of glucose transporters has been observed (35). A decrease in the inhibition of hepatic glucose production by physiological concentrations of insulin has also been implicated as one of the contributing elements (6). Its is interesting that in spite of a reduced transport in muscle of 20 months old rats, the uptake of glucose by heart at saturating insulin concentrations was similar in young and old animals, suggesting that the maximal capacity of glucose utilization remais unaltered in heart with aging (8,16). Interesting, in this study we demonstrated that in heart of old rats there is no change in insulin-induced receptor and IRS-1 tyrosine phosphorylation levels, as well as association of the latter with PI 3-kinase. This is in contrast to muscle were we previously demonstrated an important reduction in IRS-1 protein and tyrosine phosphorylation level and association with PI 3-kinase in old rats. Taken together these results suggest that tissue-specific regulation of IRS-1 may contribute to explain the differences in glucose uptake between heart and skeletal muscle of old rats.

These results suggest that the reduction in IRS-1 tyrosine phosphorylation and/or association with PI 3-kinase may there a role in the reduced glucose utilization described in heart in STZ diabetes and dexamethasone treated rats. In parallel, the absence

of changes in the early steps of insulin action is in accordance with normal insulin-induced glucose uptake in heart of aging rats.

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LEGENDS

FIG.1. Insulin receptor (IR) phosphorylation in heart of STZ diabetc rats. Normal saline (lanes 1 and 3) or 10^{-5} mM of insulin (lanes 2 and 4) were administered into the the cava vein as a bolus injection; 90 s later, the heart was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-insulin receptor antibody, and immunobloted with anti-insulin receptor (A) and antiphosphotyrosine antibody (B). The insulin receptor protein levels and phosphorylation are shown as means ± SE of the scanning densitometry of six experiments. * Significant differences at least at $p<0.05$.

FIG.2. IRS-1 phosphorylation in heart of STZ diabetc rats. Normal saline (lanes 1 and 3) or 10^{-5} mM of insulin (lanes2 and 4) were administered into the cava vein as a bolus injection; 90 s later, the heart was excised and aliquots containing the same amount of protein were immunoprecipitaded with anti-IRS-1 antibody and immunoblotted with anti-IRS-1 (A), antiphosphotyrosine (B), and anti-PI 3-kinase (C) antibodies. The IRS-1 protein level, insulin-stimulated IRS-1 phosphorylation, and PI 3-Kinase association are shown as means ± SE of the scanning densitometry of six experiments. * Significant differences at least at $p<0.05$.

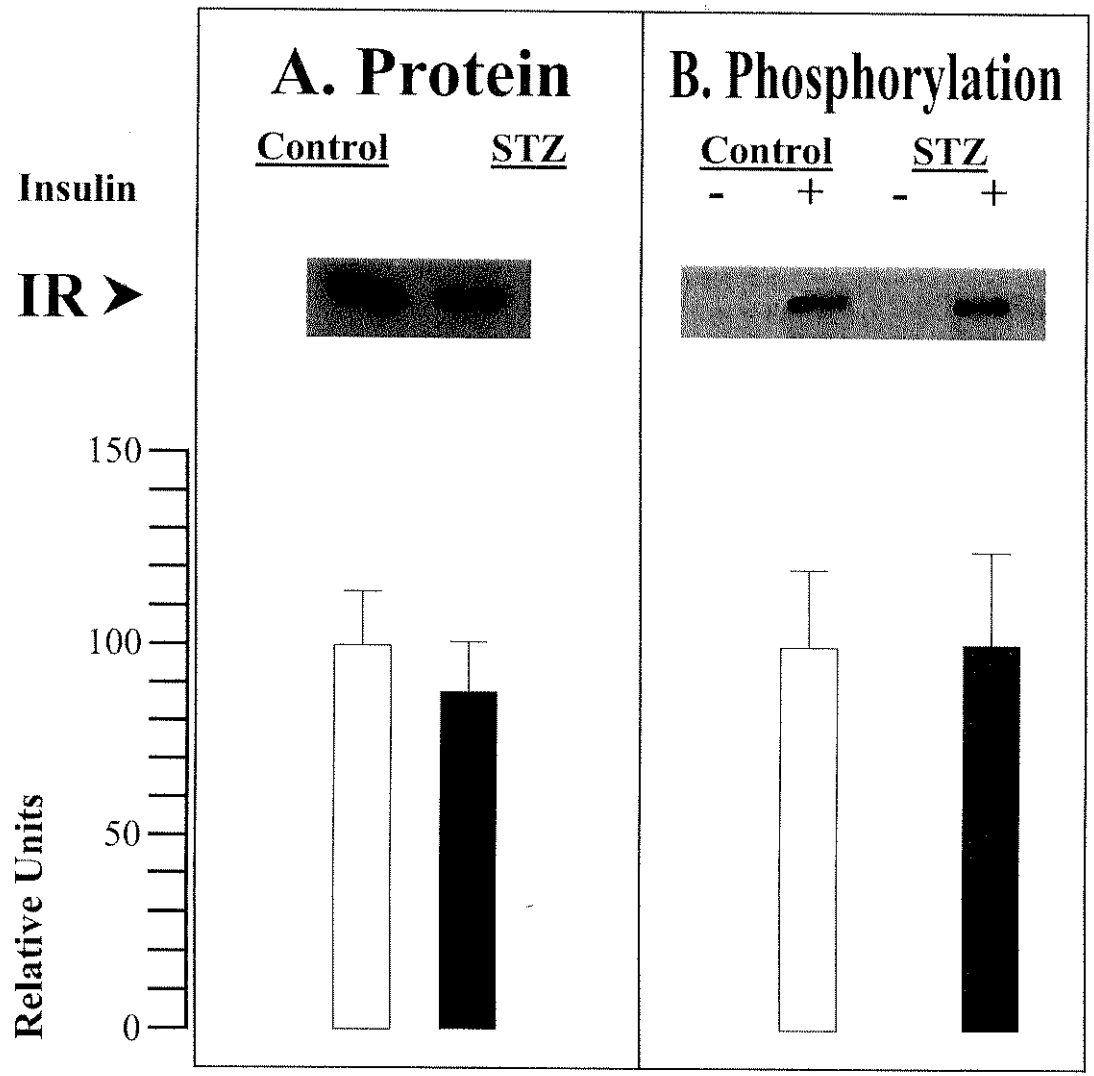
FIG.3. Insulin receptor (IR) phosphorylation in heart of dexamethasone-treated rats. Normal saline (lanes 1 and 3) or 10^{-5} mM of insulin (lanes 2 and 4) were administered into the the cava vein as a bolus injection; 90 s later, the heart was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-insulin receptor antibody, and immunobloted with anti-insulin receptor (A) and antiphosphotyrosine antibody (B). The insulin receptor protein levels and phosphorylation are shown as means ± SE of the scanning densitometry of four experiments. * Significant differences at least at $p<0.05$.

FIG.4. IRS-1 phosphorylation in heart of dexamethasone-treated rats. Normal saline (lanes 1 and 3) or 10^{-5} mM of insulin (lanes2 and 4) were administered into the cava vein as a bolus injection; 90 s later, the heart was excised and aliquots containing the same amount of protein were immunoprecipitaded with anti-IRS-1 antibody and immunoblotted with anti-IRS-1 (A), antiphosphotyrosine (B), and anti-PI 3-kinase (C) antibodies. The IRS-1

protein level, insulin-stimulated IRS-1 phosphorylation, and PI 3-Kinase association are shown as means ± SE of the scanning densitometry of six experiments. * Significant differences at least at $p<0.05$.

FIG.5. Insulin receptor (IR) phosphorylation in heart of aging rats. Normal saline (lanes 1 and 3) or 10^{-5} mM of insulin (lanes 2 and 4) were administered into the the cava vein as a bolus injection; 90 s later, the heart was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-insulin receptor antibody, and immunobloted with anti-insulin receptor (A) and antiphosphotyrosine antibody (B). The insulin receptor protein levels and phosphorylation are shown as means ± SE of the scanning densitometry of six experiments. * Significant differences at least at $p<0.05$.

FIG.6. IRS-1 phosphorylation in heart of aging. Normal saline (lanes 1 and 3) or 10^{-5} mM of insulin (lanes2 and 4) were administered into the cava vein as a bolus injection; 90 s later, the heart was excised and aliquots containing the same amount of protein were immunoprecipitaded with anti-IRS-1 antibody and immunoblotted with anti-IRS-1 (A), antiphosphotyrosine (B), and anti-PI 3-kinase (C) antibodies. The IRS-1 protein level, insulin-stimulated IRS-1 phosphorylation, and PI 3-Kinase association are shown as means ± SE of the scanning densitometry of nine experiments. * Significant differences at least at $p<0.05$.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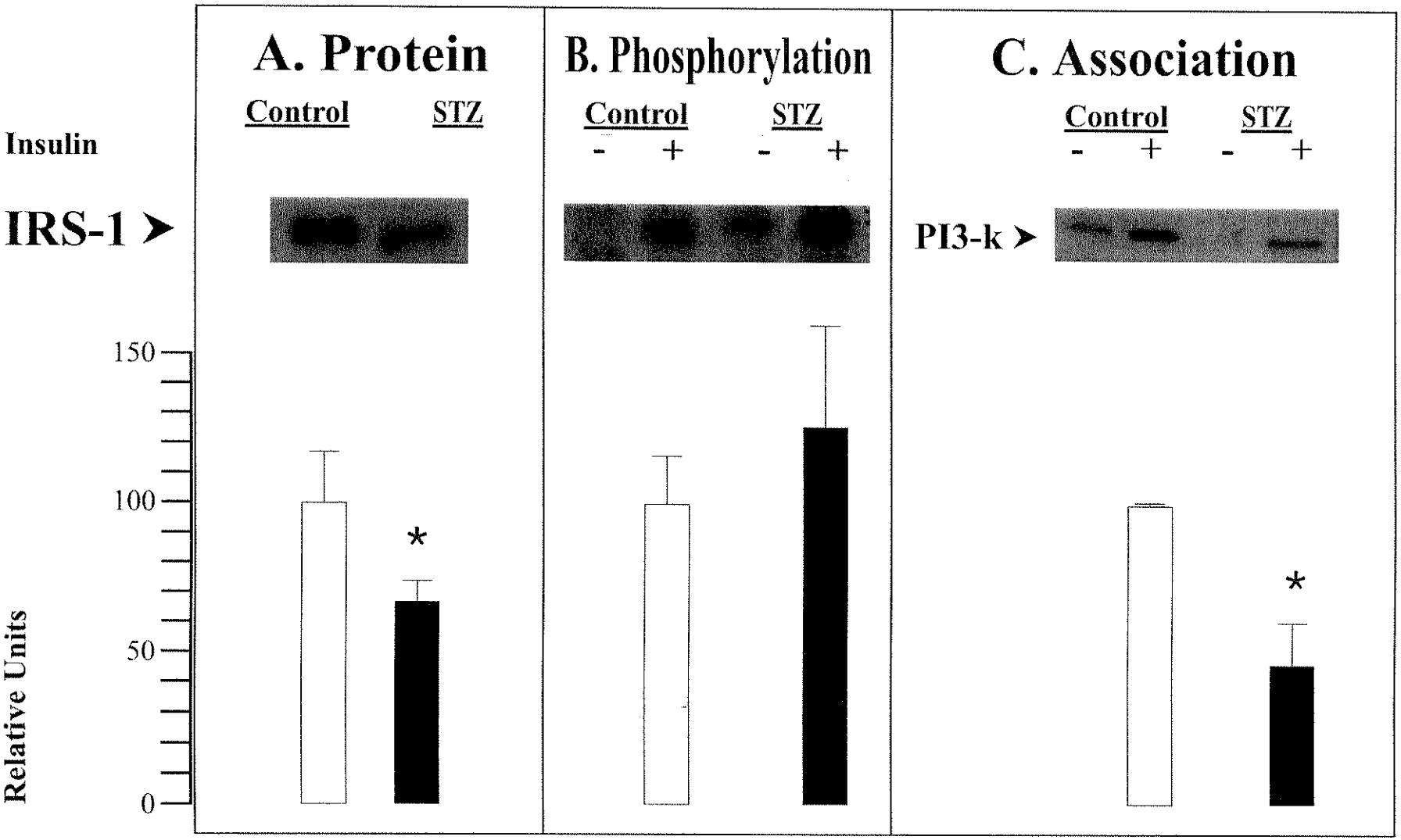
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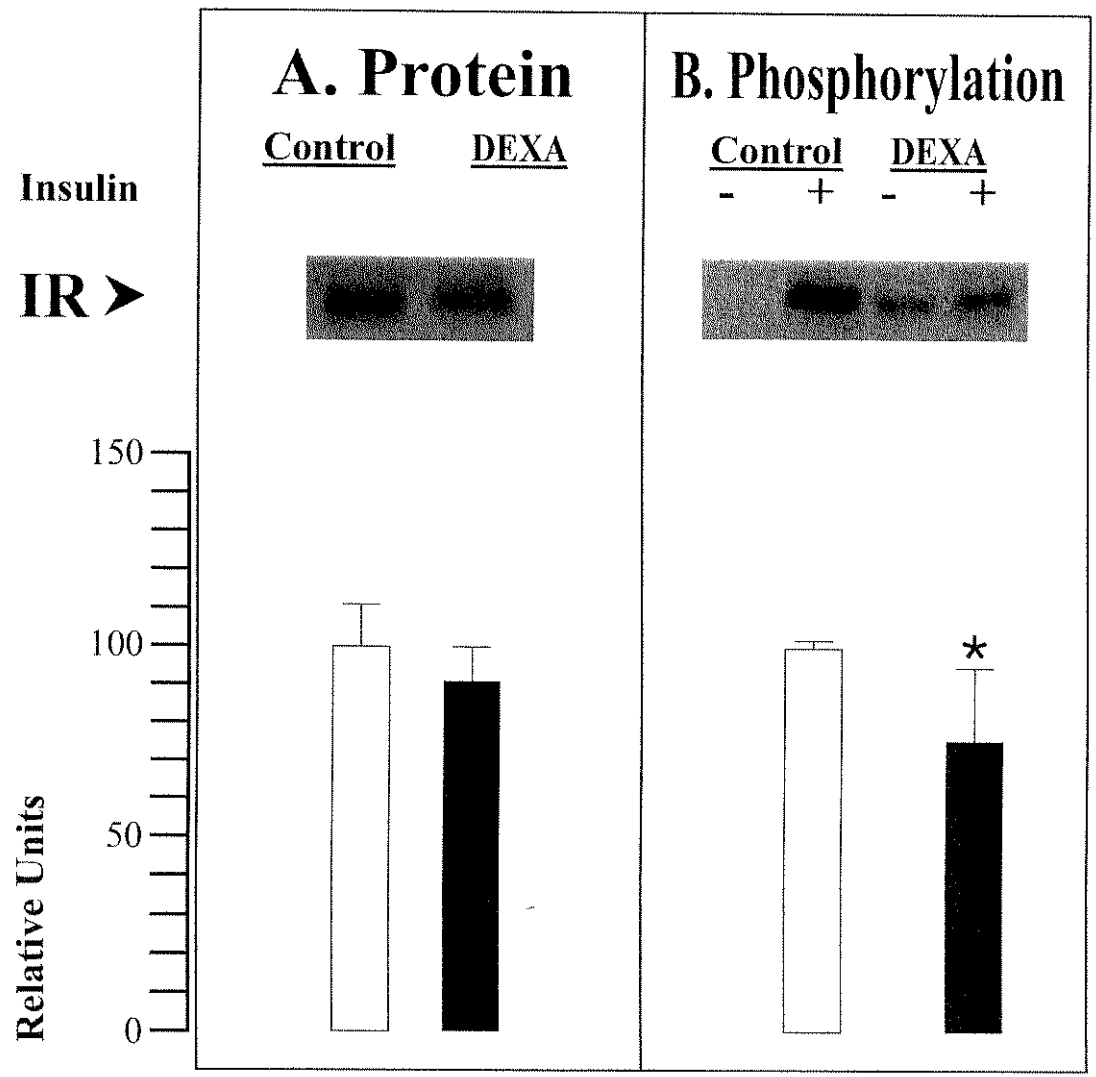
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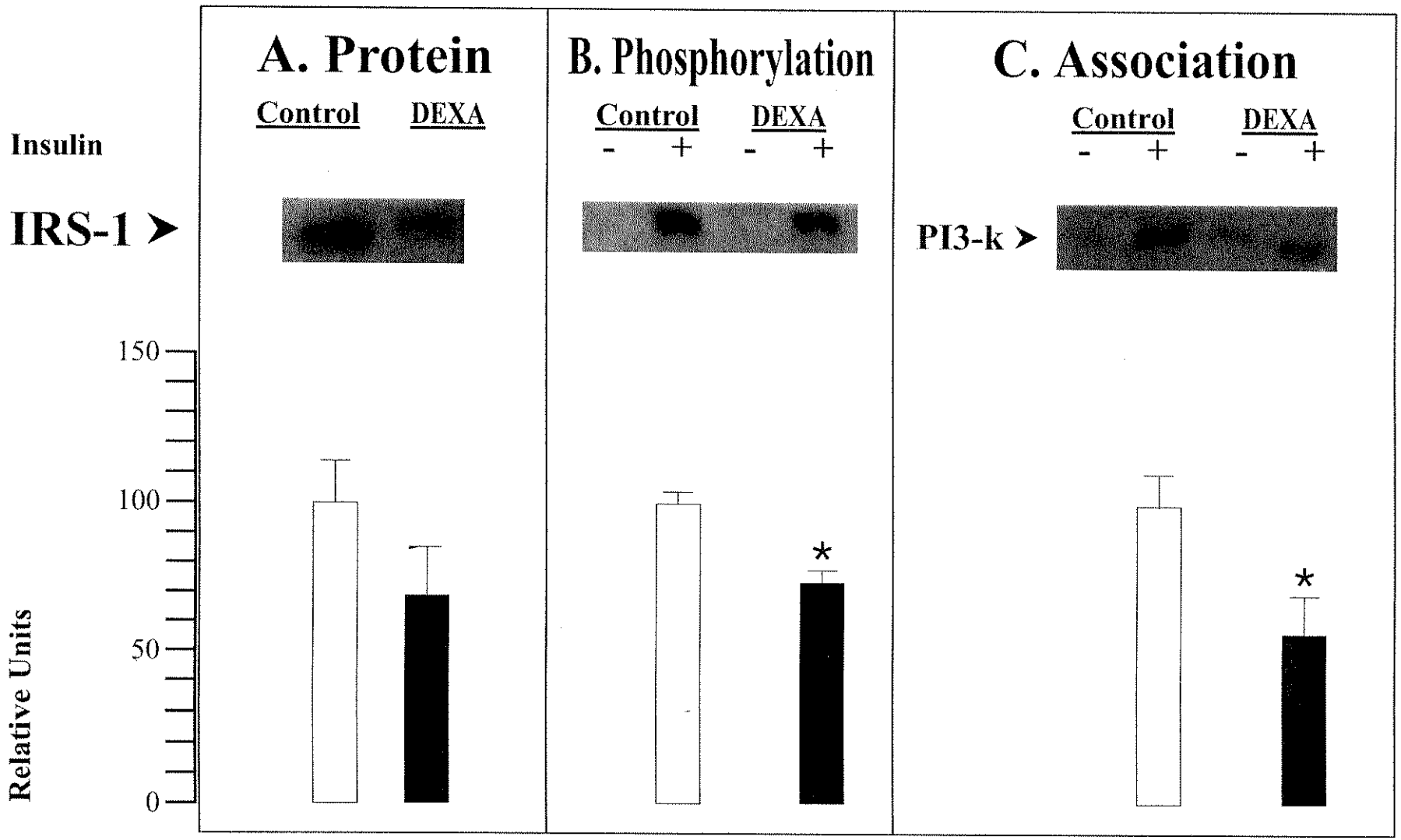
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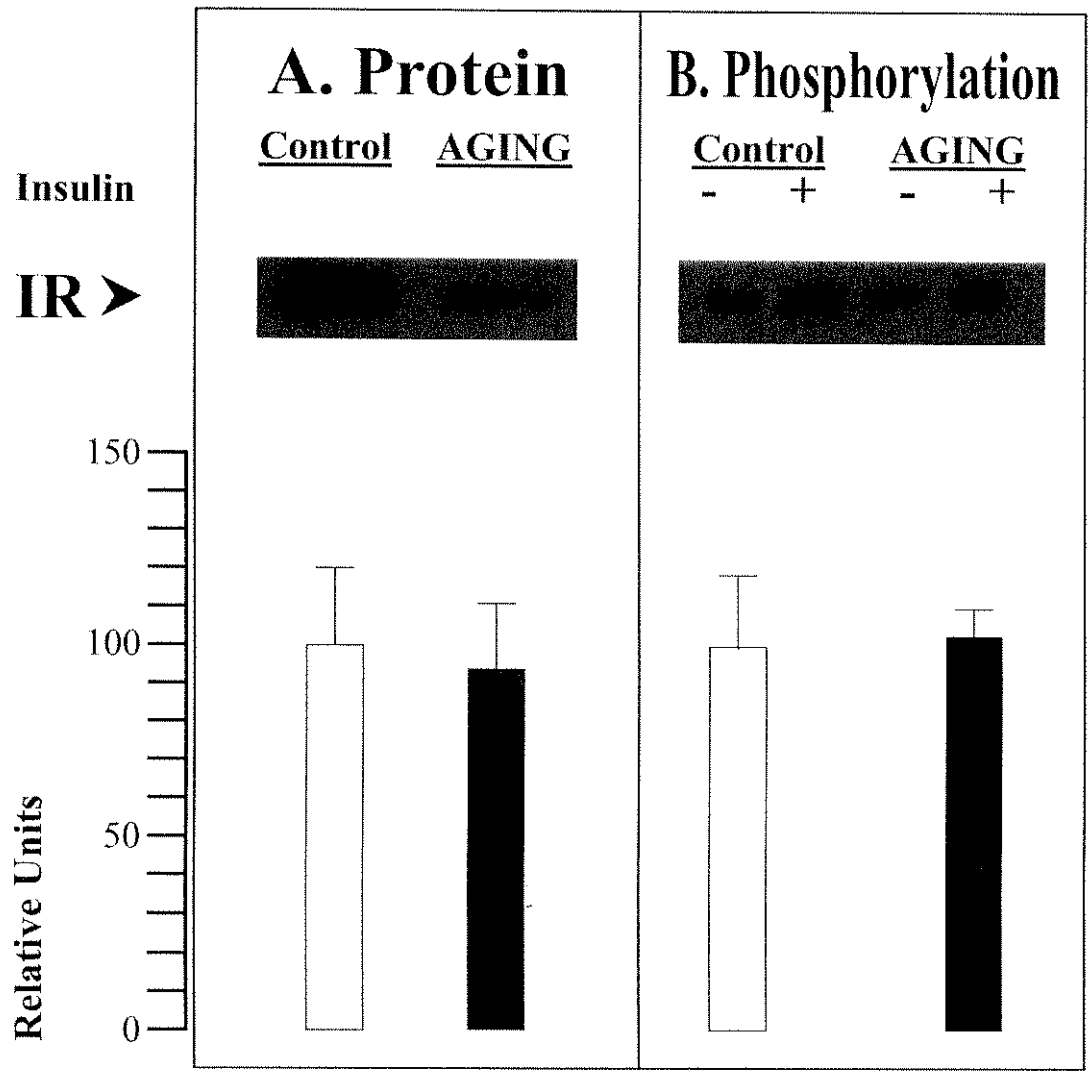
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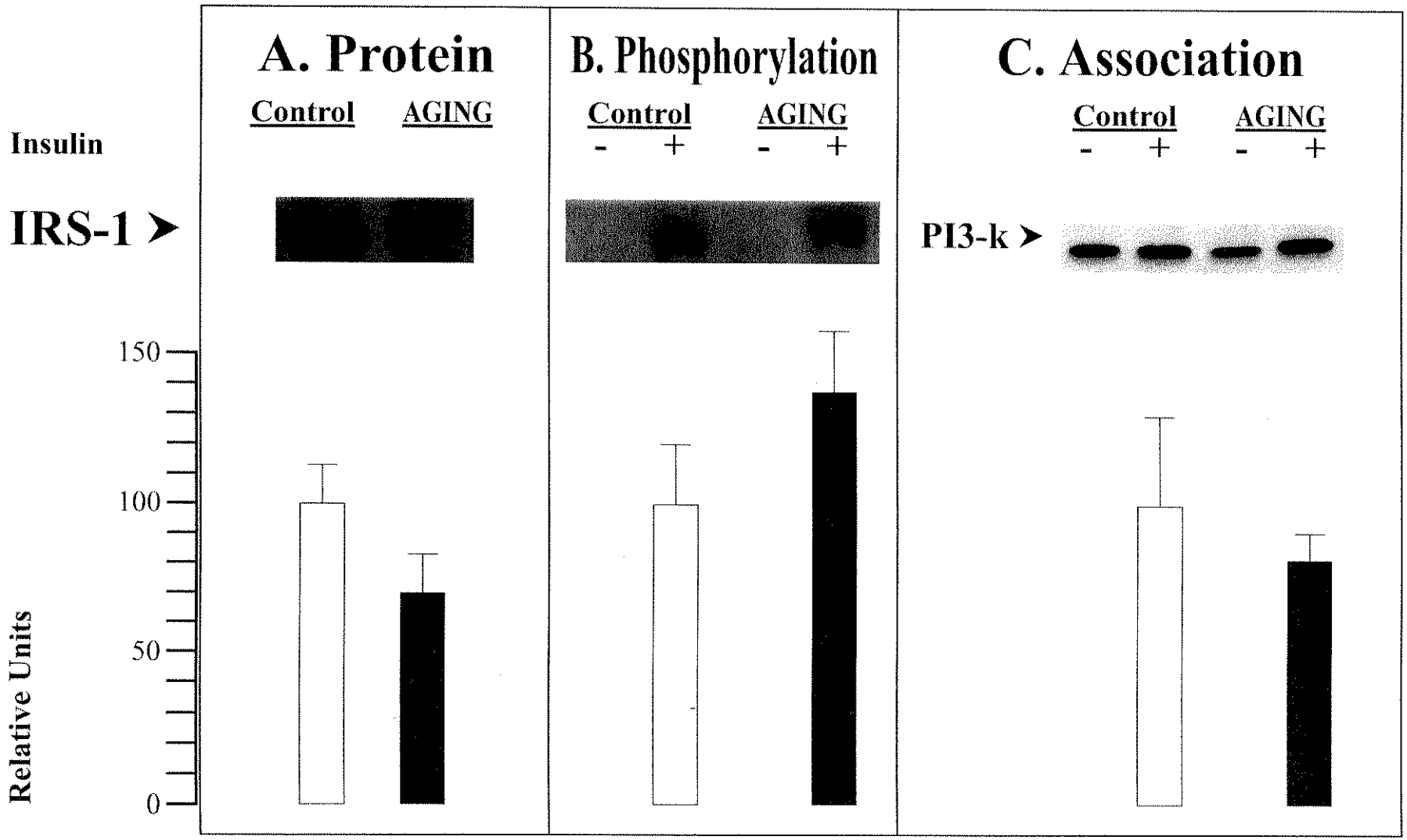
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EFFECTS OF FASTING AND EPINEPHRINE TREATED RATS ON INSULIN SIGNALING IN THE HEART OF INTACT RATS.

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ABSTRACT

In the present study we have examined the levels and phosphorylation state of the insulin receptor and insulin substrate 1 (IRS-1) as well as the association between IRS-1 and phosphatidylinositol 3-kinase(PI 3-kinase) in the heart of animal models of insulin resistance: fasting and epinephrine treated rats. The results demonstrated an increase in insulin stimulated receptor and IRS-1 phosphorylation levels which was accompained by an increased in the association of IRS-1/PI 3-kinase in vivo in heart of fasting rats. In epinephrine treated rats showed there was a decrease in insulin stimulated receptor and IRS-1 phosphorylation levels which was paralleled by a reduced association between IRS-1 and PI 3-kinase in the heart.

These results are similar to what was described for liver and muscle of animals in identical conditions, and suggest that least in these two animals models of insulin resistance the regulation of IRS-1 tyrosine phosphorylation and association with PI 3-kinase did not show a tissue-specific regulation.

INTRODUCTION

Protein tyrosine phosphorylation is an important mechamism of intracellular signalling. Insulin action in target tissues is iniciated by binding of the ligand to its receptor (IR), promoting the autophosphorylation of its β subunit and subsequently triggering a cascade of intracellular signalling (4,14). In most cells, this primary event leads to the subsequent tyrosyl phosphorylation of a citoplasmatic proteins called insulin receptor substrates (IRS-1, IRS-2, IRS-3 and IRS-4) (19,20,37). Following theirs phosphorylation, at least IRS-1 and IRSs can associate with proteins containing Src homology 2 (SH2) domains through specific tyrosyl phosphorylation sites (8,17). This association leads to activation of the enzyme phosphatidylinositol 3-kinase (PI 3-kinase) (2,11). Thus, the insulin receptor, IRS-1 and PI 3-kinase represent three of the earliest steps in insulin action, each of which can be demonstrated in vivo in two of the main target tissues for the metabolic actions of insulin, namely liver and muscle (11). Previous studies demonstrat that IRS-1 and PI 3-kinase have a tissue specific regulation, which can contribute to explain differential effects of insulin in sutiations of insulin resistance.

Prolonged fasting in rats is characterized by insulin deficiency and insulin resistance (1,3). Saad et al. (27) observed that IRS-1 phosphorylation increases in liver and muscle, whereas the level of IRS-1 protein increases in liver but decreases in muscle after 72 h of fasting, demonstrating this tissue specific regulation (28).

The excess of epinephrine causes insulin resistance (31). Catecholamines antagonize the action of insulin by stimulating gluconeogenesis, glycogenolysis and lipolysis and by inhibiting peripheral glucose use via a β-adrenergic mechanism that may also involve a decrease incellular glucose transport . Recentely, we demonstrated a decrease in insulin stimulated receptor and IRS-1 phosphorylation levels which PI 3-K in vivo in liver and muscle of epinephrine treated rats (29,31).

Heart is a insulin sensitive tissue (25), but the regulation and phosphorylation of insulin receptor and of IRS-1 as well as the association of the latter with PI 3-K in the heart in vivo, have not been examined. The aim of the present study was to determine the

level and phosphorylation state of IR, IRS-1 and IRS-1/PI 3-k in two well established situations of insulin resistance: rats fasted for 72 hs and rats treat with epinephrine. (23,25).

MATERIALS AND METHODS

Materials: The reagent for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA, U.S.A). Tris, phenylmethylsulfonyl fluoride (PMSF), aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol were obtained from Sigma Chemical Co.(St Louis, MO). Protein A Sepharose 6MB was obtained from Pharmacia (Uppsala, Sweden), [I^{125}] protein A from Amersham (Avlesbury,U.K.), and nitrocellulose paper (BA85, 0.2mm) from Schleicher & Schuell (Keene, NH). Sodium thiopental and human recombinant insulin (Humulin R) were obtained from Eli Lilly (Indianapolis, IN). Male rats were obtained from the UNICAMP Central Breeding Center .Monoclonal antiphosphotyrosine antibodies and anti PI 3-kinase (p85) antibody were obtained from Upstate Biochnology (Lake Placid, NY), and IRS-1 and anti-insulin receptor antibody, from Santa Cruz Technology (Santa Cruz, CA).

Animals and tissue extrats: Male Wistar rats (150-180 g) were provided with standard rodent diet and water ad libitum. Food was withdrawn 12-14 h before experiments. The studies were performed in parallel for the controls and experimentals rats in each pair. In the experiments on starvation, the rats were fed (controls) or fasted for 72 hs. The others animals were injected i.p. with epinephrine 25 μg/100g body wt or an equal volume of saline (controls), and utilized five minutes later.

Rats were anaesthesed with sodium amobarbital (15 mg/kg bw, i.p.) and used experiments 10 - 15 minutes later i.e. as soon as anaesthesia was assured by lossof pedal and corneal reflexes. The abdominal cavity was opened, the cava vein was exposed, and 0.5 ml normal saline (0.95 NaCl) with or without 10^{-5} M insulin was injected. After 90 sec., the piece heart was removed, minced coarsely, and homogenized immeditely in aproximately 6 volumes of solubilization extration Buffer B using a polytron PTA 20S generator (Brinkmann Instruments model pT 10/35, Westbury, NY) operating at maximum

speed for 30 sec. The buffer B contained 1% triton X-100, 10 mM Tris (pH 7.4), 100 mM sodium pirophosphatase, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PSMF and 0.1 mg/ml aprotinin at 4°C. After centrifugation, the supernatant solution of the tissue was than used for protein determination the Bradford (4) dye binding method using the Bio-Rad reagent and bovine serum albumin (BSA) as the standard. The supernatants used for immunoprecipitation with α IR, αIRS-1 and α PI 3-kinase antibodies and protein A-Sepharose 6 MB.

Protein analysis by immunoblotting: The precipitated proteins were treated with Laemmli sample buffer (16) containing 100 mmol/1dithiothreitol and heated in a boiling water bath for 4 min., after with they subjected to SDS-PAGE (6% bis-acrylamide) in a Bio-Rad miniture apparatus (Mini-Protean, Bio-Rad Laboratories, Richmond, CA).

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 describe by Towbin et al.(34), except for addition of 0.02% SDS to the transfer buffer to enhance the elution of high M, proteins. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4°C in blocking buffer (5% nonfat dry milk,10 mmol/1 Tris, 150 mmol/1 NaCl, and 0.02% Tween 20). The nitrocellulose blot was incubated with antiphosphotyrosine antibodies (1μg/ml) or with PI 3-kinase (1:500) diluted in blocking buffer (0.3% BSA insted of non fat dry milk) overnight at 4°C and then washed for 60 min. with the blocking buffer without milk. The blots were subsequently incubated with 2 μCi of [^{125} I]protein A (30μCi/μg) in 10 ml of blocking buffer for 2 h at room temperature, and then washed again for 30 min.as describe above. [^{125} I]Protein A bound to the antiphosphotyrosine and antipeptide antibodies was detect by autoradiography using preflashed Kodak XAR film (Eastman Kodak,Rochester, NY) with Cronex lightning Plus intensifying screens (DuPont, Wilmington, DE) at -80°C for 12-48 h. Band intensities were quantitated by optical densitometry (Eagle Eye) of the developed autoradiographs.

Statistical analysis: Experiments were always performed by stuying groups of animals in parallel. For comparisons, Student's unpaired *t* test was used. The levels of significance was set at $P<0.05$.

RESULTS

Caracteristics of insulin-stimulated insulin receptor and IRS-1 phosphorylation in the heart of 72 h of fasting rats.

There was no change in the insulin receptor level of heart from 72 h of fasting rats, as determined by immunoblotting with an antibody to the COOH-terminus of the insulin receptor (Fig. 1A). In heart samples previously immunoprecipitated with anti-insulin receptor antibody and immunoblotted with antiphodphotyrosine antibody, there was an increase to 143 ± 7% ($p<0,05$, n=6) in the insulin stimulated phosphorylation of 95 kDa β-subunit of the nsulin receptor in 72 h of fasting 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 heart of rats fasted (Fig.2A). Fig 2B shows that there was an increase to 147 ± 17% ($p<0,05$, n=6) in insulin-stimulated IRS-1 phosphorylation in the heart of fasting rats.

Previous studies (ana) 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. After stimulation with insulin, a band with the expected molecular mass (85 kDa) of the PI 3-kinase regulatory subunit was observed in ati-IRS-1 antibody immunoprecipitates of heart from rats in both groups. This finding is consistent with a stable association between IRS-1 and PI 3-kinase. There was an increased to 135 ± 9% ($p<0,05$, n=6) of PI 3-kinase associated with IRS-1 in rats of fasting.

Caracteristics of insulin-stimulated insulin receptor and IRS-1 in the heart of epinephrine treated rats.

As in 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 65 ± 9% ($p<0,05$, n=13) in epinephrine treated rats compared to the controls (Fig. 3B).

Similary, no significant change occurred in the level of IRS-1 protein in the heart of rats treated with epinephrine when compared to the controls (Fig.4 A). To define better the 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 group groups of animals. Fig. 4B shows that there was a marked reduction to 33 ±17% ($p<0,05$, $n=6$) in insulin-stimulated IRS-1 phosphorylation in the heart of animals pretreated with epinephrine. To examine the association of 85 kDa subunit PI 3-kinase with IRS-1, blots of samples which had been previously immunoprecipitated with IRS-1 antibodies were incubated with PI 3-kinase antibosy. As expected, in both groups na 85 kDa band was present in the IRS-1 immunoprecipitates after exposure to insulin and there was a decrease to 57 ± 32% ($p<0,05$, $n=5$) in the intensity of this band in epinephrine-treated rats (Fig. 4C).

DISCUSSION

Glucose utilization in the heart serves at least three major purposes: 1) it can serve as a major substrate to supply reducing equivalents to generate ATP in the mitochondria for contractile work (1); 2) it can be used to generate ATP via glycolysis for ion pumps to maintain transmembrane potential (2-4); and 3) it can be used to form glycogen that serves the heart during times of high levels of cardiac work, ischemia, and hypoxia (5,6).

The molecular mechanism by which insulin stimulates glucose utilization in heart is not completely understood, but the three early steps of insulin action, i.e.; insulin receptor and IRS-1 tyrosine phosphorylation levels and the association activation of IRS-1 with PI 3-kinase have an important role in this metabolic process. The study of the regulation of these early steps in insulin action in heart may contribute to our understading of glucose utilization in insulin sensitive tissues. In addition, different studies have indicated that in states of insulin resistance, alteration of these steps may contribute to the altered insulin action profile (37,42,43).

In the present study, we investigated insulin induced receptor and IRS-1 tyrosine phosphorylation and also IRS-1/PI 3-kinase association in heart of intact rat *in vivo*, in two situation that altered insulin action: 72 h fasting and epinephrine treatment.

Prolonged fasting in rats is characterized by insulin deficiency and insulin resistance (5,37). In vivo both skeletal muscle and hepatic insulin resistance on glucose metabolism have been observed (37). Interesting in fasting there is an increase in myocardial glycogen in spite of a reduction in liver and skeletal muscle.

The mechanism by which insulin resistance occurs appears to differ from tissue to tissue. Insulin receptors solubilized and partially purified from 72-h fasting rats show a decreased insulin-stimulated autophosphorylation and exogenous tyrosine kinase activation in liver, but not in muscle (6). Our results (normalized by total protein) showed an increase of 40% in insulin receptor autophosphorylation in heart of 72-h of fasting. The *in vivo* phosphorylation of IRS-1 increased by 45%, similar to liver and muscle (37). It is

interesting that IRS-1 phosphorylation increases in liver (37), muscle (37), aorta (44) and heart, whereas the level of IRS-1 protein as determined by direct immunoblotting increased in liver but decreased in muscle and heart during fasting. These data indicate that in fasting IRS-1 has a tissue specific regulation, but that IRS-1 phosphorylation in skeletal muscle and heart depends more on insulin receptor phosphorylation and kinase activity than IRS-1 protein levels.

This increase in IRS-1phosphorylation and association with PI 3-kinase in fasting occurs despite a state of decreased insulin responsiveness in both liver and peripheral tissue of fasted animals (33). The fact that euglycemic clamp studies show decreased insulin-stimulated glucose uptake in muscle *in vivo* with fasting suggests that tissue or circulating factors such as fatty acids, ketones, counterregulatory hormones, and acidosis may antagonize the stimulatory action of insulin on glucose transport in muscle as previously discussed (11). It is possible that the increase in IRS-1 phosphorylation in heart (as well as in liver, muscle, aorta) during 3-d fast could be a response to the impairment of insulin action.

We also invetigated the acute effect of epinephrine on signal transduction in the heart. It is known that epinephrine induces marked insulin resistance (12) and that this is accompained by a reduced glucose uptake in periphereal tissues and an increase in hepatic glucose output. Huang at al. demonstrated that epinephrine reduces glucose uptake in heart rats *in vivo*. The molecular mechanism that account for this reducedglucose uptake is not completely understood.

This study demonstrated ~ 35% reduction in insulin receptor and IRS-1 tyrosine phosphorylation in heart after estimulation with insulin, in animals previously treated with epinephrine, PI 3-kinase associated with IRS-1 was also reduced in the heart of animals exposed to an excess of catecholamine. These results are similar to what was observed in liver and muscle of rats (39) treated with epinephrine a make identical conditions.

The mechanism (s) whereby epinephrine induces these alterations are unknown but at least two possibilities should be considered. First, it is known that agents which raise

intracellular cAMP levels increase phosphorylation of the insulin receptor at serine and threonine residues, reduce insulin-mediated receptor phosphorylation on tyrosine, and inhibit the insulin-dependent tyrosine protein kinase activity of the receptor. Thus, cAMP may attenuate insulin actions by altering the state of phosphorylation of The insulin receptor (14,16). It is not known wether an increase in intracellular cAMP also induces a serine phosphorylation in IRS-1. However, since insulin receptor kinase activity is reduced, a reduction in IRS-1 phosphorylation and hence in the association between IRS-1/PI 3-kinase is also expected. The second possibility arises from recent data showing that an increase in cellular cAMP through activating protein kinase A (PKA) increases the activity of endogenous phosphatase (PTPase), thus leading to a sequence of dephosphorylation (30).

These alterations in the three early steps of insulin action in heart epinephrine treated rats may explain the reduced glucose uptake by this hormone in the heart (39). Kolter et al demonstrated that reduced sensitivity of glucose transport at low insulin levels was found to correlate to a completely blunted response of IRS-1 associated PI 3-kinase activity in cardiomyocytes from obese rats. In addition, distinct experimental approaches have also demonstrated between PI 3-kinase activity and glucose transport (40). Thus, IRS-1/PI 3-kinase patway may be linked to the activation of glucose transport, and a reduction in these associations in heart of epinephrine treated rat may havea role in the reduced glucose uptake described in thhis situation.

These results are similar to what was described for liver and muscle of animals in identical conditions, and suggest that at least in these two animal models of insulin resistance the regulation of IRS-1 tyrosine phosphorylation and association with PI 3-kinase did not show a tissue-specific regulation.

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LEGENDS

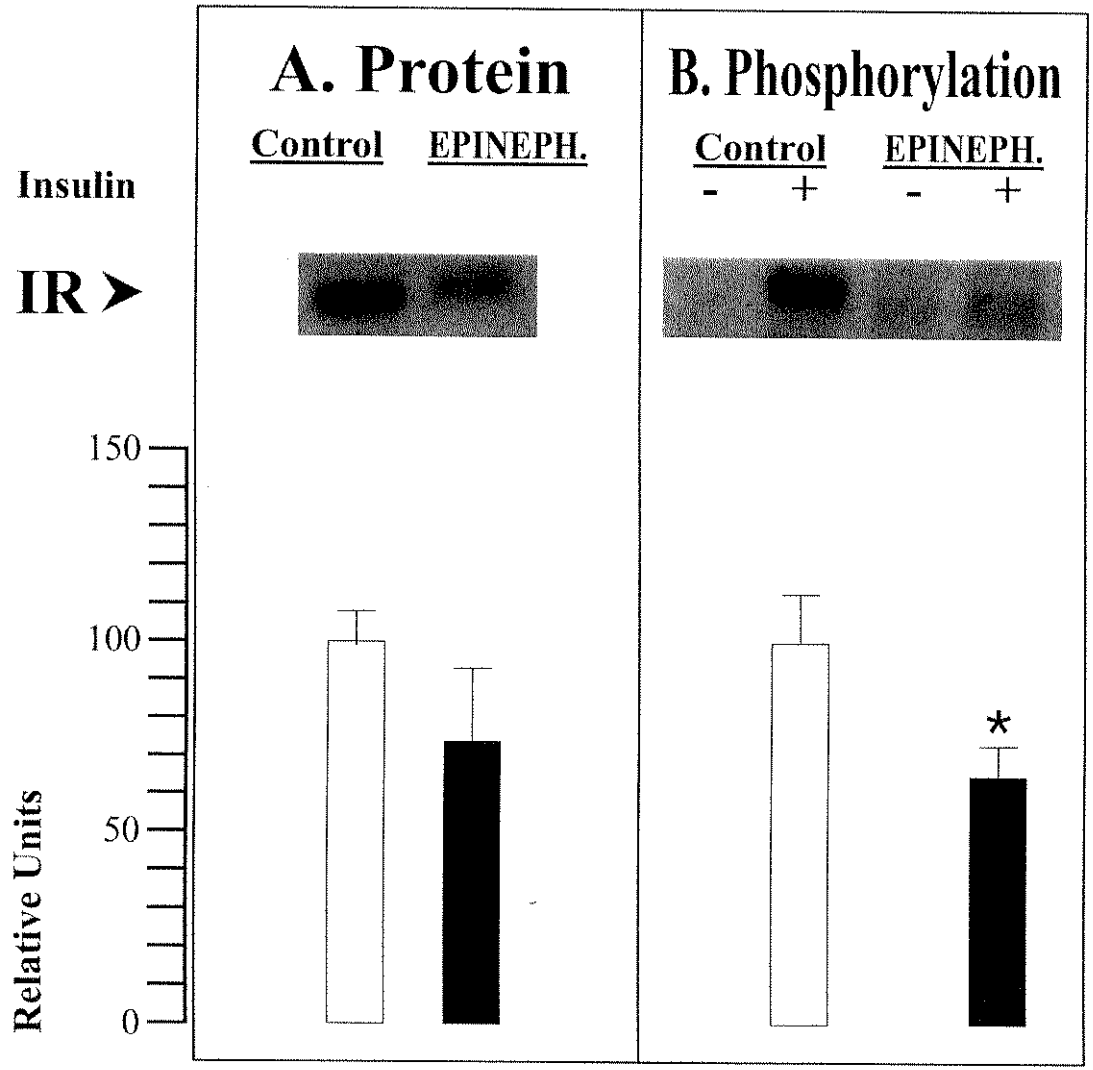
FIG.1. Insulin receptor (IR) phosphorylation in heart of fasted (72-h) rats. Normal saline (lanes 1 and 3) or 10^{-5} mM of insulin (lanes 2 and 4) were administered into the the cava vein as a bolus injection; 90 s later, the heart was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-insulin receptor antibody, and immunobloted with anti-insulin receptor (A) and antiphosphotyrosine antibody (B). The insulin receptor protein levels and phosphorylation are shown as means ± SE of the scanning densitometry of four experiments. * Significant differences at least at $p<0.05$.

FIG.2. IRS-1 phosphorylation in heart of fasted (72-h) rats. Normal saline (lanes 1 and 3) or 10^{-5} mM of insulin (lanes2 and 4) were administered into the cava vein as a bolus injection; 90 s later, the heart was excised and aliquots containing the same amount of protein were immunoprecipitaded with anti-IRS-1 antibody and immunoblotted with anti-IRS-1 (A), antiphosphotyrosine (B), and anti-PI 3-kinase (C) antibodies. The IRS-1 protein level, insulin-stimulated IRS-1 phosphorylation, and PI 3-Kinase association are shown as means ± SE of the scanning densitometry of six experiments. * Significant differences at least at $p<0.05$.

FIG.3. Insulin receptor (IR) phosphorylation in heart of acute administration of ephinephrine in rats. Normal saline (lanes 1 and 3) or 10^{-5} mM of insulin (lanes 2 and 4) were administered into the the cava vein as a bolus injection; 90 s later, the heart was excised and aliquots containing the same amount of protein were immunoprecipitated with anti-insulin receptor antibody, and immunobloted with anti-insulin receptor (A) and antiphosphotyrosine antibody (B). The insulin receptor protein levels and phosphorylation are shown as means ± SE of the scanning densitometry of eight experiments. * Significant differences at least at $p<0.05$.

FIG.4. IRS-1 phosphorylation in heart of acute administration of epinephrine in rats. Normal saline (lanes 1 and 3) or 10^{-5} mM of insulin (lanes2 and 4) were administered into the cava vein as a bolus injection; 90 s later, the heart was excised and aliquots containing the same amount of protein were immunoprecipitaded with anti-IRS-1 antibody and immunoblotted with anti-IRS-1 (A), antiphosphotyrosine (B), and anti-PI 3-kinase (C)

antibodies. The IRS-1 protein level, insulin-stimulated IRS-1 phosphorylation, and PI 3-Kinase association are shown as means ± SE of the scanning densitometry of six experiments. * Significant differences at least at $p<0.05$.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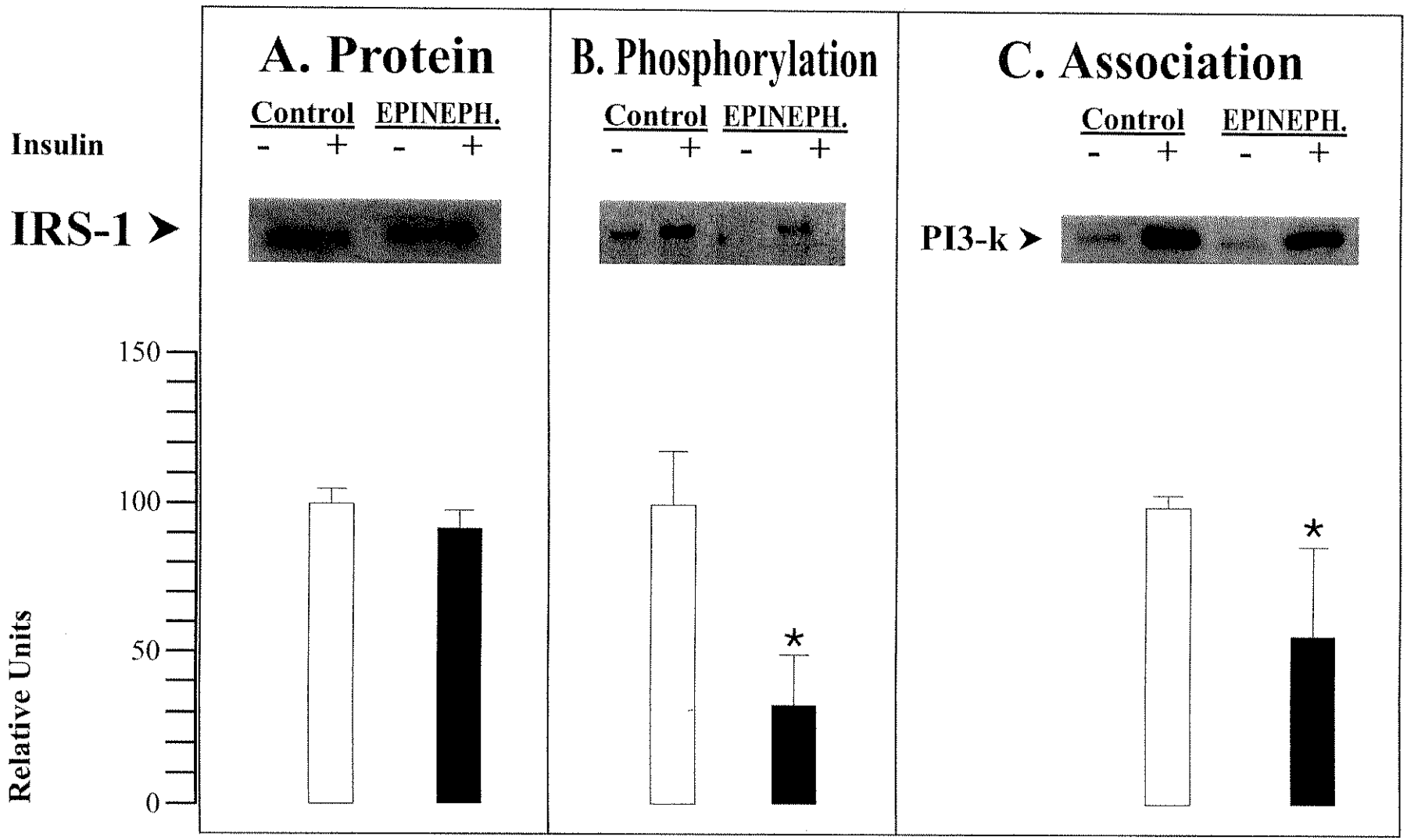
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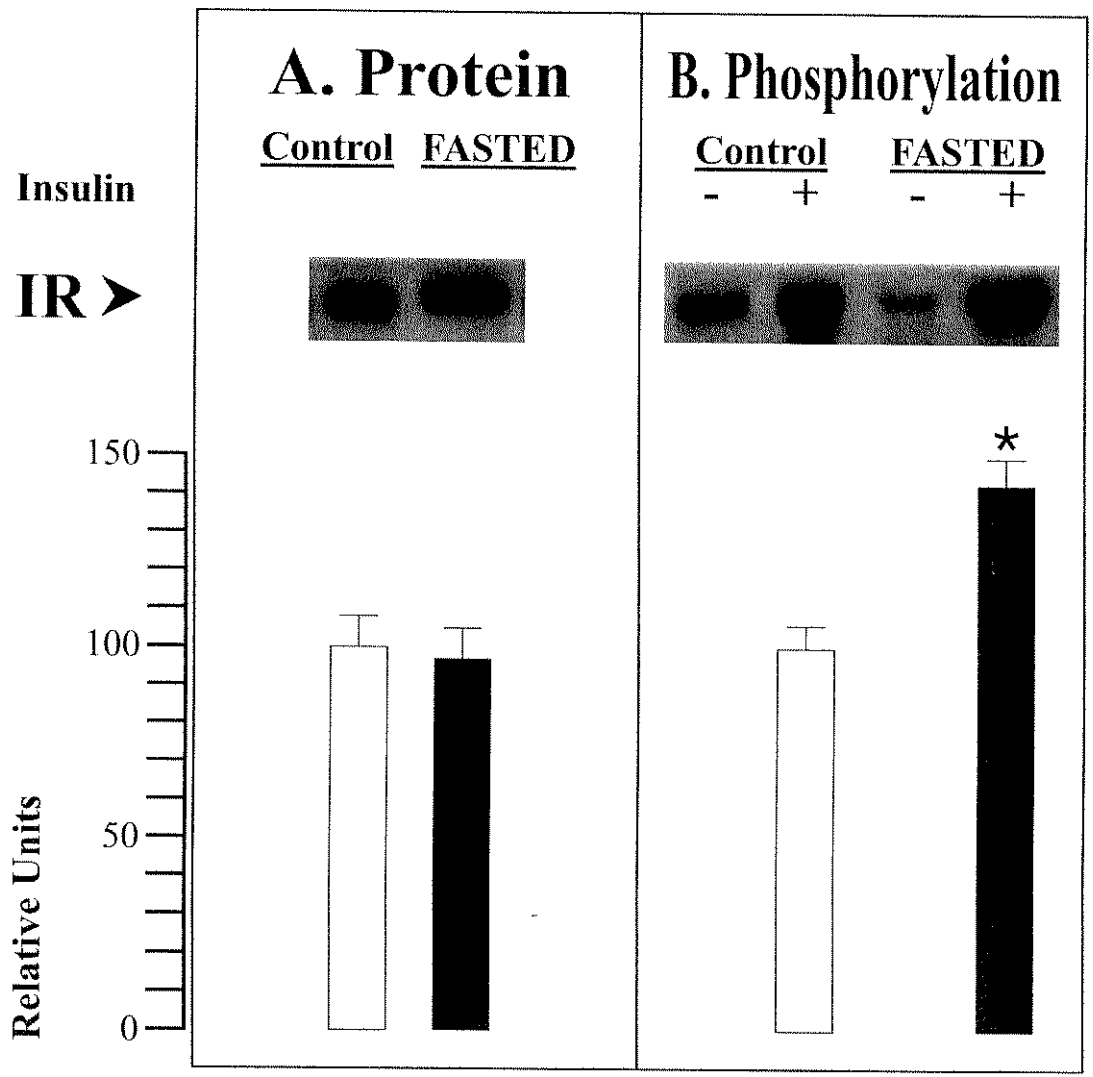
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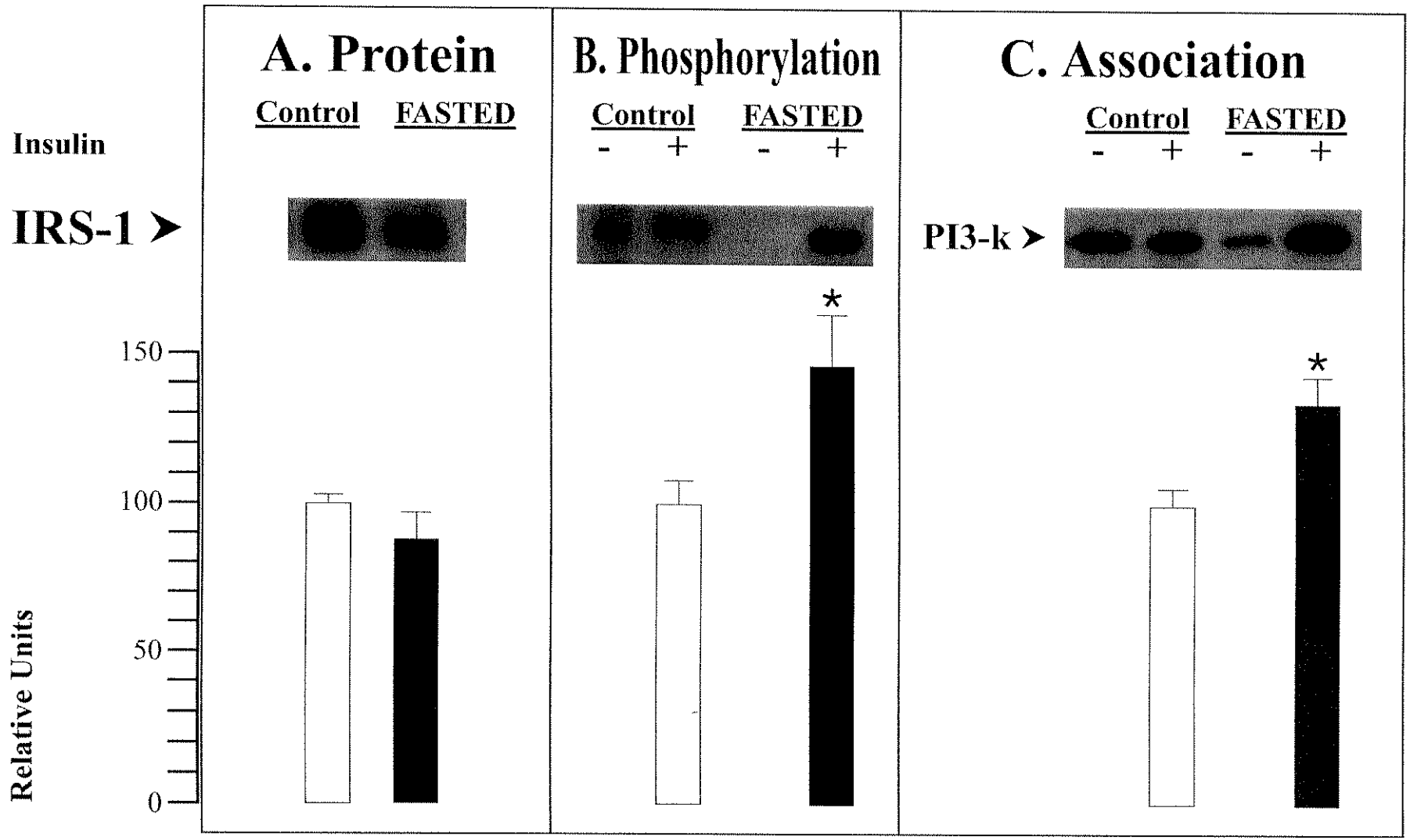
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Effect of fasting on insulin signaling in the aorta of intact rats

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Abstract

Insulin stimulates the tyrosine kinase activity of its receptor, resulting in the phosphorylation of its cytosolic substrate, insulin receptor substrate 1 (IRS-1). Previous studies have demonstrated a tissue-specific regulation of IRS-1. In the present study we investigated the levels and phosphorylation state of IRS-1 after insulin stimulation in the rat aorta *in vivo*, and the modulation of this protein after 72 h of fasting, using immunoprecipitation and immunoblotting with anti-insulin receptor, anti-IRS-1 and antiphosphotyrosine antibodies. We show that IRS-1 is present in rat aorta, and is tyrosine phosphorylated after insulin stimulation. After insulin stimulation, rats fasted for 72 h showed an increase in insulin receptor (100 ± 45%, $P<0.05$) and IRS-1 phosphorylation (68 ± 24%, $P<0.05$) in aorta, compared to fed rats. There was no change in insulin receptor or IRS-1 protein levels in fasted rats. In summary, the present study demonstrated that proteins involved in the early steps of insulin signal transduction are present in the rat aorta and can be modulated by fasting. It will be of interest to study the regulation of these proteins in the aorta of animal models of hypertension and/or atherosclerosis.

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Insulin regulates cell growth and metabolism upon binding to its own receptor through the activation of tyrosine kinase activity in the ß-subunit (1). Based on studies in which tyrosine kinase activity is inhibited by antibodies or reduced by *in vitro* and natural mutations of the receptor (2,3), evidence has accumulated that tyrosine kinase activity is essential for most of the complex cellular responses to insulin. In most cells, this primary event leads to the subsequent tyrosyl phosphorylation of a cytoplasmic protein with an apparent molecular weight of 160-180 kDa, called insulin receptor substrate 1 (IRS-1) (3-5). The insulin receptor and IRS-1 represent the earliest steps in insulin action, and each of them can be demonstrated and regulated in two of the main target tissues for the metabolic actions of insulin *in vivo*, namely liver and muscle (6-8). Previous studies demonstrated that IRS-1 has a tissue-specific regulation, and this can contribute to the different effects of insulin in situations of insulin resistance (9). In this regard, it is important to determine the presence and regulation of IRS-1 in rat aorta because insulin resistance has been implicated as a possible risk factor for atherosclerosis (10). The aim of the present study was to determine the levels and phosphorylation

state of IRS-1 after insulin stimulation of the rat aorta *in vivo*, and the modulation of this protein after 72 h of fasting which reflects a situation of insulin resistance (11).

Male rats had free access to a standard rodent chow and water. The rats were anesthetized intraperitoneally with sodium amobarbital (15 mg/kg body weight) and used in the experiments 10-15 min later, as soon as anesthesia was assured by the loss of foot and corneal reflexes. The abdominal cavity was opened, the vena cava exposed and 0.5 ml saline (0.9% NaCl) with or without 10 µM insulin was injected. After 90 sec or at the indicated time, the aorta was removed, minced coarsely and immediately homogenized in approximately 6 volumes of solubilization buffer A using a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operating at maximum speed for 30 sec in a water bath maintained at 100°C as previously described (6-8). The solubilization buffer A consisted of 1% SDS, 50 mM Hepes (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA and 10 mM sodium vanadate. The homogenate was then boiled for 10 min and cooled in an ice bath for 40 min. In some experiments, the aortas were excised and homogenized with a polytron in 6 volumes of homogenization buffer B cooled in an ice bath. The composition of buffer B was the same as that of buffer A except that 1% Triton X-100 replaced 1% SDS and 2 mM PMSF and 0.1 mg/ml aprotinin were added. Both extracts were centrifuged at 100,000 *g* at 4°C using a Beckman 70.1 Ti rotor for 30 min to remove insoluble material, and the resulting supernatant was used for the experiments. The aortas homogenized in buffer B were used for immunoprecipitation with anti-IRS-1 antibody and protein A-Sepharose 6 MB.

The samples were treated with Laemmli sample buffer (12) containing 100 mM DTT and heated in a boiling-water bath for 4 min. SDS PAGE (6.5% Tris/acrylamide) was carried out on total extracts (150 µg protein) in a Bio-Rad miniature slab gel apparatus (13). Electrotransfer of proteins from the gel to nitrocellulose was performed for 2 h at 100 V (constant) in the Bio-Rad miniature transfer apparatus (Mini-protean), as described by Towbin et al. (14) but with 0.02% SDS added to the transfer buffer to enhance the elution of high-molecular mass protein. Nonspecific protein binding to nitrocellulose was reduced by pre-incubating the filter overnight at 4°C in blocking buffer (3% BSA, 10 mM Tris, 150 mM NaCl and 0.02% Tween 20). The pre-stained molecular mass standards used were myosin (205 kDa), ß-galactosidase (116 kDa), BSA (80 kDa) and ovalbumin (49.5 kDa). The nitrocellulose blot was incubated with anti-phosphotyrosine (1 µg/ml) or anti-IRS-1 (1:100) antibodies for 4 h at 22°C. Monoclonal anti-phosphotyrosine antibody was obtained from Upstate Biotechnology (Lake Placid, NY) and anti-IRS-1 and anti-insulin receptor antibodies were raised in rabbits as previously described (9) and kindly provided by Dr. Morris F. White and X.J. Sun (Joslin Diabetes Center, Boston, MA). The blots were subsequently incubated with 2 µCi [^{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.

Figure 1A shows the presence of IRS-1 in different tissues. The tissues were extracted in buffer A and after SDS-PAGE the proteins were transferred to nitrocellulose and incubated with anti-IRS-1 antibody. The amount of IRS-1 varied in different tissues, with the highest level observed in liver, and the lowest levels in aorta and heart, with some variation from animal to animal. Figure 1B shows that in the aorta from normal rats, after stimula-

tion with insulin, a phosphotyrosine band of 95 kDa, previously identified as insulin receptor ß-subunit (1,9,15), appeared weakly after 90 sec. In addition, after injection of insulin, a phosphotyrosyl protein migrating between 165 and 185 kDa, corresponding to the size of IRS-1, an endogenous substrate, was also demonstrable. The phosphorylation of this protein started at 60 sec and increased at 90 sec after insulin stimulation (Figure 1B). In order to confirm that this high molecular weight phosphotyrosine band corresponded to IRS-1, we first immunoprecipitated aorta extracts with anti-IRS-1 antibody and then immunoblotted with anti-phosphotyrosine antibody. The result is presented in Figure 1C and shows that IRS-1 is tyrosine phosphorylated after insulin stimulation in the aorta of intact rats, suggesting that IRS-1 is at least a component of the high molecular weight phosphotyrosine band.

The regulation of the insulin receptor and IRS-1 is controlled by different mechanisms such as fasting, obesity, hormones and diabetes (6-9). Futhermore, animal models of insulin resistance show a tissue-specific regulation of IRS-1. During fasting an increase in tyrosine phosphorylation of IRS-1 in liver and muscle, and an increase in protein level in liver and a decrease in muscle were clearly demonstrated (9). We investigated the influence of fasting on protein and phosphorylation levels of insulin receptor and IRS-1 in the rat aorta. Figure 2 shows an increase of 100 ± 45% ($P<0.05$) in insulin receptor and of 68 ± 24% ($P<0.05$) in IRS-1 phosphoryl-

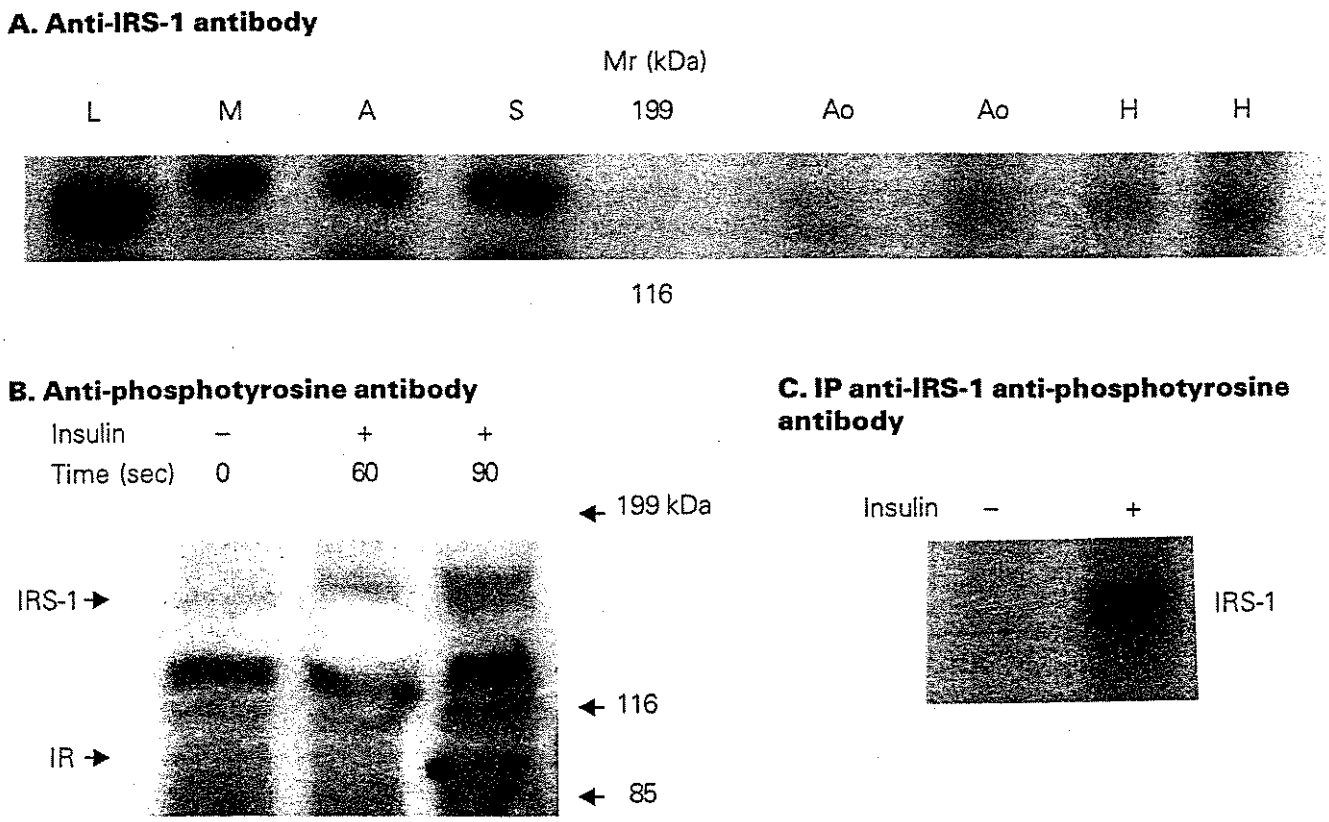

Figure 1 - Detection of insulin receptor substrate 1 (IRS-1) phosphorylation levels in rat aorta. The proteins from the aorta were isolated from anesthetized rats after normal saline or insulin infusion into the vena cava. *A*, Immunoblotting with anti-IRS-1 antibody in different tissues: liver (L), skeletal muscle (M), adipose tissue (A), spleen (S), aorta (Ao) and heart (H). *B*, Immunoblotting with anti-phosphotyrosine antibody before (0) and 60 and 90 sec after insulin infusion. *C*, Immunoprecipitation (IP) with anti-IRS-1 antibody followed by immunoblotting with anti-phosphotyrosine antibody before and after insulin stimulation. IR, Insulin receptor.

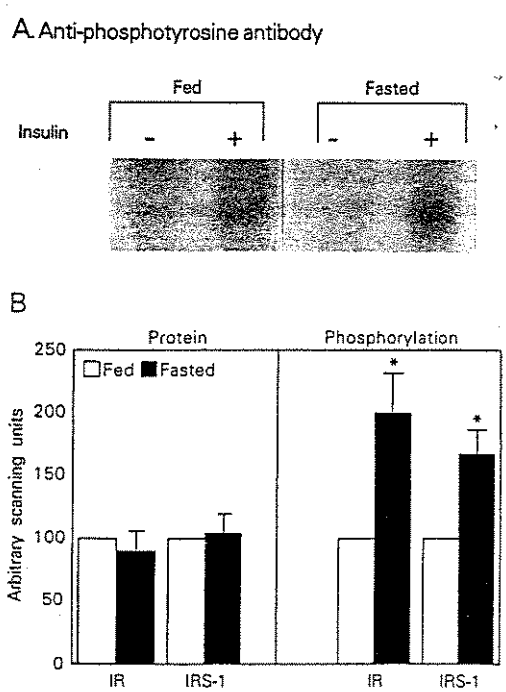

Figure 2 - Effect of fasting on insulin receptor substrate 1 (IRS-1) in the rat aorta. *A*, The aortas from fed and fasting (72 h) rats were immunoprecipitated with anti-IRS-1 antibody and then immunoblotted with anti-phosphotyrosine antibody. *B*, Scanning densitometry was performed on autoradiograms from 4 separate experiments to determine insulin receptor (IR) and IRS-1 concentration and tyrosine phosphorylation of proteins. Results are reported as mean ± SEM. *P<0.05 compared to fed rats (Student *t*-test).

ation levels after insulin stimulation in the aorta of fasting rats compared to fed rats. The insulin receptor and IRS-1 protein levels did not change during fasting (Figure 2B).

IRS-1 is a substrate of the insulin receptor and is also phosphorylated after stimulation with angiotensin II (16). In this regard, the observation that IRS-1 is regulated in the aorta may be important because insulin resistance has been implicated as a risk factor for atherosclerosis and hypertension (17). Epidemiologic studies indicate that fasting insulin levels are significantly elevated in hypertension (17,18) and atherosclerosis (19), and some studies have shown an inverse relation between blood pressure and insulin-mediated glucose disposal (20). The relationship between insulin resistance (and compensatory hyperinsulinemia) and hypertension is not completely clear, but all of them can lead to an increased cardiovascular risk (17). The molecular mechanism of insulin resistance in hypertension is poorly understood, but we have previously demonstrated in an animal model of hypertension (spontaneously hypertensive rats, SHR) that insulin receptor substrate-1 phosphorylation is reduced in liver and muscle, pointing to a post-receptor defect in insulin action in this animal (21).

In summary, the present study demonstrated that proteins involved in the early steps of insulin signal transduction are present in the rat aorta. These proteins are quickly phosphorylated to tyrosine after insulin stimulation, and during fasting they showed increased phosphorylation after insulin stimulation, without changes in protein level. It will be of interest to investigate the regulation of these proteins in the aorta of animal models of hypertension and/or atherosclerosis.

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3. DISCUSSÃO

A ligação da insulina na subunidade α do seu receptor causa uma rápida autofosforilação da subunidade β, incrementando sua capacidade tirosina quinase (WHITE, 1994; CHEATHAM & KAHN,1995). Isto desencadeia uma série de fosforilações em tirosina dos substratos, incluindo IRS-1, cuja fosforilação é de grande importância nas ações biológicas desse hormônio (CLAUSEN *et al.*, 1995). O IRS-1 é fosforilado em aminoácido tirosina e se associa à enzima PI 3-quinase, propagando o sinal intracelular. Esta associação ocorre através das seqüências repetitivas de aminoácidos YMXM ou YXXM do IRS-1 com o domínio SH2 da subunidade regulatória da PI 3-quinase, resultando na ativação catalítica da enzima (MYERS & WHITE, 1996). O tratamento de cultura de células com *wortmannin,* um potente inibidor da PI 3-quinase, inibe a captação de glicose estimulada pela insulina (OKADA, *et al.*, 1994; HARA *et al.*,1994). Estes estudos sugerem que a atividade da PI 3-quinase é importante na manutenção da homeostase da glicose.

Várias outras proteínas tem sido descritas como sendo fosforiladas após estímulo insulínico como IRS-2 (SUN *et al.*,1995), IRS-3 (SMITH-HALL *et al.*,1997; LAVAN, et al., 1997), IRS-4 (LAVAN et al.,1997), SHC (SKOLNIK, *et al.*,1993; OKADA *et al.*,1995) e JAK2 (SAAD *et al* ,1996). Além da PI 3-quinase, outras proteínas com domínio SH2 podem ser ativadas pelo IRS-1 como Fyn, SHP2, Nck e Grb2. Diante disto o IRS-1 pode ser considerada uma molécula "ancoradouro" direcionando as ações insulínicas (KUHNE *et al.*, 1993; YAMAUCH & PESSIN, 1994).

É interessante investigar a regulação da transmissão do sinal insulínico em coração, pois tem sido demonstrado uma correlação entre resistência à insulina e ou hiperinsulinemia com hipertrofia do miocárdio (KATZ *et al.* 1995). Além disso, em algumas situações de resistência à insulina existe uma diminuição da captação de glicose estimulada pela insulina em músculo esquelético, mas não em coração (HUANG *et al.*,1997). Entretanto, os mecanismo moleculares responsáveis por essas diferenças não são conhecidos. Assim, é importante a investigação da regulação das etapas iniciais da ação insulínica em coração em situações de resistência ao hormônio. Neste estudo, nós investigamos os níveis protéicos e graus de fosforilação do receptor de insulina, do seu primeiro substrato IRS-1 e a associação IRS-1 com a PI 3-quinase em coração intacto de

rato, após estímulo insulínico *in vivo*, em cinco modelos animais de resistência à insulina: ratos diabéticos por estreptozotocina, administração crônica de dexametasona, administração aguda de adrenalina, envelhecimento e jejum prolongado.

O diabetes induzido por STZ é um modelo experimental para estudo da resistência à insulina, sendo caracterizado pela redução da ação insulínica tanto em tecidos periféricos quanto em tecido hepático (ALMIRA & REDDY,1979; OKAMOTO *et al.*1986; PENICAUD et al., 1985). No coração ocorre uma diminuição da captação e metabolização de glicose induzida pela insulina em ratos diabéticos. O mecanismo molecular responsável por esta diminuição da sensibilidade à insulina ainda não foi esclarecido (KOLTER, *et al.*, 1997).

Em nosso estudo demonstramos que não houve mudança no grau de fosforilação estimulado pela insulina do receptor e do IRS-1 em ratos diabéticos. Entretanto, observamos uma redução de ~ 40% nos níveis protéicos do IRS-1. Esses resultados sugerem que no coração destes animais, uma diminuição no nível protéico do IRS-1 não é suficiente para induzir uma redução nos níveis de fosforilação desta proteína, que é regulado principalmente pelo receptor de insulina. Por outro lado, ocorreu uma redução na associação IRS-1 com a PI 3-quinase. Podemos considerar que os níveis protéicos do IRS-1 podem ter um papel nesta interação, uma vez que existem tirosinas específicas que estão envolvidas na associação IRS-1/PI 3-quinase (TILL *et al.*, 1997), e um aumento estoiquiométrico da fosforilação do IRS-1 pode não ser acompanhado por um aumento na associação com a PI 3-quinase.

Um possível mecanismo que pode desempenhar um papel nesta redução de associação, é um aumento na fosforilação em aminoácidos serina e ou treonina do IRS-1. Nesta linha, já está bem estabelecido que um aumento na atividade da PKC no coração de ratos diabéticos (INOGUCHI, *et al.*,1992) pode induzir fosforilação do IRS-1 em serina e ou treonina, sem afetar a fosforilação em tirosina. Recentemente KOLTER *et al.*(1997) demonstraram que em cardiomiócitos ventriculares isolados de ratos obesos ocorreu um aumento nos níveis de fosforilação do IRS-1 em serina, sem mudar os níveis de fosforilação em tirosina o qual reduz a interação com a PI 3-quinase. Esta diminuição na associação IRS-1/PI 3-quinase é diferente dos dados encontrados em fígado e músculo,

onde observamos um aumento nos níveis de fosforilação do receptor de insulina e do IRS-1 acompanhado pelo aumento na associação IRS-1/PI 3-quinase (FOLLI *et al.*, 1993), demonstrando uma regulação tecido-específica nas etapas iniciais da ação insulinica em ratos com diabetes.

É fato bem estabelecido que o excesso de glicocórticoides causa resistência à insulina (AMATRUDA, *et al.*,1985). A hipercortisolemia está associada com o aumento da produção de glicose pelo fígado, diminuição da utilização e do transporte periférico de glicose (DePIRRO, *et al.*,1981; OLEFSKY *et al.*,1979), diminuição da síntese protéica e aumento da degradação protéica no músculo (RANNELS & JEFFERSON, 1980). Estudos anteriores, usando fluorodeoxiglicose (FDG), demonstraram que a dexametasona provoca uma redução na captação de FDG em músculos esquelético e cardíaco de modo semelhante (KUBOTA, *et al.*,1996). Os mecanismos moleculares responsáveis pela diminuição da captação de glicose não estão bem estabelecidas. Em ambos os tecidos, depois do tratamento com dexametasona, não houve mudanças na quantidade de GLUT 4 (KUBOTA *el al.*, 1996). Nossos resultados demonstram que após 5 dias de tratamento com dexametasona há uma diminuição nos níveis protéicos e na fosforilação do IRS-1, acompanhado de redução na associação IRS-1/PI 3-quinase. Esta diminuição na associação foi também observada em outros tecidos, sugerindo que há uma alteração no mecanismo molecular comum na via de transmissão do sinal insulinico após o tratamento com dexametasona. Essas alterações podem ser decorrentes de um efeito direto da dexametasona, mas deve-se considerar também que podem ser secundárias à hiperinsulinemia que esses animais apresentam. A exposição crônica de diferentes linhagens celulares à insulina induz uma redução nos níveis protéicos e de fosforilação do IRS-1, bem como na associação IRS-1/PI 3-quinase (SAAD, *et al.*, 1994). Em situações de hiperinsulinemia uma diminuição no níveis protéicos do IRS-1 pode ser conseqüência de um aumento na taxa de degradação das proteínas (CLARK *et al.*,1986).

A elevacão dos níveis circulantes de catecolaminas induz resistência a insulina (CHIASSON *et al.*,1991). Este hormônio antagoniza a ação da insulina, estimulando a neoglicogenêse, a glicogenólise e inibindo a captação periférica de glicose. HUANG *et al.*(1997) demostraram que a adrenalina reduz a captação de glicose no coração de ratos

in vivo. O mecanismo molecular responsável por esta redução da captação de glicose ainda não é completamente conhecido.

Os resultados deste estudo demonstraram uma redução de aproximadamente 35% no grau de fosforilação do receptor de insulina e do IRS-1, após o tratamento agudo com adrenalina. A associação IRS-1/PI 3-quinase também estava reduzida no tecido cardíaco de ratos tratados com esta catecolamina. O mecanismo pela qual a adrenalina exerce este efeito nas etapas iniciais da ação insulínica em coração não está completamente elucidado, mas pelo menos duas possibilidades devem ser consideradas. (SAAD *et al.*, 1995). Primeiro, é bem estabelecido que agentes que elevam as concentrações intracelulares de AMPc, induzem a fosforilação do receptor de insulina em serina e treonina, o que reduz a autofosforilação em tirosina do receptor e sua capacidade tirosina-quinase (STADMAUER & ROSEN, 1986; ROTH & BEAUDOIN, 1987; TAKAYAMA *et al.*, 1988). Assim, o AMPc pode atenuar a ação insulínica alterando o estado de fosforilação do receptor de insulina (SAAD *et al.*, 1995b, SAAD *et al.*, 1995c). Não está ainda estabelecido se o aumento do AMPc intracelular induz a fosforilação em serina do IRS-1, mas como a atividade quinase do receptor está reduzida, espera-se uma menor fosforilação do IRS-1, bem como da associação IRS-1/PI 3-quinase. A segunda possibilidade deriva de uma descrição relativamente recente, demonstrando que o aumento na concentração intracelular de AMPc, ativando a proteína quinase A (PKA), que ativa fosfatases fosfotirosinas (PTAse), resultando em uma seqüência de desfosforilações (GOLDSTEIN., 1992).

Estas alterações nas três primeiras etapas da ação insulínica em coração de ratos tratados com adrenalina pode explicar uma redução na captação de glicose provocada por este hormônio. KOLTER *et al.* (1997) demonstraram que a redução no transporte de glicose estimulado por níveis fisiológicos de insulina em cardiomiócitos de ratos obesos, estava relacionado a uma diminuição da associação IRS-1/PI 3-quinase. Em adição, protocolos experimentais distintos tem demonstrado correlação a entre atividade da PI 3-quinase e transporte de glicose (SAAD *et al.*,1995a, SAAD *et al* 1995 b). Assim, a associação IRS-1/PI 3-quinase parece exercer um papel importante na ativação do transporte de glicose, e uma redução nesta associação em coração de ratos tratados com

adrenalina pode estar relacionada a uma diminuição da captação da glicose descrito nesta situação.

Em resumo, a redução na associação IRS-1/PI 3-quinase induzida pela insulina em ratos diabéticos , tratados com dexametasona ou com adrenalina deve ter um papel na diminuição da captação de glicose no coração destes animais. A regulação da translocação do GLUT 4 para a membrana plasmática é essencial para que haja um rápido efeito da insulina sobre a captação de glicose no coração. Esta proteína transportadora é armazenada em compartimentos vesiculares intracelulares em condições basais, e após estímulo insulínico, migram para a membrana plasmática e permitem a entrada da glicose Evidências de diferentes fontes indicam que a ativação da PI 3-kinase durante a associação com IRS-1 proporciona um sinal essencial para a translocação do GLUT 4 (TILL *et al.*, 1997).

O envelhecimento é uma situação de resistência à insulina bem estabelecida em humanos e em animais. A resistência à insulina desenvolvida durante o envelhecimento está associada a um aumento na intolerância à glicose (BARNARD *et al*, 1992). A síntese de glicogênio em resposta a estimulação com insulina é marcadamente diminuída no tecido muscular esquelético de ratos Wistar com 85 semanas (GOODMAN *et al.*,1983), e a captação de glicose estimulada pela insulina está prejudicada em relação direta com a depleção do *pool* dos transportadores de glicose (GIORGINO *et al.*, 1993). Demonstrou-se ainda uma menor inibição da produção hepática de glicose por concentrações fisiológicas de insulina (YOSHIMASA *et al.*, 1988). Interessante que apesar de ocorrer uma redução no transporte de glicose em músculo esquelético de ratos com 20 meses de idade, a captação de glicose pelo coração após infusão de dose máxima de insulina foi semelhante tanto em ratos jovens como em ratos velhos, sugerindo que a capacidade máxima da utilização de glicose permanece inalterada em coração de animais envelhecidos (ESCRIVA *et al.*, 1997). No nosso estudo demonstramos que em coração de ratos velhos não houve mudanças no nível protéico e na fosforilação do IRS-1, bem como na associação com a PI 3-quinase. Em contraste, no músculo esquelético foi demonstrado uma importante redução nos níveis protéicos e na fosforilação do IRS-1,bem como na associação com a PI 3-quinase em animais velhos (CARVALHO *et al*,1996). Estes resultados sugerem que uma regulação tecido-especifica do IRS-1 pode contribuir para

explicar as diferenças na captação de glicose entre coração e músculo esquelético em ratos velhos.

O jejum prolongado em ratos é caracterizado por hipoinsulinemia e resistência à insulina. Nessa situação descreve-se redução da ação insulínica tanto em tecidos periféricos quanto em tecido hepático. No jejum de 72 horas há uma redução do conteúdo de glicogênio em tecido muscular e hepático, mas em tecido cardíaco há um aumento.

O mecanismo de resistência à insulina parece diferir de tecido para tecido. Receptores de insulina parcialmente purificados e solubilizados de fígado e músculo esquelético de ratos submetidos a jejum de 72 horas, mostram uma redução da autofosforilação e da atividade tirosina quinase em fígado mas não em músculo (BALAGE *et al.*, 1990).

Nossos resultados demonstraram que o jejum induz um aumento de ~ 40% na fosforilação do receptor de insulina, em coração de ratos e do IRS-1 de ~ 45%, semelhante ao observado em fígado e músculo. (SAAD *et al*, 1992). É interessante que o grau de fosforilação do IRS-1 aumentou em todos os tecidos estudados no jejum, mas os níveis protéico aumentaram no fígado e reduziram em músculo esquelético e cardíaco. Estes dados confirmam que o IRS-1 tem uma regulação tecido-específica e que a fosforilação desta proteína em musculatura esquelética e cardíaca depende mais da fosforilação e atividade quinase do receptor do que dos níveis teciduais do IRS-1. O aumento no grau de fosforilação do IRS-1 e na associação com a PI 3-quinase ocorreram a despeito de um estado de menor responsividade a insulina em fígado e tecidos periféricos nos animais em jejum (PENICAUD *et al.*,1985). O fato de estudos de "clamp" euglicêmico demonstrarem uma redução na captação de glicose estimulada pela insulina *in vivo* neste modelo animal sugere que fatores circulantes ou teciduais, como ácidos graxos livres, corpos cetônicos, hormônios contra-reguladores da insulina ou acidose podem antagonizar a ação estimulatória deste hormônio no transporte de glicose em músculo esquelético (CHARRON & KAHN, 1990). Neste sentido é possível que o incremento observado na atividade das proteínas envolvidas nas etapas iniciais da ação insulínica reflita uma compensação molecular na situação de resistência à insulina.

Demonstramos que as proteínas envolvidas nas etapas iniciais de transmissão do sinal insulínico estão presentes em aorta de ratos e que podem ser moduladas pelo jejum. Estas proteínas são rapidamente fosforiladas após estímulo insulínico, sem mudar os níveis protéicos, entretanto observamos um aumento no grau de fosforilação do receptor de insulina e IRS-1, quando comparados com controles (SILVA *et al.*, 1996).

Estes resultados são semelhantes ao descrito em fígado e músculo de animais nas mesmas condições, e sugerem que neste modelo experimental tanto em aorta quanto em coração a regulação da fosforilação do IRS-1 e sua associação com PI 3-quinase não se mostram como uma regulação tecido-específica. Efeitos semelhantes foram descritos em animais tratados agudamente com adrenalina.

Nossos resultados demonstram que as proteínas envolvidas nas etapas iniciais da transmissão do sinal insulínico estão presentes em coração e aorta de ratos, e que apresentam uma regulação diferenciada dependendo do modelo analisado, contribuindo para explicar a modulação específica da utilização da glicose em coração nesses modelos animais.

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