HENRIQUE GOTTARDELLO ZECCHIN

TRANSMISSÃO DO SINAL DE INSULINA E ACETILCOLINA NA AORTA DE MODELOS ANIMAIS DE RESISTÊNCIA À INSULINA

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

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aos meus irmãos, Victor e Karina,
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Toda a nossa ciência, comparada com a realidade,
é primitiva e infantil – e, no entanto, é a coisa
maispreciosa que temos.

Albert Einstein (1879-1955)

PÁG.

μCi	microCuri
αPY	anticorpo anti-fosfotirosina
AII	angiotensina II
ATP	adenosina trifosfato
bcr	*breakpoint cluster region*
BSA	albumina sérica bovina
DNA	ácido desoxiribonucléico
DTT	ditiotreitol
EDTA	ácido etilenodiaminotetracético
eNOS	óxido nítrico sintase endotelial
EPM	erro padrão da média
ERK	quinase reguladora da sinalização extracelular
GLUT	transportador de glicose
GRB2	proteína ligadora do receptor para fator de crescimento
GSK-3	glicogênio sintase quinase-3
HE	hematoxilina e eosina
HUVEC	células endoteliais de veia umbilical humana
Ig	imunoglobulina
IGF	fator de crescimento “insulina-like”
IR	receptor de insulina
IRS	substrato do receptor de insulina
IRβ	subunidade β do receptor de insulina

ITT	teste de tolerância à insulina
JAK2	*Janus Kinase 2*
kDa	quilodalton
Kitt	constante de desaparecimento da glicose
MAP kinase	proteína quinase ativadora da mitogênese
MEK	MAP/ERK quinase
MKP-1	MAP quinase fosfatase-1
NO	óxido nítrico
PAI-1	inibidor 1 do ativador do plasminogênio
PDGF	fator de crescimento derivado de plaquetas
PDK	proteína quinase dependente de fosfatidilinositol
PFK2	6-fosfofrutose 2-quinase
PH	domínio com homologia à plecstrina
PI	fosfatidilinositol
PI 3-K	fosfatidilinositol 3-quinase
PKB	proteína quinase B ou Akt
PKC	proteína quinase C
PMSF	fenilmetilsulfonilfluoreto
PTB	domínio de ligação à fosfotirosina
Raf	serina-quinase citoplasmática ativada pelo Ras e ativadora da MAP quinase
SDS-PAGE	eletroforese em gel de poliacrilamida com dodecil-sulfato de sódio
SH2	*src-homology 2 domain*
SH3	*src-homology 3 domain*
Shc	*src-homology 2 domain-containing*

SHR	ratos espontaneamente hipertensos
SOS	fator ativador do Ras, com homologia a *Son-of-sevenless*
Src	oncogene definido como produto do sarcoma-vírus Rous
STAT	*Signal Transducers and Activators of Transcription*
TCA	ácido tricloroacético
TNF	fator de necrose tumoral
t-PA	ativador do plasminogênio tecidual
TRIS	tri-hidroximetil-aminometano
VEGF	fator de crescimento do endotélio vascular
VSMC	células musculares lisas vasculares

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

RESUMO

A resistência seletiva à insulina através da via IRS/PI3-K/Akt/eNOS associada à ativação normal ou exacerbada da via de crescimento MAPK tem sido proposta como um possível elo entre situações de resistência à insulina e doença cardiovascular. Inicialmente demonstramos que animais com resistência à insulina e doença cardiovascular (o rato espontaneamente hipertenso, SHR) apresentam menor ativação da via IRS/PI3-K/Akt/eNOS e hiperativação/hiperexpressão da via da MAPK na aorta torácica, enquanto a ativação normal da via IRS/PI3-K/Akt/eNOS pode proteger o animal obeso, resistente à insulina e que não apresenta doença cardiovascular. Posteriormente, outras vias estimulatórias do crescimento celular, como a via JAK/STAT, foram estudadas no vaso de outro modelo animal de resistência à insulina e doença cardiovascular - o rato com obesidade induzida por dieta. Este modelo demonstrou que a redução da ativação da via PI3-K/Akt/eNOS ocorre em paralelo à hiperativação das vias da MAPK e JAK/STAT, e isso pode desempenhar função importante da patogênese da doença cardiovascular neste estado patológico. Em outro estudo foi demonstrado pela primeira vez que a acetilcolina pode ativar a eNOS de maneira cálcio-independente, através da via IRS/PI3-K/Akt utilizando para isso uma tirosina quinase intracelular, a JAK2. Em ratos com obesidade induzida por dieta, resistentes à insulina e com disfunção endotelial, foi demonstrado que há resistência na via da PI3-K/Akt/eNOS tanto em resposta à insulina quanto à acetilcolina, em decorrência de maior fosforilação inibitória do IRS-1 e da redução dos níveis teciduais da eNOS. Assim, o desequilíbrio entre a ativação reduzida da via IRS/PI3-K/Akt/eNOS e a maior ativação das vias de crescimento (MAPK e JAK/STAT) pode contribuir para o desenvolvimento de doença cardiovascular em estados de resistência à insulina.

ABSTRACT

The actions of acetylcholine on endothelium are mainly mediated through muscarinic receptors, which are members of the G protein-coupled receptor family. In the present study we show that acetylcholine induces rapid tyrosine phosphorylation and activation of Janus kinase 2 (JAK2) in rat aorta. Upon JAK2 activation, tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) is detected. In addition, acetylcholine induces JAK2/IRS-1 and IRS-1/phosphatidylinositol (PI) 3-kinase associations, downstream activation of Akt/protein kinase B, endothelial cell-nitric oxide synthase (eNOS), and extracellular signal-regulated kinase (ERK1/2). The pharmacological blockade of JAK2 or PI 3-kinase reduced acetylcholine-stimulated eNOS phosphorylation, NOS activity and aorta relaxation. These data indicate a new signal transduction pathway for IRS-1/PI 3- kinase/Akt/eNOS activation and ERK1/2 by means of JAK2 tyrosine phosphorylation stimulated by acetylcholine in vessels. Moreover, we demonstrate that, in aorta of obese rats (high-fat diet), there is an impairment in insulin- and acetylcholine-stimulated IRS-1/PI 3-kinase pathway, leading to reduced activation with lower protein levels of eNOS associated with a hyperactivated ERK/MAP kinase pathway. These results suggest that in aorta of obese rats, there is not only insulin resistance, but also acetylcholine resistance, probably mediated by a common signaling pathway that controls the activity and the protein levels of eNOS.

1- INTRODUÇÃO

A insulina é o principal hormônio anabólico do organismo. Seus efeitos mais conhecidos estão associados ao metabolismo energético, especialmente a regulação do metabolismo de glicose, ácidos graxos e aminoácidos. Assim, este hormônio facilita o armazenamento de macromoléculas no fígado, músculo e tecido adiposo.

Importantes síndromes clínicas estão associadas a um estado definido como "resistência à insulina", tais como a obesidade, o *diabetes mellitus* tipo 2 (DM2), a síndrome dos ovários policísticos e a esteatose hepática não-alcoólica (Reaven, 1988; Reaven, 2005). A resistência à insulina tem despertado grande interesse médico e científico devido a sua associação com a doença cardiovascular aterosclerótica (Ginsberg, 2000b; Reaven, Abbasi, & McLaughlin, 2004). O termo "resistência à insulina" enfatiza a incapacidade da insulina de manter a homeostase normal da glicose. Em outras palavras, a reduzida ação biológica da insulina demanda a presença de concentrações mais elevadas deste hormônio a fim de manter a normoglicemia e a utilização normal de glicose pelos tecidos-alvo da insulina (Reaven, 1991a). Portanto, o termo "resistência à insulina" implica a existência de resistência aos efeitos metabólicos da insulina, o que reflete o efeito inadequado da insulina sobre o metabolismo de glicose, mas não lida diretamente com outros aspectos da ação da insulina. No entanto, a insulina exerce inúmeros efeitos sobre o metabolismo de lipídios e proteínas, sobre o transporte de íons e aminoácidos, sobre a proliferação e o ciclo celular, sobre a diferenciação celular e sobre a síntese de óxido nítrico (Steinberg, Brechtel, Johnson, Fineberg, & Baron, 1994; Zeng & Quon, 1996; Cardillo et al., 1999). Nas últimas duas décadas, ficou claro que as ações da insulina não estão restritas às desempenhadas por tecidos-alvo clássicos. Receptores de insulina foram descritos no cérebro e nas células beta do pâncreas, onde a insulina parece desempenhar funções importantes no controle do metabolismo e da secreção de insulina, respectivamente (Aspinwall, Lakey, & Kennedy, 1999; Kulkarni et al., 1999; Freychet, 2000; Schulingkamp, Pagano, Hung, & Raffa, 2000). Além disso, receptores de insulina também foram encontrados em células endoteliais e células musculares lisas vasculares (Jialal et al., 1985; King, Goodman, Buzney, Moses, & Kahn, 1985; Velloso, Carneiro, Crepaldi, Boschero, & Saad, 1995), além de ter sido demonstrado que a insulina pode modular o tônus vascular e o fluxo sangüíneo tecidual (Baron, 1994).

A resistência à insulina é, portanto, caracterizada pela redução das ações biológicas clássicas da insulina de estimular a captação de glicose no músculo esquelético e células adiposas, e de inibir a lipólise do tecido adiposo e a produção hepática de glicose, resultando em elevação da glicemia e dos ácidos graxos livres plasmáticos, além de hiperinsulinemia compensatória (Dunaif, 1997; Kahn & Flier, 2000). Os mecanismos envolvidos na patogênese da resistência à insulina não são ainda totalmente conhecidos mas são provalvalmente multifatoriais (Kahn, Hull, & Utzschneider, 2006). Embora os efeitos da resistência à insulina nos órgãos clássicos tenham sido extensivamente descritos, o resultado da resistência à ação da insulina sobre sistemas efetores não-clássicos são bem menos conhecidos.

Embora a resistência à insulina tenha recebido muita atenção com respeito ao papel central que parece desempenhar na etiopatogênese do DM2, atualmente está claro que a resistência à insulina, independente da hiperglicemia, está associada ao aumento de duas a três vezes no risco de mortalidade cardiovascular, sendo que somente 50% deste excesso de mortalidade pode ser explicado por fatores de risco cardiovasculares clássicos, como a hipertensão arterial e elevação do colesterol (Laakso, 1996; Van Gaal, Mertens, & De Block, 2006). Tem sido sugerido que a reduzida ação da insulina na vasculatura pode contribuir para a aterosclerose mais grave observada em situações clínicas de resistência à insulina (Fontbonne & Eschwege, 1991; Fontbonne et al., 1991).

A resistência à insulina tem sido considerada um importante fator de risco para o desenvolvimento da aterosclerose (Howard et al., 1996; Reaven et al., 2004). A doença vascular é a principal causa de mortes e responde por um grande percentual da morbidade em pacientes diabéticos tipo 1 ou tipo 2 (Stern, 1995). O risco de complicações cardiovasculares em diabéticos é duas a seis vezes maior que em indivíduos não-diabéticos e, devido a isso, a expectativa de vida é sete a dez anos menor em diabéticos (Ginsberg, 2000a).

No DM2 há o acometimento crônico de pequenos e grandes vasos (respectivamente, micro e macroangiopatia) (Skyler, 1996). A doença microvascular é demonstrada pela retinopatia, neuropatia e nefropatia, sendo o *diabetes mellitus* a principal causa de cegueira em adultos (Aiello, 2005; Aiello et al., 1998), de insuficiência renal

crônica terminal (Rossing et al., 2004; Molitch et al., 2004) e de amputações não-traumáticas de membros inferiores (Cianci, 2000). Ao contrário da doença microvascular, que ocorre apenas em pacientes com *diabetes mellitus*, a doença macrovascular assemelha-se à que ocorre em indivíduos não-diabéticos. A macroangiopatia diabética é caracterizada pela aterosclerose acelerada, mais precoce e mais extensa do que a encontrada em indivíduos não-diabéticos (Granger et al., 1993), e acomete órgãos vitais como cérebro e coração (Lewinter, 2005). A aterosclerose no *diabetes mellitus* é multifatorial e inclui interações complexas entre hiperglicemia, hiperlipidemia, estresse oxidativo, envelhecimento acelerado, hiperinsulinemia e/ou hiper-pró-insulinemia, e alterações na coagulação e fibrinólise (Calles-Escandon, Garcia-Rubi, Mirza, & Mortensen, 1999; Van Gaal et al., 2006).

Embora a dislipidemia e a hipertensão arterial sistêmica ocorram com grande freqüência em populações com DM2, ainda existe excesso de risco de morte por doença cardiovascular nos diabéticos quando estes fatores são corrigidos (Stamler, Vaccaro, Neaton, & Wentworth, 1993). O risco absoluto de morte por doença cardiovascular é muito maior – cerca de três vezes maior - em homens diabéticos do que em não-diabéticos, independente da faixa etária, etnia, nível de colesterol sérico, pressão arterial sistólica e tabagismo (Vaccaro, Stamler, & Neaton, 1998). Adicionalmente, a presença de *diabetes* agrava acentuadamente os efeitos deletérios dos demais fatores de risco cardiovasculares (Fuller, Shipley, Rose, Jarrett, & Keen, 1980; Rosengren, Welin, Tsipogianni, & Wilhelmsen, 1989).

Em indivíduos com ou sem *diabetes* a lesão inicial da aterosclerose é a disfunção endotelial (Ross, 1999; Lusis, 2000), fenômeno que pode ser definido pragmaticamente por menor vasodilatação em resposta à acetilcolina (ACh) ou hiperemia, por redução na produção de óxido nítrico pelo endotélio e por elevação dos níveis plasmáticos de fator de von Willebrand, trombomodulina, selectina, PAI-1 (*inibidor 1 do ativador do plasminogênio*), colágeno tipo IV e t-PA (*ativador do plasminogênio tecidual*) (Calles-Escandon & Cipolla, 2001). A disfunção endotelial expressa um "mau funcionamento" do endotélio vascular e geralmente reflete uma menor biodisponibilidade do óxido nítrico. Muitos componentes do *diabetes* causam disfunção endotelial de forma

independente: hiperglicemia, resistência à insulina, aumento dos produtos finais de glicosilação avançada e dislipidemia. Antes de tentar entender como pode ocorrer a associação entre resistência à insulina e disfunção endotelial / doença cardiovascular é necessário entender o papel fisiológico do endotélio na homeostase vascular.

Funções da célula endotelial e óxido nítrico

As células endoteliais delimitam o lúmen de toda a vasculatura constituindo uma barreira entre o sangue circulante e as células musculares lisas vasculares (VSMC). As pesquisas realizadas durante as duas últimas décadas evidenciaram que, além de servir como barreira física, a célula endotelial atua sobre as células sangüíneas e componentes plasmáticos, bem como sobre as VSMC, através da produção de vários mediadores químicos, como prostaglandinas e óxido nítrico (Vanhoutte, Luscher, & Graser, 1991).

O endotélio pode promover uma ou mais das seguintes alterações: (1) vasodilatação ou vasoconstricção, para regular o fluxo sangüíneo; (2) crescimento e/ou alterações nas características fenotípicas das VSMC; (3) alterações pró-inflamatórias ou anti-inflamatórias e (4) manutenção da fluidez do sangue e controle de hemorragias (De Meyer & Herman, 1997; Calles-Escandon et al., 2001). A célula endotelial normal mantém, portanto, um equilíbrio entre forças opostas resultando na manutenção de suprimento sangüíneo adequado para os tecidos e regulando a inflamação e coagulação.

O óxido nítrico (NO) é um segundo mensageiro gasoso produzido pelo endotélio, fundamental para a manutenção da homeostase cardiovascular, regulando a pressão arterial sistêmica, a remodelação vascular e a angiogênese (Huang et al., 1995; Murohara et al., 1998; Rudic et al., 1998; Shesely et al., 1996).

Após a descoberta de Furchgott e Zawadski em 1980 de que a presença do endotélio é obrigatória para o relaxamento vascular induzido pela acetilcolina (Furchgott & Zawadzki, 1980), estudos de bioensaio demonstraram que uma substância muito lábil, difusível e não-prostanóide era responsável por mediar o relaxamento vascular dependente do endotélio, e esta substância foi denominada “fator de relaxamento derivado do endotélio”, ou

EDRF (Rubanyi & Vanhoutte, 1986). Entre os diversos estímulos que podem provocar a liberação de EDRF estão os produtos plaquetários, trombina, hormônios e peptídeos vasoativos (acetilcolina, serotonina, bradicinina, insulina), neurotransmissores, autacóides locais, alterações na tensão de oxigênio local e aumento no fluxo sangüíneo (*shear stress* – força de cisalhamento) (Vanhoutte, Rubanyi, Miller, & Houston, 1986). Baseado nas grandes similaridades entre o NO autêntico e o EDRF, incluindo a capacidade de ambos de estimular a guanilato-ciclase solúvel (Rapoport, Draznin, & Murad, 1983) e de serem inativados pela hemoglobina (Martin, Villani, Jothianandan, & Furchgott, 1985) e por ânions superóxido (Rubanyi et al., 1986), foi proposto que EDRF e NO correspondessem à mesma molécula (Furchgott, 1987; Rubanyi & Vanhoutte, 1987; Moncada, Radomski, & Palmer, 1988; Furchgott, 1990; Furchgott, 1996).

A produção de NO endógeno ocorre a partir da conversão do aminoácido L-arginina em L-citrulina pela enzima óxido nítrico sintase (NOS) (Schmidt et al., 1991). Até o momento foram isoladas, purificadas e clonadas quatro isoformas desta enzima.

A óxido nítrico sintase tipo III, isolada a partir de células endoteliais, produz níveis picomolares de NO dos quais apenas uma pequena fração desencadeia respostas fisiológicas (Palmer, Ashton, & Moncada, 1988). Essa isoforma é regulada pelo sistema cálcio-calmodulina com NADPH, flavina adenina dinucleotídeo / mononucleotídeo (FAD/FMN) e tetra-hidrobiopterina (HB_4) como cofatores. Funcionalmente, a óxido nítrico sintase endotelial (eNOS) é essencial para o controle do tônus vascular em resposta a vários estímulos: mecânicos (*shear stress*), dependentes de receptores (ex.: acetilcolina) e independentes de receptores (ex.: cálcio) (Furchgott, 1993). O óxido nítrico é um gás e, portanto, após ser produzido pela célula endotelial, difunde-se localmente, atingindo as células musculares lisas vasculares onde ativa a enzima guanilato-ciclase. O concomitante aumento no GMP cíclico induz o relaxamento das VSMC, promovendo vasodilatação. Ocorre também uma produção basal de NO pela eNOS (Vallance, Collier, & Moncada, 1989).

A vasodilatação contínua produzida pelo NO é importante para a regulação da pressão arterial (Luscher, 1990). Muitos estudos demonstraram que a infusão sistêmica de inibidores da NOS pode elevar a pressão arterial (Sakai, Hara, Tsai, Tsuchida, & Intaglietta,

2000; Sekiguchi et al., 2001). O óxido nítrico também contribui para a prevenção da agregação plaquetária anormal e da remodelação vascular (Radomski, Palmer, & Moncada, 1987; Lopez-Jaramillo, Gonzalez, Palmer, & Moncada, 1990; Radomski, Palmer, & Moncada, 1990b; Radomski, Palmer, & Moncada, 1990a; Moncada & Higgs, 1993; Wennmalm, 1994; Cohen, 1995; Griendling & Alexander, 1996; Cooke & Dzau, 1997; Murohara et al., 1998; Rudic et al., 1998). A redução na biodisponibilidade do óxido nítrico é uma característica de pacientes com doença coronariana (Zeiher, 1996) e está associada ao desenvolvimento de lesões ateroscleróticas (Moroi et al., 1998). O óxido nítrico dilata os vasos sangüíneos e inibe a função plaquetária e leucocitária, enquanto os inibidores da NOS elevam a pressão arterial e reduzem o fluxo sangüíneo (Papapetropoulos, Rudic, & Sessa, 1999). Muitos desses efeitos biológicos foram confirmados através da produção de camundongos que não expressam a eNOS (Huang et al., 1995; Shesely et al., 1996; Godecke et al., 1998). Além de seus efeitos cardiovasculares, estudos sugerem que o NO desempenha um papel importante na modulação da sensibilidade à insulina e metabolismo de carboidratos, e que a eNOS tem função metabólica predominante sobre as outras isoformas (Shankar, Wu, Shen, Zhu, & Baron, 2000), ou seja, camundongos que não expressam a eNOS apresentam resistência à insulina no fígado e tecidos periféricos.

Por outro lado, a disfunção endotelial está associada ao aumento da secreção de quimocinas, como a MCP-1 (*monocyte chemoattractant protein 1*), ao aumento da expressão de moléculas de adesão endotelial para leucócitos e plaquetas e ao aumento da permeabilidade para lipoproteínas e outros componentes do plasma. Isto induz ao recrutamento de monócitos-macrófagos para o espaço subendotelial e à infiltração de moléculas de LDL-colesterol plasmático, as quais se ligam aos proteoglicanos arteriais (Lusis, 2000; Skalen et al., 2002). O LDL retido sofre oxidação e é captado pelos macrófagos (Libby, 2002). Os macrófagos e outros leucócitos ativados, bem como as plaquetas agregadas e aderidas ao vaso estimulam a proliferação de VSMC e a produção de matriz extracelular, culminando na formação de uma lesão complexa preenchida com material pró-trombótico contido por uma cápsula de fibrina. A ruptura desta cápsula de fibrina por metaloproteinases de matriz (MMP) leva à formação do trombo e subseqüentemente à oclusão arterial (Davies, 1990; Ross, 1993). Como a doença

macrovascular também ocorre em indivíduos não-diabéticos, acredita-se que a resistência à insulina e o *diabetes* acelerem este processo através do agravamento da disfunção endotelial e da dislipidemia .

A patogênese da disfunção endotelial no DM2 parece envolver tanto a resistência à insulina quanto a hiperglicemia. Diversos estudos sugerem que a insulina apresenta potencialmente tanto efeitos anti-aterogênicos quanto pró-aterogênicos. Um dos principais efeitos anti-aterogênicos da insulina é o estímulo da produção endotelial de NO, o qual é um potente inibidor da agregação plaquetária e da adesão de plaquetas à parede vascular. O NO endotelial também controla a expressão de genes envolvidos na aterogênese. Ele reduz a expressão de MCP-1 e de moléculas de adesão de superfície como CD11/CD18, P-selectina, VCAM-1 (*vascular cell adhesion molecule-1*) e ICAM-1 (*intercellular adhesion molecule-1*) e também reduz a permeabilidade vascular e a taxa de oxidação da lipoproteína de baixa densidade (LDL) para a sua forma pró-aterogênica (Wang, Goalstone, & Draznin, 2004; Zeng et al., 1996).

As ações biológicas da insulina nas células vasculares incluem aumento no transporte de aminoácidos, síntese de glicogênio, síntese de DNA e expressão gênica. Fisiologicamente, a insulina também tem efeitos vasculares específicos, como o aumento da liberação de óxido nítrico, a regulação da expressão de mRNA de proteínas da matriz e a ativação da eNOS (King et al., 1985; Obata et al., 1996). Nas células sensíveis à insulina, as ações deste hormônio iniciam-se com a sua ligação a receptores específicos de membrana, desencadeando a transmissão do sinal molecular através de vias específicas até os efetores finais.

Vias de sinalização da insulina

A insulina é um hormônio polipeptídico anabólico produzido pelas células β do pâncreas, cuja síntese é ativada pela elevação da glicemia. A insulina circulante age em vários tecidos periféricos, incluindo músculo, fígado e tecido adiposo. Seus efeitos metabólicos imediatos incluem: aumento da captação de glicose, aumento da síntese de

proteínas, ácidos graxos e glicogênio, bem como bloqueio da neoglicogênese, glicogenólise, lipólise e proteólise. Além disso, a insulina tem efeitos tardios na expressão de genes e síntese protéica, assim como na proliferação e diferenciação celulares. Uma função recém-descoberta da insulina e dos fatores de crescimento "insulina-like" (IGFs) envolve a prevenção da apoptose ou morte celular, promovendo a sobrevida celular.

Os eventos que ocorrem após a ligação da insulina ao seu receptor são altamente regulados e específicos (Pessin & Saltiel, 2000). A ação da insulina na célula se inicia através da sua ligação a um receptor de membrana plasmático (Freychet, Roth, & Neville, Jr., 1971; Cuatrecasas, 1972). Os receptores de insulina estão presentes em todas as células do organismo, em concentrações variáveis, incluindo as células endoteliais e células da camada muscular lisa do vaso (Jialal et al., 1985; King & Johnson, 1985). Suas concentrações variam de 40 receptores por eritrócito circulante a mais de 200.000 receptores por adipócito ou hepatócito. O receptor de insulina é uma glicoproteína heterotetramérica constituída por duas subunidades α, cada uma com 135 kDa e duas subunidades β, cada uma com 95 kDa, unidas por ligações dissulfeto (Kahn, 1985). A subunidade α é inteiramente extracelular e contém o domínio de ligação da insulina ou "domínio regulatório". A subunidade β é uma cadeia peptídica transmembrana e é a responsável pela transmissão do sinal, contendo o domínio tirosina-quinase ou "domínio catalítico".

Para os receptores transmembrana da família das tirosina-quinases a união de um ligante a sua porção extracelular (domínio regulatório) catalisa a transferência do grupo (γ)-fosfato terminal do ATP para resíduos de tirosina em proteínas-alvo selecionadas. O aumento da carga negativa atribuída aos fosfoésteres formados em um ou mais domínios na proteína muda a sua forma e, conseqüentemente, seu estado funcional. A fosforilação de uma proteína é transitória; na célula há fosfatases, que hidrolisam a forma fosforilada do substrato protéico de volta a sua forma original. A alteração no estado de fosforilação de uma proteína é uma das principais vias de que as células dispõem para que sinais externos (hormônios, fatores de crescimento, neurotransmissores, drogas) possam regular as vias metabólicas intracelulares (Virkamaki, Ueki, & Kahn, 1999).

O receptor de insulina pertence à família de receptores de fatores de crescimento com atividade tirosina-quinase intrínseca. Após a ligação da insulina às subunidades α, o receptor se autofosforila rapidamente em múltiplos resíduos de tirosina (1158, 1162, 1163) nas subunidades β (Ebina et al., 1985) tornando-se capaz de fosforilar outros substratos em resíduos de tirosina (Kasuga, Karlsson, & Kahn, 1982). Tem sido postulado que a subunidade α mantém um tônus inibitório sobre a atividade quinase da subunidade β, que é aliviado após a ligação da insulina (White, Shoelson, Keutmann, & Kahn, 1988). A ligação da insulina induz uma alteração conformacional que modifica a interação entre os heterodímeros α-β, permitindo a transfosforilação das subunidades β, ou seja, uma subunidade β fosforila os resíduos de tirosina da outra subunidade β (Baron & Van Obberghen, 1995).

A maior evidência de que a atividade tirosina-quinase do receptor de insulina é necessária para a ação da insulina provém de experimentos com mutagênese *in vitro*, nos quais um resíduo de lisina na posição 1030 foi trocado por qualquer outro aminoácido. Com base na analogia de outras quinases, este resíduo é o sítio de união do ATP na quinase (Ebina et al., 1985; Ullrich et al., 1985). Quando essas mutações são expressas em células, a ligação da insulina ocorre normalmente mas, como quinases, são totalmente inativas e ineficientes na mediação da estimulação da insulina no metabolismo celular (Ebina et al., 1987). Isto é verdadeiro para todos os efeitos da insulina, tanto os efeitos metabólicos agudos quanto a promoção do crescimento. Mutações nos principais sítios de autofosforilação do receptor de insulina produzem efeitos similares, porém menos intensos (Wilden et al., 1992).

Após a ativação da tirosina quinase do receptor de insulina, este é capaz de fosforilar uma série de proteínas, dentre as quais as conhecidas por IRS (*substratos do receptor de insulina*) estão melhor caracterizadas. Essas proteínas-substratos que não têm atividade quinase são comumente denominadas proteínas de acoplamento pois, após a fosforilação, podem se ligar a várias outras proteínas intracelulares com domínios específicos para a transmissão do sinal de insulina. A fosforilação e as interações proteína-proteína são essenciais para transmitir e compartimentalizar o sinal da insulina (Virkamaki et al., 1999).

A fosfotirosina no domínio 960 do receptor de insulina autofosforilado na seqüência NPXpY (asparagina – prolina – qualquer aminoácido – fosfotirosina) serve de reconhecimento para o domínio PTB (*domínio de ligação à fosfotirosina*) das proteínas IRS. A modificação desta tirosina inibe completamente a fosforilação subseqüente do IRS-1 e de outros substratos do receptor de insulina e leva à perda das ações biológicas dependentes de insulina (White et al., 1988).

Os substratos do receptor de insulina correspondem a uma família de proteínas fosforiladas pelo receptor de insulina ativado. Até o momento, nove membros desta família foram identificados, incluindo IRS-1 (White, Maron, & Kahn, 1985; Rothenberg et al., 1991; Sun et al., 1992), IRS-2 (Sun et al., 1995), IRS-3 (Lavan, Lane, & Lienhard, 1997b) e IRS-4 (Lavan et al., 1997a), que são vistos como os mais específicos para a sinalização da insulina; e outros substratos, incluindo Gab-1, $p60^{dok}$, Cbl, APS e isoformas da Shc (Pessin et al., 2000).

Uma proteína IRS típica apresenta alguns domínios de interação (White, 1998). Estes domínios existem na estrutura terciária natural de uma proteína ou podem ser criados por modificações covalentes da proteína, como as que ocorrem após a fosforilação de resíduos de tirosina ou serina/treonina (Virkamaki et al., 1999). Na extremidade NH2-terminal de todas as IRS, exceto da Shc, encontra-se o domínio PH (*domínio com homologia à plecstrina*), envolvido no direcionamento das IRS à membrana celular e às proximidades do receptor de insulina. O domínio PH se liga a grupos fosfatidilinositídeos específicos eletricamente carregados na membrana celular próximos ao receptor de insulina. O domínio PTB é crítico para o reconhecimento da seqüência NPXpY na subunidade β do receptor de insulina. Assim, os domínios PH e PTB contribuem para a interação de uma proteína IRS com o receptor de insulina. Durante a interação com o receptor de insulina, a proteína IRS é fosforilada em vários de seus resíduos de tirosina pelo receptor de insulina, criando sítios de ligação para proteínas com domínios SH2. O IRS-1 contém pelo menos vinte domínios de tirosina potencialmente susceptíveis à fosforilação, incluindo seis em seqüências YMXM e três em seqüências YXXM (Keller, Lamphere, Lavan, Kuhne, & Lienhard, 1993). Pelo menos oito destes domínios de tirosina são fosforilados pelo receptor de insulina ativado. O IRS-1 também contém cerca de 30

resíduos serina/treonina em domínios de fosforilação para serina/treonina quinases, e vários estudos indicam que a fosforilação do IRS-1 nestes resíduos pode ter efeito inibitório na sinalização da insulina (Tanti, Gremeaux, Van Obberghen, & Marchand-Brustel, 1994; De Fea & Roth, 1997). Em células musculares lisas da aorta de ratos, o tratamento com angiotensina II reduziu a fosforilação do IRS-1 em tirosina e aumentou a sua fosforilação em resíduos de serina (Folli, Kahn, Hansen, Bouchie, & Feener, 1997).

Para estudar o papel fisiológico do IRS-1 em animais, camundongos que não expressam o gene do IRS-1 (*knockout* do gene) foram produzidos (Araki et al., 1994): surpreendentemente, camundongos homozigotos para o *knockout* do gene do IRS-1 eram viáveis e apresentavam poucas mudanças em seu metabolismo – déficit de crescimento e discreta intolerância à glicose, além do aumento de fatores de risco cardiovasculares: hipertensão arterial, hipertrigliceridemia e deficiência no relaxamento vascular dependente do endotélio (Abe et al., 1998a). Isto direcionou as pesquisas a buscar vias alternativas para a sinalização de insulina. Análises posteriores do fígado de camundongos com *knockout* do gene do IRS-1 demonstraram que a insulina estimulava a fosforilação em tirosina de uma proteína com peso molecular aparente de 190 kDa em SDS-PAGE (*eletroforese em gel de poliacrilamida com dodecil-sulfato de sódio*). Devido ao seu peso molecular semelhante ao do IRS-1 e sua aparente capacidade de compensar uma deficiência do IRS-1, essa proteína foi denominada IRS-2 e foi clonada em 1995 (Sun et al., 1995). Recentemente foi demonstrado que camundongos com *knockout* do gene do IRS-2 em homozigose desenvolvem uma síndrome muito semelhante ao *diabetes mellitus* tipo 2 (Withers et al., 1998), com resistência periférica à insulina e massa reduzida de células β pancreáticas, evoluindo para deterioração da homeostase de glicose. Posteriormente, dois novos membros da família de proteínas IRS foram clonados: o IRS-3 (PM 60 kDa) é um substrato adipócito-específico do receptor de insulina, que pode ser o principal regulador da PI 3-quinase em células adiposas (Lavan & Lienhard, 1993; Lavan et al., 1997a; Lavan et al., 1997b; Smith-Hall et al., 1997); o IRS-4 é uma proteína de 160 kDa que foi detectada em células renais embrionárias humanas – HEK (Fantin et al., 1998). O papel relativo de cada uma dessas proteínas IRS nas vias de sinalização da insulina ainda requer estudos adicionais .

Após a estimulação com insulina, as proteínas IRS e Shc são imediatamente fosforiladas em resíduos de tirosina (White, 2002). A ativação destes substratos protéicos leva à ativação de duas vias específicas de sinalização, divididas apenas para maior clareza de compreensão, já que as evidências atuais indicam que estas vias não são independentes. Uma delas, a cascata p42/p44MAPK (*proteína quinase ativadora da mitogênese*), tem início com a ativação da Ras pela Shc ou pelas IRSs ativadas, levando à estimulação seqüencial das proteínas quinases citoplasmáticas Raf-1, MEK e p42/p44MAPK (ERK1/2, *quinases reguladoras da sinalização extracelular*). Esta cascata termina por fosforilar fatores de transcrição nucleares que modulam a expressão de genes, o crescimento, a proliferação e a diferenciação celulares (L'Allemain, 1994; Davis, 1993). A outra cascata é conhecida como via da PI 3-quinase / PKB (*fosfatidilinositol 3-quinase / proteína quinase B*) e, além de também exercer ações sobre o crescimento celular, parece ser a responsável pelo controle do transporte de glicose, glicólise, síntese de glicogênio, síntese protéica e inibição da apoptose (Shepherd, Withers, & Siddle, 1998).

Muitas proteínas intracelulares que interagem com as IRSs contêm domínios SH2 (*Src homology 2*), que são módulos de ligação a fosfotirosinas, identificados inicialmente em 1991 (Koch, Anderson, Moran, Ellis, & Pawson, 1991). Os domínios SH2 têm maior afinidade de ligação às fosfotirosinas que os domínios PTB e reconhecem padrões específicos de aminoácidos, tornando possível uma interação proteína-proteína mais estável. As proteínas adaptadoras, que contêm domínios SH2, geralmente possuem domínios SH3 para ligação a seqüências ricas em prolina PXXP com uma estrutura em hélice específica. Esses domínios SH3 constituem uma ligação entre as proteínas adaptadoras e seus alvos intracelulares ou subunidades catalíticas associadas. A subunidade p85 da PI 3-quinase não possui atividade enzimática intrínseca mas é responsável pela transmissão de etapas do sinal insulínico devido a sua capacidade de interação com outras proteínas fosforiladas em resíduos de tirosina. A subunidade regulatória da PI 3-quinase, assim como a Grb2, são exemplos bem conhecidos de proteínas adaptadoras (Virkamaki et al., 1999).

A PI 3-quinase é composta de duas subunidades: uma subunidade catalítica (p110), com peso molecular de 110 kDa, e uma subunidade regulatória (p85) com 85 kDa.

Em condições de "repouso", isto é, na ausência de estímulo com insulina, a subunidade regulatória p85 serve para estabilizar e inativar a subunidade catalítica p110. A atividade inibitória da p85 é liberada quando o domínio SH2 da porção NH2-terminal é ocupado por um resíduo de tirosina fosforilado (Cuevas et al., 2001). A subunidade p85 é uma proteína com vários domínios: dois domínios SH2, um domínio inter-SH2, um domínio bcr (*breakpoint cluster region*) e um domínio SH3 (Carpenter & Cantley, 1990). Os domínios SH2, localizados na extremidade C-terminal da proteína, podem interagir com seqüências de fosfotirosina específicas em moléculas sinalizadoras, como o IRS-1 (White, 1998). O domínio inter-SH2 permite a associação entre a p85 e a extremidade N-terminal da p110 (Dhand et al., 1994a). O papel do domínio bcr ainda é pouco conhecido e o domínio SH3, localizado na extremidade N-terminal da subunidade p85, interage com seqüências ricas em prolina de proteínas sinalizadoras. A subunidade p110 possui duas atividades enzimáticas. Possui atividade catalítica quinase direcionada contra a posição D3 de vários fosfatidilinositídeos, gerando os segundos mensageiros restritos à membrana PI(3,4,5) tri-fosfato – PIP3 - e PI(3,4)bi-fosfato – PIP2. Possui também uma atividade serina-quinase capaz de fosforilar p85 e IRS-1 em resíduos de serina podendo exercer papel modulatório negativo na sinalização da insulina (Tanti et al., 1994; Dhand et al., 1994b).

Em resposta à insulina, a subunidade p85 da PI 3-quinase se associa ao IRS-1 fosforilado em tirosina através da interação entre os domínios SH2 da p85 e as seqüências YMXM do IRS-1 (Myers, Jr. et al., 1992; Rordorf-Nikolic, Van Horn, Chen, White, & Backer, 1995) ativando a função catalítica da p110. A PI 3-quinase também é recrutada pelo IRS-2 e IRS-3 fosforilados em tirosina e podem ocorrer diferenças funcionais na capacidade destes substratos do receptor de insulina de ativar a PI 3-quinase (Smith-Hall et al., 1997).

A PI 3-quinase ativada age rapidamente sobre uma serina/treonina quinase conhecida por c-Akt ou PKB, localizada primariamente no citosol (90%). Esta enzima corresponde ao homólogo celular humano da v-Akt, um oncogene viral detectado inicialmente em células AKR de ratos, as quais apresentam alta incidência de leucemias e linfomas a partir de timomas espontâneos e nas quais pôde ser isolado um retrovírus tumorigênico denominado AKT8 (Staal, Hartley, & Rowe, 1977; Staal & Hartley, 1988). O

locus da Akt humana foi mapeado no cromossomo 14q32, próximo ao locus das cadeias pesadas de imunoglobulinas (Staal, Huebner, Croce, Parsa, & Testa, 1988). Em 1991 a Akt humana foi clonada e teve seu peso molecular determinado em 57 kDa (Bellacosa, Testa, Staal, & Tsichlis, 1991). Esta quinase apresenta alta homologia com a PKA (*proteína quinase A*) e com a PKC (*proteína quinase C*) e, por conseqüência, também foi denominada de PKB. O genoma de mamíferos contém três genes codificando Akt ou PKBs: Akt1 (PKBα), Akt2 (PKBβ) e Akt3 (PKBγ) (Coffer, Jin, & Woodgett, 1998). Os três homólogos têm alta conservação evolutiva (Franke, Tartof, & Tsichlis, 1994). A extremidade N-terminal da Akt contém um domínio PH (Haslam, Koide, & Hemmings, 1993; Mayer, Ren, Clark, & Baltimore, 1993) e o domínio catalítico central tem muita semelhança com a PKA e a PKC (Bellacosa et al., 1993). Todos os tecidos contêm pelo menos uma isoforma de Akt/PKB: Akt1 (PKBα) e Akt2 (PKBβ) têm níveis de expressão mais altos no cérebro, timo, coração e pulmões, enquanto a Akt3 (PKBγ) é expressa de forma mais restrita, com níveis mais elevados no cérebro e testículos (Coffer & Woodgett, 1991; Jones, Jakubowicz, Pitossi, Maurer, & Hemmings, 1991; Bellacosa et al., 1993). Parece que a expressão de Akt é tanto maior quanto mais diferenciada for a célula (Coffer et al., 1998).

A insulina age de forma diferente sobre as três isoformas da Akt, de acordo com o tecido e a espécie em estudo. A administração de insulina em ratos rapidamente ativa a Akt1 no músculo esquelético, com efeitos mínimos sobre a Akt2 e sem efeitos sobre a Akt3 (Walker et al., 1998). Em humanos, a insulina ativa as três isoformas no músculo, embora o efeito sobre a Akt3 seja pequeno (Kim, Nikoulina, Ciaraldi, Henry, & Kahn, 1999). A incubação de adipócitos de ratos com insulina *in vitro* resulta em ativação da Akt1 e Akt2, mas não da Akt3, enquanto em hepatócitos de ratos *in vitro* a insulina ativa principalmente a Akt1, com pouco efeito sobre a Akt2 e nenhum efeito sobre a Akt3 (Walker et al., 1998). Em adipócitos humanos, a insulina ativa a Akt2 (Rondinone, Carvalho, Wesslau, & Smith, 1999), mas os efeitos deste hormônio sobre as outras isoformas da Akt no tecido adiposo ainda não foram individualmente estudados.

Apesar da Akt ter potencial oncogênico (Aoki, Batista, Bellacosa, Tsichlis, & Vogt, 1998), sua regulação e função normais eram desconhecidas até que se descobriu ser um alvo de ação da PI 3-quinase (Burgering & Coffer, 1995; Kohn, Kovacina, & Roth,

1995). Os produtos lipídicos 3'-fosforilados da PI 3-quinase (PIP2, PIP3) medeiam a ativação da Akt, interagindo com seu domínio PH (Coffer et al., 1998; Lietzke et al., 2000). Através desta interação, a Akt é translocada do citosol para a membrana celular, tornando-se substrato de duas proteínas quinases, também ligadas à membrana pela interação entre seus domínios PH e os fosfatidilinositídeos gerados pela PI 3-quinase - denominadas PDK1 e PDK2 (*proteína quinase dependente de fosfatidilinositol 1* e *2*), as quais fosforilam a Akt seqüencialmente em treonina 308 e serina 473 (Stephens et al., 1998; Currie et al., 1999; Alessi et al., 1997). Somente quando fosforilada nestes dois resíduos é que a Akt se torna ativa, deslocando-se da membrana para fosforilar seus substratos celulares (Alessi et al., 1996).

Como a atividade da Akt é estimulada por uma variedade de fatores de crescimento e insulina, muito se tem estudado sobre seus efeitos no metabolismo intermediário, na síntese de proteínas e na sua capacidade de afetar o potencial de crescimento celular, induzir diferenciação celular e inibir a apoptose.

Vários trabalhos têm demonstrado uma possível associação entre a ativação da Akt e a estimulação da translocação de GLUT4 (*transportador de glicose 4*) dos estoques intracelulares para a superfície celular (Tanti et al., 1997; Wang et al., 1999).

O aumento da síntese de glicogênio em alguns tecidos pode ser regulado pela Akt pois foi demonstrado que a GSK-3 (*glicogênio sintase quinase-3*) é um de seus substratos. A inibição da GSK-3 ativa a síntese de glicogênio e o início da produção de certos mRNA (Alessi et al., 1996; Coffer et al., 1998).

Um substrato direto da Akt é a PFK2 (*6-fosfofrutose 2-quinase*) e a fosforilação da PFK2 pode explicar como a glicólise é estimulada pela insulina (Deprez, Vertommen, Alessi, Hue, & Rider, 1997).

O estímulo da síntese protéica em resposta à insulina em diversos tipos de células, principalmente músculo esquelético e tecido adiposo, também pode estar sob controle da Akt, a qual controla a atividade da quinase mTOR e da p70S6 quinase de maneira ainda não totalmente esclarecida (Burgering et al., 1995).

A morte celular programada, ou apoptose, é fundamental na regulação do desenvolvimento e no controle da homeostase tecidual em condições de estresse celular. Fatores de crescimento como PDGF (*fator de crescimento derivado de plaquetas*), IGF-1 e a insulina podem promover a sobrevida celular ativando a PI 3-quinase e a Akt (Ataliotis & Mercola, 1997; Kulik & Weber, 1998). A ativação da Akt induz um forte sinal anti-apoptótico. Parte deste efeito é, provavelmente, mediado pela fosforilação da BAD, um membro da família da Bcl-2. A proteína BAD no estado não-fosforilado interage com Bcl_{XL} para induzir apoptose. Após ser fosforilada em serina pela Akt, BAD se dissocia de Bcl_{XL} e forma um complexo inativo com a proteína 14.3.3 (Datta et al., 1997). Tem sido encontrada superexpressão da Akt em grande variedade de tumores humanos, o que pode ser interpretado como uma conseqüência da promoção da sobrevida de células transformadas em condições nas quais a apoptose normalmente evitaria o seu crescimento (Coffer et al., 1998).

Experimentos recentes demonstraram que a Akt pode fosforilar e ativar diretamente a eNOS em resíduos de serina (1177 e 1179), levando à produção endotelial de óxido nítrico (Dimmeler et al., 1999; Fulton et al., 1999). O estímulo fisiológico mais importante para a produção contínua de NO no endotélio é o estresse mecânico gerado pela corrente sangüínea sobre a camada endotelial. Até então, desconhecia-se como o estresse gerado pelo movimento do sangue sobre o endotélio poderia regular a fosforilação da eNOS. Uma forma mutante de eNOS (S1179A) é resistente à fosforilação e ativação pela Akt (Fulton et al., 1999). Através da transferência de genes com adenovírus, uma Akt constitucionalmente ativada (myr-Akt) aumenta a liberação basal de NO pelas células endoteliais, enquanto uma Akt inativa (AA-Akt) atenua a produção de NO estimulada por VEGF (*fator de crescimento do endotélio vascular*) (Fulton et al., 1999). A inibição da via da PI 3-quinase/Akt ou uma mutação na eNOS, no domínio de ligação com a Akt (serina 1177), atenua a fosforilação em serina da eNOS evitando a sua ativação. Mimetizando-se a fosforilação da Ser 1177 na eNOS há aumento de sua atividade enzimática, a qual passa a apresentar atividade máxima em concentrações sub-fisiológicas de cálcio (Dimmeler et al., 1999). Assim, ficou demonstrado que a eNOS é um substrato da Akt e que a fosforilação da eNOS pela Akt é um novo mecanismo de regulação da ativação da eNOS independente do cálcio (Dimmeler et al., 1999; Fulton et al., 1999).

Outro estudo documentou novo papel fisiológico para a Akt: a regulação do tônus vasomotor (Luo et al., 2000). A expressão endotelial da forma mutante da Akt constitucionalmente ativada aumentou o diâmetro de repouso de artérias femorais de coelhos com conseqüências funcionais no fluxo sangüíneo. Esta vasodilatação pôde ser bloqueada completamente pela administração do inibidor da eNOS L-NAME.

Bases moleculares da associação entre resistência à insulina e doença vascular

A maioria dos efeitos biológicos da insulina foi caracterizada no músculo esquelético, tecido adiposo e fígado, alvos tradicionais do hormônio (Kahn, 1994; White, 1997). Estes estudos demonstraram que a insulina é um mensageiro pleiotrópico e suas ações são tecido-específicas. No entanto, tecidos que não são alvos tradicionais da insulina, como por exemplo o coração (Velloso et al., 1996), também podem sofrer alguma regulação pelo hormônio (controle do transporte de glicose, síntese de glicogênio e crescimento celular). É bem conhecida, clínica e epidemiologicamente, a associação entre as síndromes de resistência à insulina e hiperinsulinemia (como o DM2 e a obesidade) com a hipertensão arterial sistêmica, aterosclerose e hipertrofia cardíaca (Feener & King, 1997; Ferrannini et al., 1987; Reaven, 1991b; Sasson, Rasooly, Bhesania, & Rasooly, 1993; Zavaroni et al., 1989). Assim, o papel da resistência à insulina e da hiperinsulinemia na patogênese da doença cardiovascular foi proposto, mas os estudos prévios enfatizavam apenas as anormalidades no metabolismo de lipoproteínas e a hiperglicemia como possíveis efetores (Reaven, 1991b; Sasson et al., 1993). O efeito direto da insulina no crescimento do miocárdio e da camada muscular arterial também foi proposto (Straus, 1984), mas pouco se sabe ainda sobre os seus mecanismos moleculares. Estudos recentes demonstraram um *cross-talk* entre os sistemas de sinalização de angiotensina II (AII) e insulina: a AII induz a fosforilação de IRS-1, IRS-2 e STAT1 (*Signal Transducer and Activator of Transcription 1*) no coração de ratos mas, ao invés de ativação, ocorre inibição da atividade da PI 3-quinase (Saad, Velloso, & Carvalho, 1995; Velloso et al., 1996; Velloso, Carvalho, Rojas, Folli, & Saad, 1998). Foi demonstrado ainda que a AII pode modular a expressão da eNOS nas células miocárdicas e, conseqüentemente, a função do sistema do óxido nítrico no miocárdio (Tambascia et al., 2001).

A etiopatogenia do DM2 envolve a resistência à insulina. Humanos obesos têm diminuição do nível de expressão e da atividade tirosina-quinase do receptor de insulina no músculo esquelético (Caro et al., 1987) e adipócitos (Olefsky, 1976), ambos restaurados pela perda de peso (Freidenberg, Reichart, Olefsky, & Henry, 1988), a qual melhora também a sensibilidade à insulina. Em modelos animais de obesidade genética ou adquirida, o número de receptores de insulina no fígado está reduzido e pode ser corrigido com redução da hiperinsulinemia (Hurrell, Pedersen, & Kahn, 1989). A resistência à insulina associada à obesidade também pode ser causada por alterações nos lipídios das membranas celulares secundárias à hiperlipidemia (Cremel et al., 1993). Em pacientes extremamente obesos (índice de massa corporal de 52 kg/m^2), o nível de expressão de IRS-1 no músculo esquelético está reduzido em 54% em relação a pacientes não-obesos (Goodyear et al., 1995). O achado mais uniforme com relação aos substratos do receptor de insulina na obesidade é a redução da fosforilação em resíduos de tirosina do IRS-1 e da atividade da PI 3-quinase no músculo esquelético e adipócitos estimulados pela insulina (Goodyear et al., 1995; Zierath, Krook, & Wallberg-Henriksson, 1998). Em modelos animais de obesidade, genéticos e induzidos, a fosforilação do IR e IRS-1 estimulada pela insulina está diminuída no músculo esquelético (Anai et al., 1998; Folli, Saad, Backer, & Kahn, 1993; Friedman et al., 1997; Heydrick, Gautier, Olichon-Berthe, Van Obberghen, & Marchand-Brustel, 1995; Kerouz, Horsch, Pons, & Kahn, 1997). Ratos Wistar–Hannover com 12 meses de idade apresentam obesidade e resistência à insulina. Nesses animais os níveis de IRS-1 estão reduzidos no músculo e os graus de fosforilação do receptor e do IRS-1 diminuídos no fígado e no músculo (Carvalho et al., 1996). Entretanto, a regulação das etapas iniciais da ação insulínica no tecido vascular desse modelo animal de resistência à insulina não foi ainda investigada.

A descoberta de que a Akt fosforila e ativa a eNOS (Dimmeler et al., 1999; Fulton et al., 1999), a qual produz NO para controle da pressão arterial, da remodelação vascular e da angiogênese, pode explicar melhor as bases moleculares da associação entre resistência à insulina e doença cardiovascular.

Há evidências crescentes de que a resistência à insulina possa ser diretamente aterogênica (Howard et al., 1996) devido à capacidade da insulina de promover o crescimento da musculatura vascular lisa (King et al., 1985), à produção de matriz

extracelular (Tamaroglio & Lo, 1994), à reabsorção renal de sódio (DeFronzo, Cooke, Andres, Faloona, & Davis, 1975) e à estimulação adrenérgica (Anderson, Hoffman, Balon, Sinkey, & Mark, 1991). Entretanto, para que essas hipóteses pudessem ser válidas, a resistência à insulina não poderia ser um fenômeno universal, mas sim restrito apenas ao músculo e tecido adiposo, enquanto os tecidos vascular e renal deveriam permanecer sensíveis à insulina. Estudos em pacientes resistentes à insulina e diabéticos tipo 2 sugerem que as células vasculares também são resistentes à insulina ao documentar que algumas funções endoteliais estão reduzidas nesses indivíduos, como a vasodilatação arterial induzida pela infusão de insulina (Steinberg et al., 1996; Williams, Cusco, Roddy, Johnstone, & Creager, 1996). Para explicar estes achados, Jiang e colaboradores (1999a) sugeriram que algumas ações da insulina, como os efeitos estimuladores sobre a síntese de NO e seus efeitos metabólicos, estariam seletivamente inibidos em estados de resistência à insulina, enquanto seus efeitos sobre o crescimento da musculatura vascular lisa estariam preservados, podendo resultar em aceleração da aterogênese em animais resistentes à insulina. Neste estudo, em aortas torácicas e microvasos epididimais de ratos Zucker obesos (*fa/fa*) e magros, demonstrou-se que a fosforilação em tirosina do IRβ e IRS-1/2 estimuladas pela insulina, bem como a atividade PI 3-quinase associada ao IRS-1/2 e a fosforilação em serina da Akt estavam reduzidas nos vasos dos animais obesos em comparação aos magros. Entretanto, a fosforilação em tirosina das isoformas ERK1 e ERK2 do complexo MAP quinase foi estimulada pela insulina igualmente nos microvasos do epidídimo dos animais obesos e magros, embora os níveis basais de fosforilação em tirosina da MAP quinase estivessem significativamente mais altos nas aortas e microvasos dos animais obesos em relação aos magros. Assim, no rato Zucker obeso, o qual apresenta resistência à insulina e hipertensão arterial, preserva-se o estímulo desencadeado pela insulina na via da MAP quinase (e até mesmo ocorre aumento na sua fosforilação basal) na presença de resistência na via da PI 3-quinase, ocorrendo um desequilíbrio entre estímulos pró-aterogênicos mediados pela cascata da MAP quinase (expressão de genes, proliferação e crescimento das células musculares lisas vasculares e produção de matriz extracelular) e estímulos anti-aterogênicos mediados pela via da PI 3-quinase-Akt-eNOS (regulação da pressão arterial sistêmica, inibição da remodelação vascular e da angiogênese).

Este fenômeno de resistência seletiva na cascata da PI 3-quinase associada à manutenção da sensibilidade à insulina na via da MAP quinase também foi documentado em músculos de pacientes obesos, resistentes à insulina, diabéticos ou não. Após *clamps* euglicêmicos seguidos por biópsias musculares, o estímulo desencadeado pela insulina na via da PI 3-quinase, avaliada pela fosforilação do IR e IRS-1 e pela associação entre IRS-1 e a subunidade p85 da PI 3-quinase, estava bastante reduzida em obesos não-diabéticos e virtualmente ausente em diabéticos tipo 2, em comparação aos controles magros. Por outro lado, a sensibilidade ao estímulo insulínico na via da MAP quinase estava completamente normal em indivíduos obesos e diabéticos (Cusi et al., 2000).

Tem sido demonstrado que o efeito estimulatório da insulina sobre a produção de óxido nítrico nos vasos pode estar seletivamente reduzido em modelos animais de resistência à insulina que apresentam doença cardiovascular. No entanto, os mecanismos através dos quais os efeitos biológicos normais da insulina estão preservados nos vasos de outros modelos animais que apresentam resistência à insulina mas que não desenvolvem doença cardiovascular ainda não foram estudados. **O primeiro objetivo** deste estudo foi investigar *in vivo* as vias de sinalização de insulina no músculo esquelético e na aorta torácica de ratos adultos obesos (12 meses de idade), os quais apresentam resistência à insulina mas não apresentam doença cardiovascular, e de ratos espontaneamente hipertensos (SHR), os quais, além de resistência à insulina, apresentam disfunção endotelial e hipertensão arterial.

Posteriormente, as vias estimulatórias do crescimento celular, como a da MAP quinase e também a da JAK/STAT, foram estudadas na aorta de outro modelo animal de resistência à insulina e doença cardiovascular - o rato com obesidade induzida por dieta.

Do exposto observa-se que a insulina fosforila e ativa a eNOS na vasculatura através de uma via dependente da PI 3-quinase, e uma menor ativação desta via tem sido associada à disfunção endotelial e hipertensão arterial em estados de resistência à insulina, embora o papel fisiológico da insulina sobre o controle vascular ainda não esteja completamente elucidado. Alguns estudos demonstraram que o bloqueio da via da PI 3-quinase com um inibidor farmacológico específico resultou em menor resposta vasodilatadora da acetilcolina na artéria basilar *in vivo* (Kitayama et al., 2000), sugerindo

que a acetilcolina também poderia fazer uso desta via de tirosina-quinases para ativar a eNOS. Além disso, o camundongo que não expressa o IRS-1 (*knockout* de IRS-1) apresenta disfunção endotelial (Abe et al., 1998b). No entanto, a acetilcolina inicia suas ações através de um receptor acoplado a proteína G (GPCR), o qual não tem atividade tirosina-quinase intrínseca. Outros hormônios que agem através de GPCRs, como a angiotensina (ANG) II, a vasopressina e o LH podem induzir a fosforilação de proteínas citoplasmáticas em resíduos de tirosina, dentre os quais o IRS-1 (Gallo-Payet & Guillon, 1998; Marrero et al., 1995; Saad et al., 1995; Velloso et al., 1996). A ANG II ativa a enzima JAK2, um membro da família JAK, e provavelmente utiliza esta quinase para promover a fosforilação em tirosina de diversos substratos intracelulares. **O terceiro objetivo** deste estudo foi investigar se a acetilcolina poderia ativar a JAK2 e assim induzir a fosforilação em tirosina do IRS-1, bem como a associação IRS-1/PI 3-quinase e a fosforilação e ativação da Akt e da eNOS na aorta de ratos. A seguir, foi feita a comparação entre os níveis de ativação da via IRS-1/PI 3-quinase/Akt/eNOS, após estímulo tanto com insulina quanto com acetilcolina, em ratos normais e em ratos com obesidade induzida por 60 dias de dieta hipercalórica e hiperlipídica.

2- OBJETIVOS

Os objetivos deste trabalho foram:

1. Caracterizar a transmissão do sinal de insulina na aorta torácica de modelos animais de resistência à insulina: ratos adultos (12 meses de idade), ratos espontaneamente hipertensos (SHR) e ratos com obesidade induzida por dieta hiperlipídica e hipercalórica.

2. Avaliar se o estímulo colinérgico pode regular proteínas da via da PI 3-quinase na aorta torácica de animais intactos.

3. Avaliar os graus de ativação, associação e níveis teciduais das proteínas da via da PI 3-quinase na aorta torácica de animais obesos após estímulo com insulina e acetilcolina (ACh).

3- ARTIGOS PUBLICADOS

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Diabetologia

Insulin signalling pathways in aorta and muscle from two animal models of insulin resistance – the obese middle-aged and the spontaneously hypertensive rats

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Abstract

Aims/hypothesis. The aim of this study was to investigate insulin signalling pathways directly in vivo in skeletal muscle and thoracic aorta from obese middle-aged (12-month-old) rats, which have insulin resistance but not cardiovascular disease, and from spontaneously hypertensive rats (SHR), an experimental model of insulin resistance and cardiovascular disease.
Methods. We have used in vivo insulin infusion, followed by tissue extraction, immunoprecipitation and immunoblotting.
Results. Obese middle-aged rats and the SHR showed marked insulin resistance, which parallels the reduced effects of this hormone in the insulin signalling cascade in muscle. In aortae from obese middle-aged rats, the PI 3-kinase/Akt pathway is preserved, leading to a normal activation of endothelial nitric oxide synthase. In SHR this pathway is severely blunted, with reductions in eNOS protein concentration and activation. Both animals, however, showed higher concentrations and higher tyrosine phosphorylation of mitogen-activated protein (MAP) kinase isoforms in aortae.
Conclusions/interpretation. Alterations in the IRS/PI 3-K/Akt pathway in muscle of 12-month-old rats and SHR could be involved in the insulin resistance of these animals. The preservation of this pathway in aorta of 12-month-old rats, apart from increases in MAP kinase protein concentration and activation, could be a factor that contributes to explaining the absence of cardiovascular disease in this animal model. However, in aortae of SHR, the reduced insulin signalling through IRS/PI 3-kinase/Akt/eNOS pathway could contribute to the endothelial dysfunction of this animal. [Diabetologia (2003) 46:479–491]

Keywords Aging, obesity, insulin resistance, aorta, phosphatidylinositol 3-kinase, MAP kinase, Akt, endothelial cell-nitric oxide synthase, SHR.

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Abbreviations: αPY, Antibody against phosphotyrosine; eNOS, endothelial cell-nitric oxide synthase; ERK, extracellular signal-related kinase; IR, insulin receptor; IRβ, insulin receptor β subunit; IRS, insulin receptor substrate; MAP kinase, mitogen-activated protein kinase; MEK, MAP/ERK kinase; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; PI, phosphatidylinositol; PKB, protein kinase B; PMSF, phenylmethylsulfonyl fluoride; SH2, src-homology 2 domain; SH3, src-homology 3 domain; Shc, src-homology 2 domain-containing; SHR, spontaneously hypertensive rats; VSMC, vascular smooth muscle cell.

Introduction

Insulin regulates many vascular functions under physiological conditions. Biological actions of insulin in vascular cells include increases in amino acid transport, glycogen synthesis, DNA synthesis and gene expression [1, 2]. It also has specific vascular actions, such as enhancing the release of nitric oxide (NO) [3], regulating mRNA expression of matrix proteins [4, 5], and constitutive endothelial NO synthase (eNOS) [6, 7]. Physiologically, insulin infusion stimulates local vasodilation by enhancing the action of NO [8, 9].

Normal endothelial production of NO plays an important role in preventing vascular disease. In addition to its function as an endogenous vasodilator, NO released from endothelial cells is a potent inhibitor of

platelet aggregation and adhesion to the vascular wall and also controls the expression of proteins involved in atherogenesis [10].

In vascular cells, insulin actions are initiated through binding to the insulin receptor alfa subunit, which activates the intrinsic receptor tyrosine kinase [11], resulting in autophosphorylation of insulin receptor beta subunit (IRβ) and tyrosine phosphorylation of intracellular adapter proteins such as insulin receptor substrates (IRS-1 and IRS-2) [12, 13] and Shc [14]. Tyrosine-phosporylated IRS-1 or IRS-2 binds to src-homology 2 (SH2) domains of intracellular proteins, including the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase [15]. The interaction of IRS and p85 subunit of PI 3-kinase results in the activation of p110 catalytic subunit of PI 3-kinase. In the vasculature, the activation of PI 3-kinase increases serine phosphorylation of Akt which, in turn, directly phosphorylates eNOS on serine 1177 and activates the enzyme, leading to increased NO production [16, 17].

It has been proposed that some of the actions of insulin in the vasculature, such as the stimulatory effects on NO production, are selectively inhibited in the obese Zucker rats, which present insulin resistance and hypertension. However, other animal models of insulin resistance have normal blood pressure and no cardiovascular disease.

We have characterized insulin signalling pathways directly in vivo in skeletal muscle and thoracic aortae from two animal models of insulin resistance: the obese middle-aged Wistar rats, which do not present vascular dysfunction, and the spontaneously hypertensive rats (SHR), an animal model of insulin resistance with profound vascular dysfunction on the basis of hypertension in the absence of lipid disorders.

Materials and methods

Experimental animals. Male Wistar rats were obtained from the UNICAMP Central Animal Breeding Center (Campinas, Sao Paulo, Brazil) and divided into two groups by age (2 and 12 months old) with body weights of 137±2.4 and 432.6±2.7 g, respectively. Twenty-week-old male SHR were compared to the age-matched normotensive, insulin-sensitive Wistar-Kyoto (WKY) strain. Animals were allowed free access to standard rodent chow and water ad libitum. Food was withdrawn 12 h before the experiments. All experiments involving animals were approved by the ethics committee at the University of Campinas.

Blood samples were taken for measuring plasma concentration of total cholesterol and triglycerides. Lipids were measured in a routine diagnostic analyser (Modular, Roche Diagnostics) using enzymatic colorimetric assays (cholesterol, CHOD-PAP assay; triglycerides, GPO-PAP assay). Plasma glucose concentrations were measured by the glucose oxidase method, as described previously [18]. Serum insulin was determined by radioimmunoassay [19].

Antibodies and Chemicals. The reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, Calif., USA). TRIS, phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiothreitol, Triton X-100, Tween-20 and glycerol were obtained from Sigma Chemical (St. Louis, Mo., USA). Human recombinant insulin (Humulin R) and sodium amobarbital were purchased from Eli Lilly (Indianapolis, Ind., USA). [^{125}I]Protein A was obtained from Amersham (Aylesbury, UK), and protein A-Sepharose 6 MB was obtained from Pharmacia Biotech (Uppsala, Sweden). Nitrocellulose (BA85; 0.2 μm) was purchased from Schleicher & Schuell, (Keene, N.H., USA). Molecular weight, M_r standards were myosin (194 kDa), β-galactosidade (116 kDa), BSA (85 kDa) and ovalbumin (49.5 kDa).

Monoclonal antiphosphotyrosine (αPY) and anti-PI 3-kinase (p85) antibodies were obtained from Upstate Biotechnology (UBI – Lake Placid, N.Y., USA). Polyclonal antibodies against beta subunit of insulin receptor (C-19, sc-711), IRS-1 (C-20, sc-559), IRS-2 (A-19, sc-1556), eNOS (NOS3, C-20, sc-654), phospho-eNOS (Ser 1177, sc-12972), Akt1 (C-20, sc-1618), Shc (C-20, sc-288: specific for Shc p46, p52 and p66) and ERK1 (C-16, sc-93: reactive with ERK1 p44 and, to a lesser extent, ERK2 p42), as well as the monoclonal antibody against phospho-ERK (Tyr 204, E-4, sc-7383) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Polyclonal antibodies against phospho-Akt (Ser 473) were obtained from New England Biolabs (Beverly, Mass., USA).

Buffers. Buffer A consisted of 100 mmol/l TRIS, 1% sodium dodecyl sulphate (SDS), 50 mmol/l HEPES (pH 7.4), 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA and 10 mmol/l sodium vanadate. Buffer B was similar to buffer A except that 1% Triton X-100 replaced 1% SDS and 2 mmol/l phenylmethylsulphonic fluoride (PMSF) and 0.1 mg/ml aprotinin were added. Buffer C contained 100 mmol/l TRIS, 10 mmol/l sodium vanadate, 10 mmol/l EDTA and 1% Triton X-100.

Immunoprecipitation and Western immunoblotting. Rats were anaesthetized by intraperitoneal injection of sodium amobarbital (100 mg/kg body weight) and the experiments were done after the loss of corneal and pedal reflexes. The abdominal cavity was opened, the cava vein was exposed, and 0.5 ml of normal saline (0.9% NaCl) with or without 10^{-5} mol/l of insulin was injected. Thoracic aorta and hind limb skeletal muscle were isolated after 60, 90 or 180 s (according to the protein studied), frozen with liquid N_2, powdered with a glass Dounce homogenizer on ice for at least 80 strokes and immediately homogenized in freshly prepared, boiling buffer A when intended for immunoblots, or freshly prepared, ice-cold buffer B when intended for immunoprecipitation. The extracts were centrifuged at 30 000×g and 4°C in a Beckman Coulter 70.1 Ti rotor (Palo Alto, Calif., USA) for 45 min to remove insoluble material. Protein concentration in the supernatants was determined by the Bradford method [20].

For immunoprecipitations, samples containing 3 mg of total protein were incubated with antibodies against IRβ (0.3 mg/ml), IRS-1 (1:1000 dilution), IRS-2 (1:1000), Shc (1:1000) or eNOS (1:500) at 4°C overnight, followed by the addition of Protein A Sepharose 6 MB for 1 h. The pellets were repeatedly washed in buffer C (five times), resuspended in 50 ml of Laemmli sample buffer [21], and boiled for 5 min before loading onto the gel. For immunoblotting, similar sized aliquots (200 mg of total protein) were suspended in 50 ml of Laemmli sample buffer containing 100 mmol/l dithiothreitol and boiled for 5 min before loading onto a 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system in a miniature slab gel apparatus from Bio-Rad.

Electrotransfer of proteins from the gel to nitrocellulose was carried out for 2 h at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described previously [22] except for adding the 0.02% of SDS to the transfer buffer to enhance the elution of high molecular mass proteins. Non specific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4°C in blocking buffer (5% non-fat dry milk, 10 mmol/l TRIS, 150 mmol/l NaCl, and 0.02% Tween-20). The nitrocellulose blot was then incubated with the following antibodies: antiphosphotyrosine (1 mg/ml), anti-p85 subunit of PI 3-kinase (1:500), anti-insulin receptor (1:100), anti-IRS-1 (1:100), anti-IRS-2 (1:100), anti-Shc (1:1000), anti-ERK1/2 (p44/42 MAP kinase, 1:1000), anti-tyrosine-phosphorylated ERK1/2 (1:1000), anti-Akt1 (1:1000), anti-phospho-(ser-473)-Akt (1:1000), anti-eNOS (1:1000) and anti-phospho-(ser-1177)-eNOS (1:1000), each one diluted in blocking buffer (0.3% BSA instead of non-fat dry milk) overnight at 4°C, and then washed for 60 min with blocking buffer without milk. The blots were subsequently incubated with 0.074 MBq [^{125}I]protein A (1.11 MBq/μg) in 10 ml blocking buffer for 2 h at room temperature and then washed again for 30 min as described above. [^{125}I]Protein A bound to the antiphosphotyrosine and antipeptide antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak, Rochester, N.Y., USA) with Cronex Lightning Plus intensifying screens (DuPont, Wilmington, Del., USA) at −80°C for 12 to 48 h. Band intensities were quantitated by optical densitometry (model GS 300, Hoefer Scientific Instruments, San Francisco, Calif., USA) of the developed autoradiographs.

Fifteen-minute insulin tolerance test Rats were fasted overnight and submitted to an intravenous insulin tolerance test (IVITT; 1 U/kg body weight of insulin, i.v.) and samples for blood glucose measurements were collected at 0 (basal), 4, 8, 12, and 16 min after injection. Rats were anaesthetized with sodium amobarbital as described above, 40 μl of blood was collected from their tails and blood glucose concentration was measured by the glucose oxidase method. Thereafter, the rate constant for plasma glucose disappearance (K_{itt}) was calculated using the formula $0.693/(t_{1/2})$. The plasma glucose $t_{1/2}$ was calculated from the slope of the least squares analysis of the plasma glucose concentrations during the linear phase of decline [23].

Blood pressure measurement. A tail-cuff system (MKIV, Narco BioSystems, Austin, Tex., USA) combining a transducer or amplifier which provides output signals proportional to cuff pressure and amplified Korotkoff sounds was used to obtain blood pressure measurements in conscious rats. This indirect approach permits repeated measurements with a close correlation (correlation coefficient =0.975) compared to direct intra-arterial recording [24].

Vascular tissue preparation for morphological analysis. Rats were heparinized, deeply anaesthetized with pentobarbital sodium and killed with a lethal dose of lidocaine. Aortae were fixed with 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4, dehydrated in alcohol and xylol and routinely paraffin embedded. Step sections (5 μm) were stained with hematoxylin and eosin. At least five step sections of ascending, arch, thoracic and abdominal aorta were examined by light microscopy. Fragments were deparaffinized and routinely processed for electronmicroscopy. Ultrathin sections were stained with uranylacetate and lead citrate and examined with a ZEISS EM 10 electron microscope.

Statistical analysis. Experiments were always carried out by studying the groups of animals in parallel (2-month-old vs 12-month-old male Wistar rats and SHR vs age-matched WKY male rats). Data are means±SEM of absolute or percent values. For comparisons, unpaired Student's *t* test was used. The significance level was set at *p* value of less than 0.05.

Results

Animal characteristics. The 12-month-old rats were heavier than the control rats (2m: 137±12 g vs 12m: 432±13 g, $p<0.0001$) and had higher contents of fat mass in different sites (bilateral epididymal fat – 2m: 2.89±0.31 g vs 12m: 4.01±0.22 g, $n=10$ each, $p=0.0127$; bilateral perirenal fat – 2m: 2.17±0.05 g vs 12m: 2.77±0.03 g, $n=10$ each, $p<0.0001$; mesenteric fat – 2m: 3.26±0.30 g vs 12m: 5.03±0.41 g, $n=10$ each, $p=0.0026$; Table 1). The lengths of 2- and 12-month-old rats were 20±2 cm and 24±1 cm ($n=10$ each, $p=0.09$), respectively. The 12-month-old rats had higher plasma concentrations of total cholesterol and triglycerides than the 2-month-old rats. Fasting blood glucose and systolic blood pressure were similar in the two groups. Older animals were more insulin resistant than the young control rats, as expressed by their higher serum insulin concentrations (2m: 25±4 vs 12m: 41±6, $n=10$ each, $p<0.05$) and lower plasma glucose disappearance rates measured by the fifteen minute insulin tolerance test (K_{itt} 2m: 4.31±0.56%/min vs K_{itt} 12m: 2.34±0.38%/min, $n=10$ each, $p=0.004$).

Table 2 shows comparative data regarding SHR and WKY strains. As noticed, the SHR had a smaller body weight than the WKY rats (WKY: 369±10 g vs SHR: 315±8 g, $p<0.0001$). Consistent with previous

Table 1. Characteristics of obese middle-aged Wistar rats (12-month-old) and the young control rats (2-month-old)

Groups	Body weight (g)	Blood glucose (mg/dl)	Serum insulin (μU/ml)	Systolic blood pressure (mmHg)	K_{itt} (%/min)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
2-month-old	137±12 (n=30)	123±5 (n=10)	25±4 (n=10)	114±2 (n=10)	4.31±0.56 (n=10)	32±7 (n=10)	49±11 (n=10)
12-month-old	432±13[a] (n=30)	118±9 (n=10)	41±6[b] (n=10)	112±3 (n=10)	2.34±0.38[c] (n=10)	91±15[c] (n=10)	108±21[b] (n=10)

Data are given as means ± SEM. p values of less than 0.05 were considered to be significantly different.
[a] $p<0.0001$ vs young controls; [b] $p<0.05$ vs young controls; [c] $p<0.01$ vs young controls

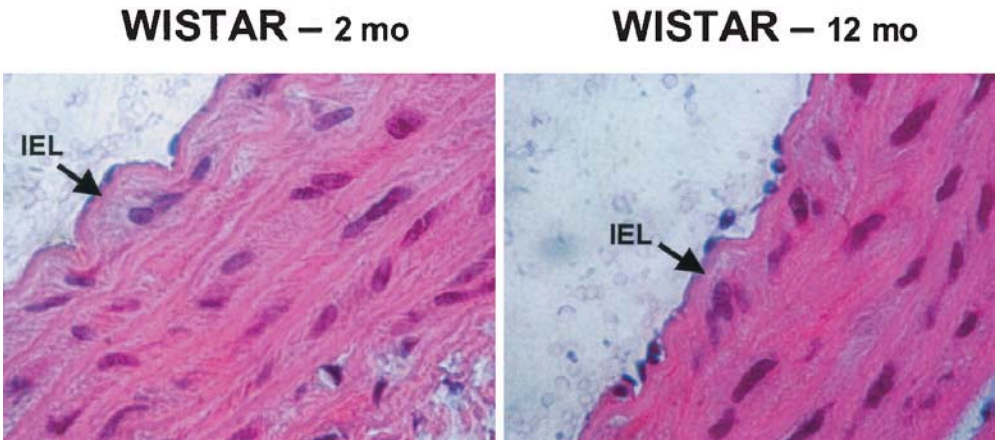

Fig. 1. Light photomicrographs of sections of HE stainings of thoracic aortae from 2- and 12-month-old Wistar rats. Magnification ×960. IEL=internal elastic lamina (*arrows*)

Table 2. Characteristics of SHR and the control rats, WKY

Groups	Body weight (g)	Blood glucose (mg/dl)	Serum insulin (μU/ml)	Systolic blood pressure (mmHg)	K_{itt} (%/min)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
WKY	369±10 (*n*=30)	142±7 (*n*=10)	18±6 (*n*=10)	115±5 (*n*=10)	4.79±0.62 (*n*=10)	83±12 (*n*=10)	70±9 (*n*=10)
SHR	315±8[a] (*n*=30)	149±5 (*n*=10)	29±4 (*n*=10)	175±5[a] (*n*=10)	1.89±0.39[b] (*n*=10)	89±8 (*n*=10)	159±26[b] (*n*=10)

Data are given as means ± SEM. *p* values of less than 0.05 were considered to be significantly different.
[a] $p<0.0001$ vs WKY; [b] $p<0.01$ vs WKY

studies, systolic blood pressure was higher in SHR compared to the WKY rats (WKY: 115±5 mmHg vs SHR: 175±5 mmHg, $n=10$ each, $p<0.0001$). There were no differences between plasma concentrations of total cholesterol and glucose when the rats from these two strains were compared. Plasma concentration of triglycerides were higher in SHR than in WKY. SHR animals were more insulin resistant than the age-matched WKY as expressed by lower plasma glucose disappearance rates (K_{itt} WKY: 4.79±0.62%/min vs K_{itt} SHR: 1.89±0.39%/min, $n=10$ each, $p<0.01$) and higher, though not statistically significant, serum insulin concentrations.

Histological examination of aortae. In all step sections examined there were no signs of atherosclerotic changes, such as lipid and foam cell deposition, intimal thickening or SMC proliferation, neither on light microscopic examination (Fig. 1) nor on ultrastructural sections in aortae from the 12-month-old rats and the control rats.

Insulin signalling in skeletal muscle from 2-month-old and 12-month-old animals. There was no difference in insulin-stimulated tyrosine phosphorylation of insulin receptor beta subunit between muscles from 2- and 12-month-old rats (2m: 100±7% vs 12m: 100±4%, $n=6$; Fig. 2), and the protein concentrations of IRβ also did not change in obese middle-aged rats (Fig. 2B).

There was a greater decrease in insulin-stimulated IRS-1 tyrosine phosphorylation in muscles from obese, older rats than from the control rats (Fig. 2C, 2m: 100±5% vs 12m: 55±4%, $n=6$, $p<0.0001$). Also, IRS-1 protein concentrations were reduced in muscle from 12-month-old rats (2m: 100±9% vs 12m: 56±7%, $n=6$, $p<0.05$; Fig. 2D).

The insulin-stimulated association between IRS-1 and the p85 regulatory subunit of PI 3-kinase was also diminished in skeletal muscle from the 12-month-old rats compared to the 2-month-old control rats (2m: 100±5% vs 12m: 30±7%, $n=6$, $p<0.0001$; Fig. 2E).

Insulin was able to stimulate the serine phosphorylation of Akt in both groups of animals, but older animals showed a reduced concentration of Akt activation after insulin infusion (2m: 100±4% vs 12m: 41±3%, $n=6$, $p<0.0001$; Fig. 2F). The blot, representative of six experiments, was obtained after incubating the membranes with anti-Akt antibodies (Fig. 2G). There was no difference between the protein concentration of Akt in skeletal muscle from these two groups.

Insulin-stimulated Shc tyrosine phosphorylation in skeletal muscle from 12-month-old Wistar rats was not different from that observed in the 2-month-old group (Fig. 2H, $n=6$), without any difference in Shc protein expression between the two groups of animals (Fig. 2I).

Insulin was able to stimulate tyrosine phosphorylation of ERK1/2 equally in skeletal muscle from 2- and

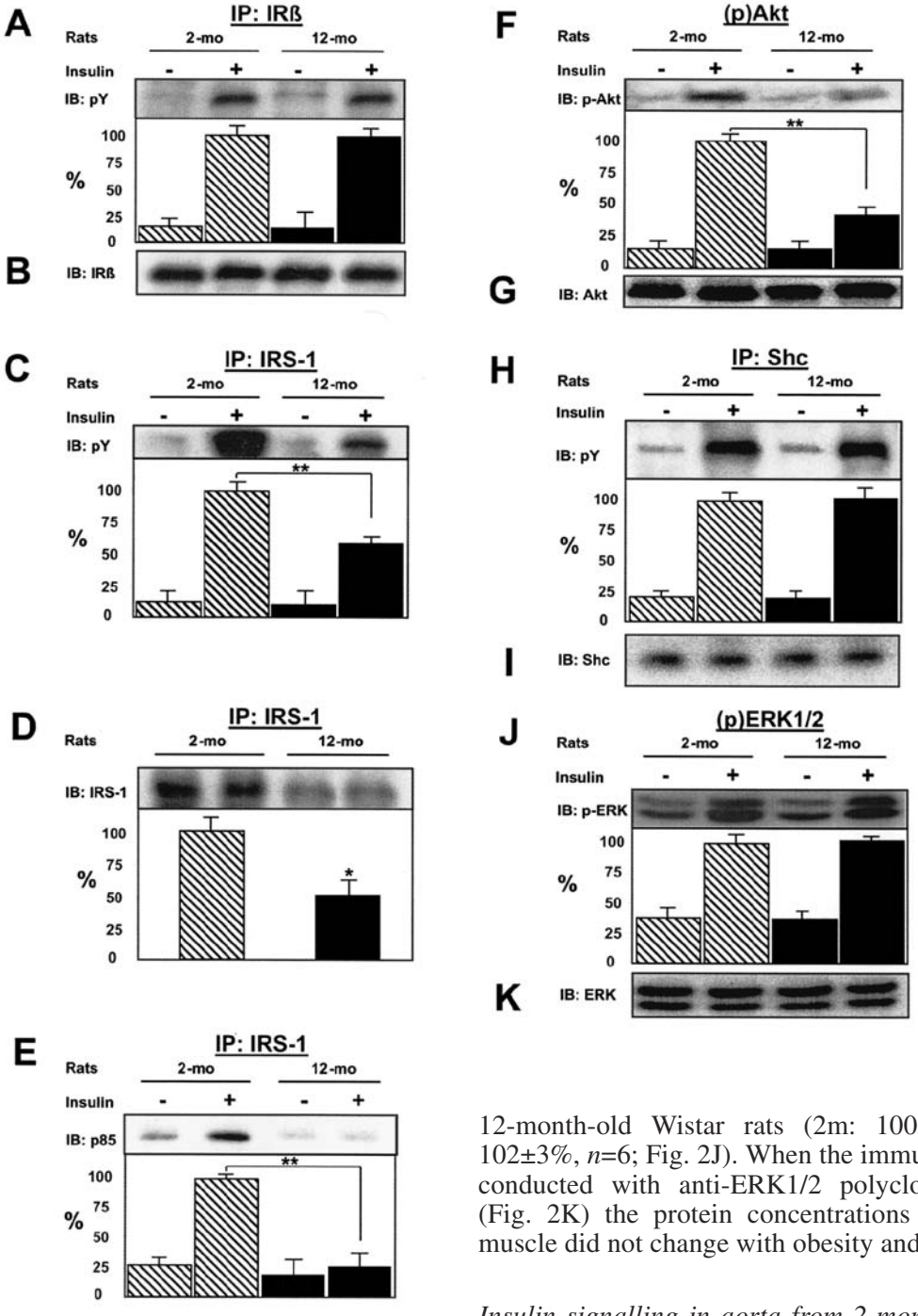

Fig. 2A–K. Insulin signalling in skeletal muscle of young (2-month-old) and middle-aged (12-month-old) Wistar rats. Insulin-stimulated insulin receptor tyrosine phosphorylation (**A**), insulin receptor protein concentrations (**B**), insulin-stimulated IRS-1 tyrosine phosphorylation (**C**), IRS-1 protein concentrations (**D**), insulin-stimulated association between IRS-1 and p85 (**E**), insulin-stimulated Akt serine phosphorylation (**F**), Akt protein concentrations (**G**), insulin-stimulated Shc tyrosine phosphorylation (**H**), Shc protein concentrations (**I**), insulin-stimulated ERK1/2 tyrosine phosphorylation (**J**) and ERK1/2 protein concentrations (**K**). Values are shown as the means ± SEM and are expressed as a percentage of the insulin-stimulated control (100%). *, $p<0.05$; **, $p<0.0001$

12-month-old Wistar rats (2m: 100±4% vs 12m: 102±3%, $n=6$; Fig. 2J). When the immunoblotting was conducted with anti-ERK1/2 polyclonal antibodies (Fig. 2K) the protein concentrations of ERK1/2 in muscle did not change with obesity and aging.

Insulin signalling in aorta from 2-month-old and 12-month-old animals. Insulin-stimulated IRβ tyrosine phosphorylation concentrations were similar in aortae from both young and middle-aged rats (representative of five different experiments, Fig. 3A). To evaluate the tissue levels of the insulin receptor beta subunit, nitrocellulose membranes of IRβ immunoprecipitates were stripped and reblotted with antibodies against IRβ (Fig. 3B). The tissue levels of IRβ did not change between the two groups.

There was no difference in insulin-stimulated IRS-1 tyrosine phosphorylation in aortae from 2- and 12-month-old rats (2m: 100±4% vs 12m: 105±3%, $n=6$; Fig. 3C). The same membranes were stripped and sub-

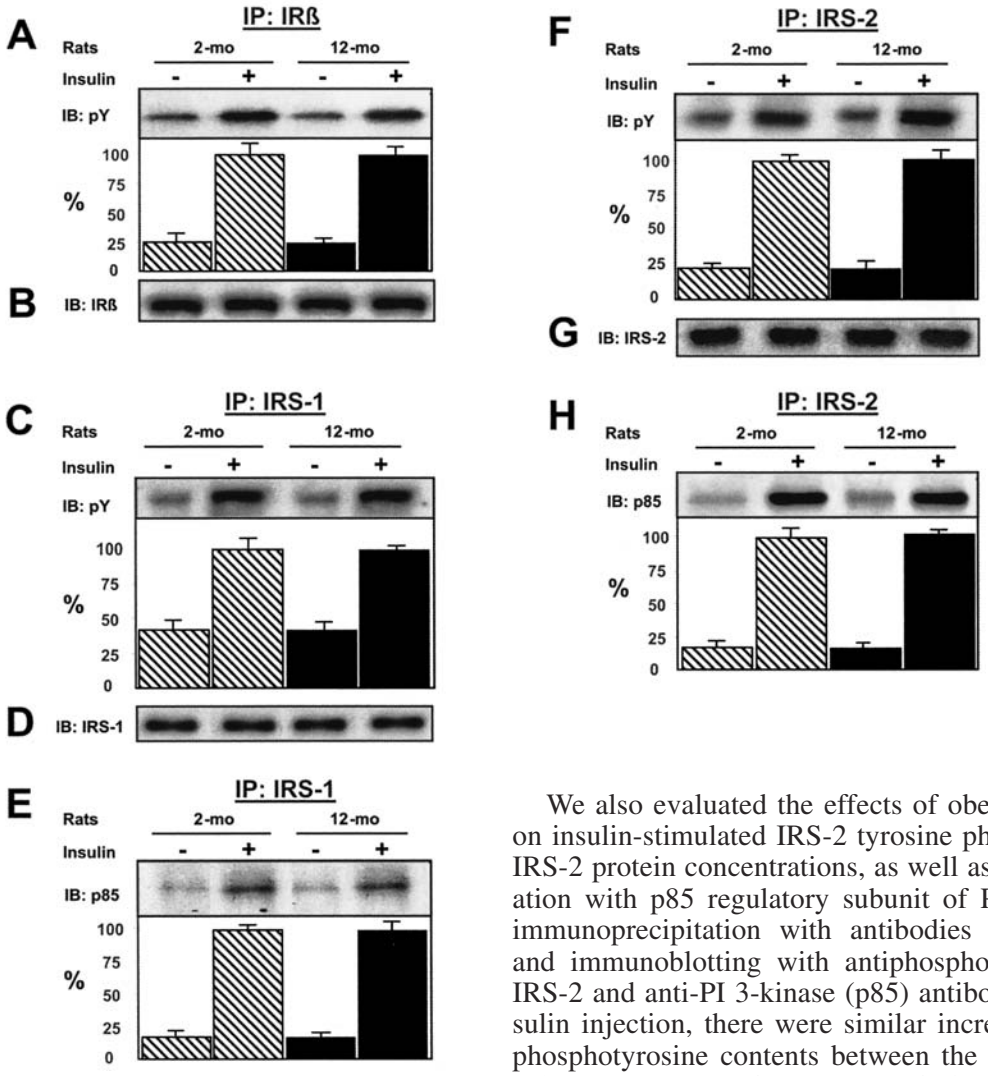

Fig. 3A–H. Insulin signalling in thoracic aortae of young (2-month-old) and middle-aged (12-month-old) Wistar rats. Insulin-stimulated insulin receptor tyrosine phosphorylation (**A**), insulin receptor protein concentrations (**B**), insulin-stimulated IRS-1 tyrosine phosphorylation (**C**), IRS-1 protein concentrations (**D**), insulin-stimulated association between IRS-1 and p85 (**E**), insulin-stimulated IRS-2 tyrosine phosphorylation (**F**), IRS-2 protein concentrations (**G**) and insulin-stimulated association between IRS-2 and p85 (**H**). Values are shown as the means ± SEM and are expressed as a percentage of the insulin-stimulated control (100%)

mitted to immunoblotting with anti-IRS-1 antibodies to evaluate the protein concentrations of IRS-1 in these tissues (Fig. 3D). No statistically significant difference was found in IRS-1 protein concentrations in the aortae of young and middle-aged rats.

The insulin-stimulated association between IRS-1 and the p85 regulatory subunit of PI 3-kinase in aorta was investigated (Fig. 3E). After stimulation with insulin, the association between IRS-1 and PI 3-kinase (p85) increased eightfold, with no difference between 2- and 12-month-old rats (2m: 100±5% vs 12m: 98±7%, n=6).

We also evaluated the effects of obesity and aging on insulin-stimulated IRS-2 tyrosine phosphorylation, IRS-2 protein concentrations, as well as IRS-2 association with p85 regulatory subunit of PI 3-kinase by immunoprecipitation with antibodies against IRS-2 and immunoblotting with antiphosphotyrosine, anti-IRS-2 and anti-PI 3-kinase (p85) antibodies. After insulin injection, there were similar increases in IRS-2 phosphotyrosine contents between the two groups of animals (2 m: 100±5% vs 12m: 95±6%, n=6; Fig. 3F). The tissue concentrations of IRS-2 were evaluated by immunoblotting with anti-IRS-2 antibodies, and showed no difference between the two groups of rats (Fig. 3G). Next, we evaluated the insulin-stimulated IRS-2 association with p85 subunit of PI 3-kinase in aortae from these animals (Fig. 3H). After insulin injection, p85 association to IRS-2 increased to 100±11% in 2-month-old rats and to 107±4% in 12-month-old rats (n=6 each, p=0.56), showing no significant difference between these two groups.

Basal concentrations of Akt serine phosphorylation in aorta were not different between the two groups of rats (2m: 53±10% vs 12m: 48±11%, n=10, p>0.05; Fig. 4A). Insulin was able to increase Akt serine phosphorylation in both groups of rats in a similar manner (2m: 100±6% vs 12m: 96±5%, n=10, p>0.05). When these membranes were submitted to immunoblotting with anti-Akt antibodies, as shown in (Fig. 4B) there was no difference between the two groups, indicating that vascular protein concentrations of Akt did not change with obesity and aging in Wistar male rats.

Akt is activated in response to insulin and so becomes capable of phosphorylating endothelial cell-

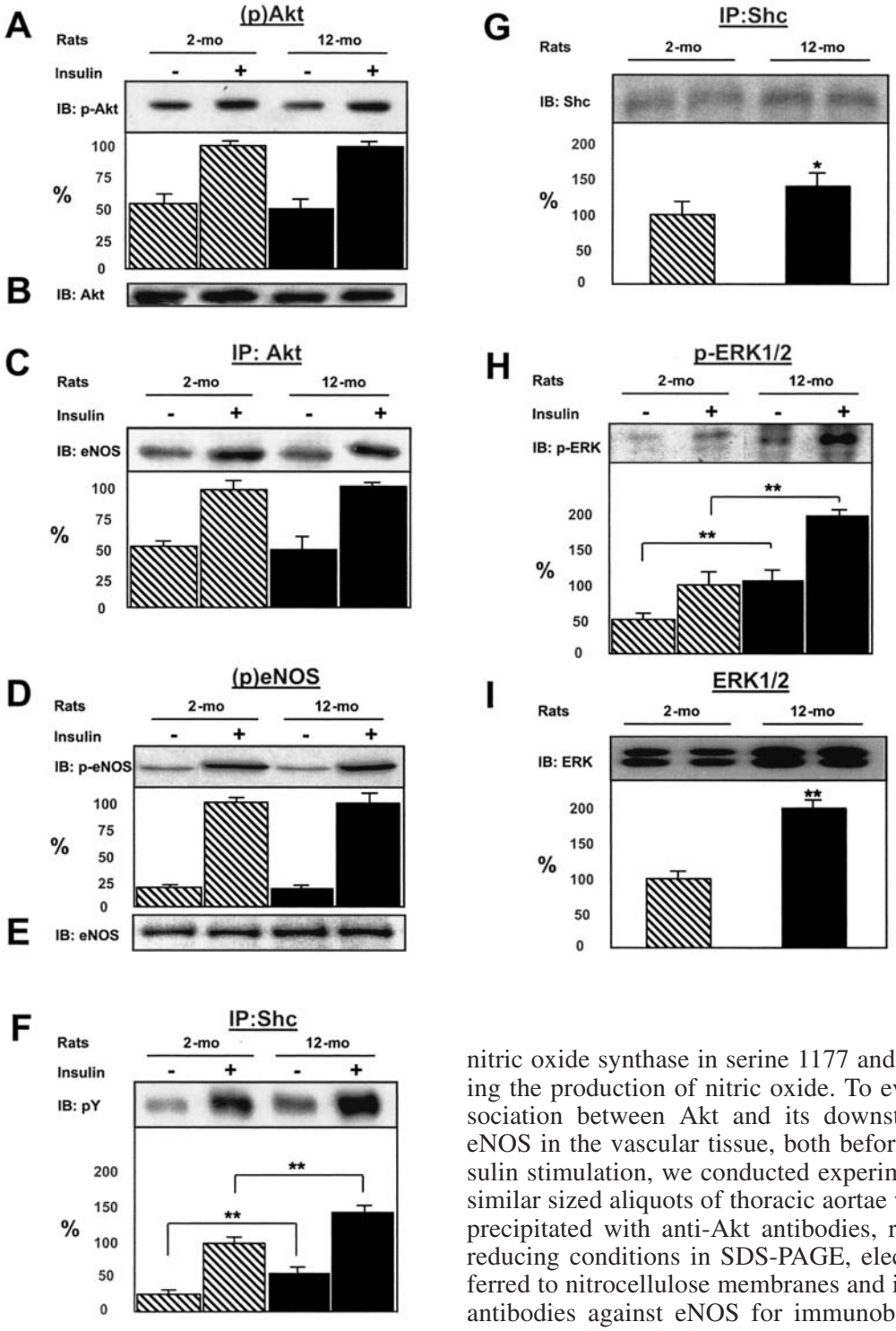

Fig. 4A–I. Insulin signalling in thoracic aortae of young (2-month-old) and middle-aged (12-month-old) Wistar rats. Insulin-stimulated Akt serine phosphorylation (**A**), Akt protein concentrations (**B**), insulin-stimulated association between Akt and eNOS (**C**), insulin-stimulated eNOS serine phosphorylation (**D**), eNOS protein concentrations (**E**), insulin-stimulated Shc tyrosine phosphorylation (**F**), Shc protein concentrations (**G**), insulin-stimulated ERK1/2 tyrosine phosphorylation (**H**) and ERK1/2 protein concentrations (**I**). Values are shown as the means ± SEM and are expressed as a percentage of the insulin-stimulated control (100%). *, $p<0.05$; **, $p<0.0001$

nitric oxide synthase in serine 1177 and 1179, activating the production of nitric oxide. To evaluate the association between Akt and its downstream effector eNOS in the vascular tissue, both before and after insulin stimulation, we conducted experiments in which similar sized aliquots of thoracic aortae were immunoprecipitated with anti-Akt antibodies, resolved under reducing conditions in SDS-PAGE, electrically transferred to nitrocellulose membranes and incubated with antibodies against eNOS for immunoblotting. In the absence of stimulation with insulin, the association between Akt and eNOS in aorta is discrete, represented by faint bands, and was not different between young and middle-aged rats (2m: 52±4% vs 12m: 50±8%, $n=6$, $p>0.5$; Fig. 4C). However, after stimulation with insulin, their association increased twofold, consistent with a stable association of Akt and eNOS, with no difference between 2- and 12-month-old rats (2m: 100±7% vs 12m: 104±3%, $n=6$, $p>0.5$).

Whole cell lysates obtained from thoracic aortae before or 3 min after insulin injection into the cava

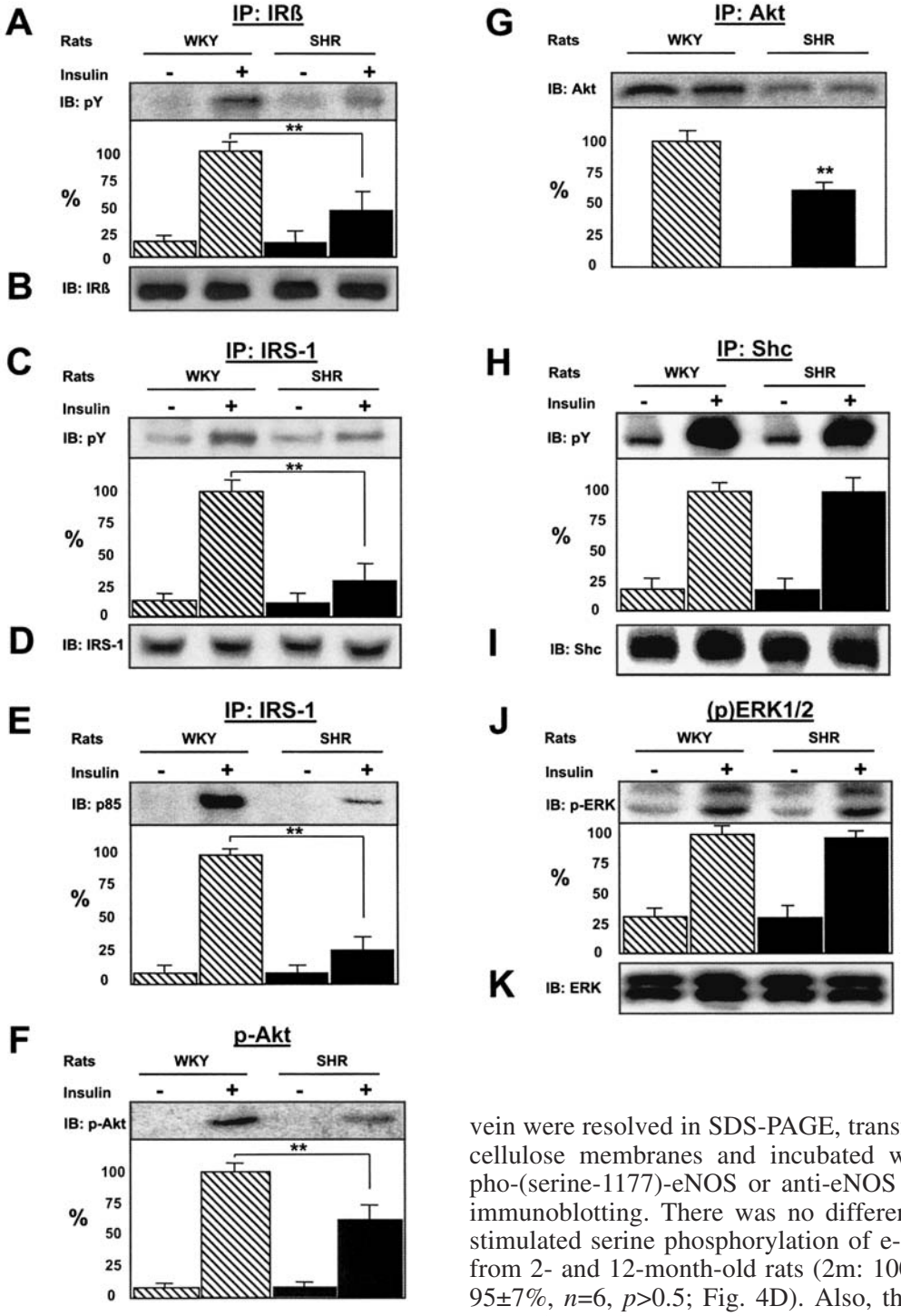

Fig. 5A–K. Insulin signalling in skeletal muscle of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Insulin-stimulated insulin receptor tyrosine phosphorylation (**A**), insulin receptor protein concentrations (**B**), insulin-stimulated IRS-1 tyrosine phosphorylation (**C**), IRS-1 protein concentrations (**D**), insulin-stimulated association between IRS-1 and p85 (**E**), insulin-stimulated Akt serine phosphorylation (**F**), Akt protein concentrations (**G**), insulin-stimulated Shc tyrosine phosphorylation (**H**), Shc protein concentrations (**I**), insulin-stimulated ERK1/2 tyrosine phosphorylation (**J**) and ERK1/2 protein concentrations (**K**). Values are shown as the means ± SEM and are expressed as a percentage of the insulin-stimulated control (100%). $^{**}p<0.0001$

vein were resolved in SDS-PAGE, transferred to nitrocellulose membranes and incubated with anti-phospho-(serine-1177)-eNOS or anti-eNOS antibodies for immunoblotting. There was no difference in insulin-stimulated serine phosphorylation of e-NOS in aortae from 2- and 12-month-old rats (2m: 100±3% vs 12m: 95±7%, n=6, p>0.5; Fig. 4D). Also, the protein concentrations of e-NOS did not change between these two groups (Fig. 4E).

Both before and after stimulation with insulin, Shc showed higher degrees of tyrosine phosphorylation in aortae from 12-month-old Wistar rats as compared to the 2-month-old animals (without insulin: 2m: 28±4% vs 12m: 56±4%, n=6, p<0.0001; with insulin: 2m: 100±4% vs 12m: 145±4%, n=6, p<0.0001; Fig. 4F). This greater level of activation was accompanied by an increase in Shc protein concentrations in aortae from 12-month-old rats (2m: 100±8% vs 12m: 136±6%, n=6, p<0.05; Fig. 4G).

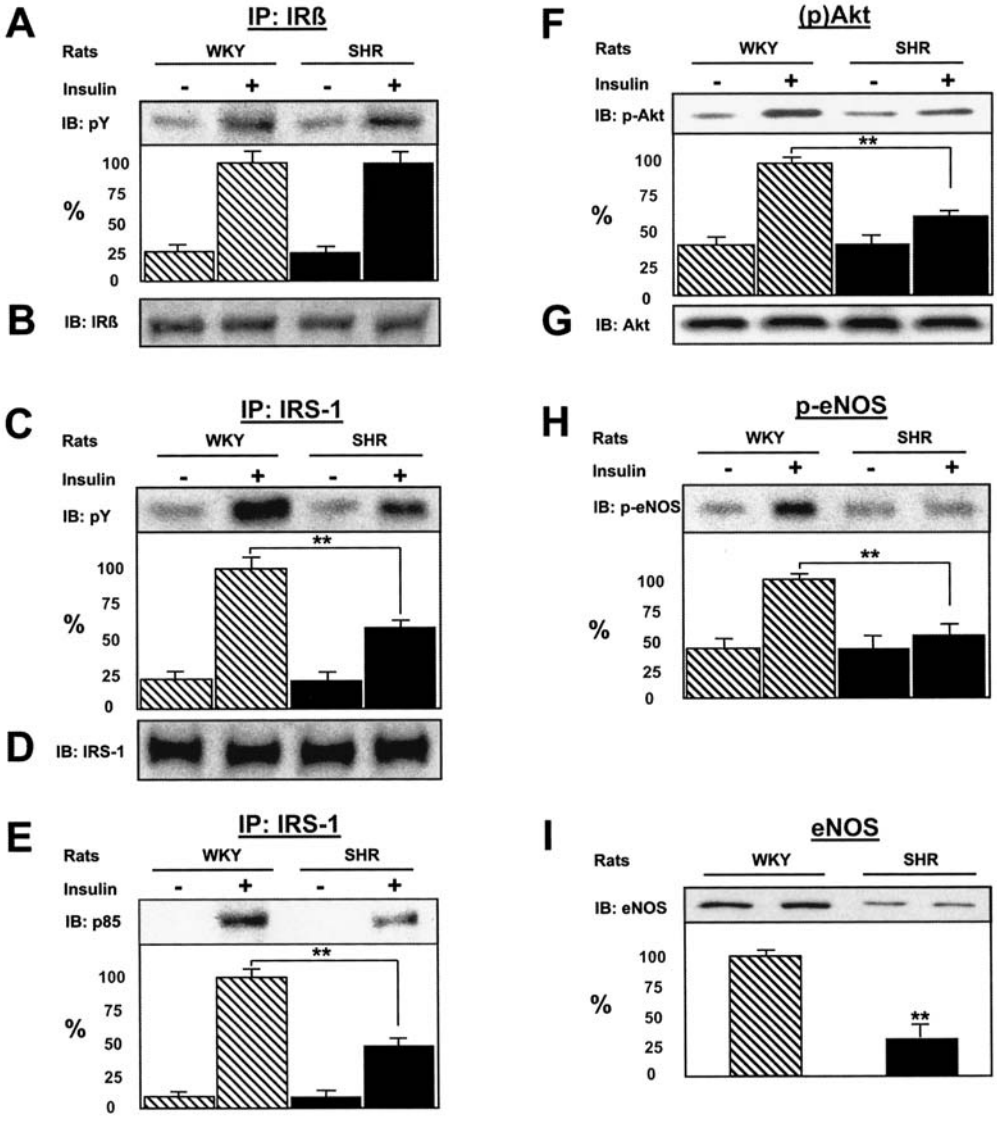

Fig. 6A–I. Insulin signalling in thoracic aortae of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Insulin-stimulated insulin receptor tyrosine phosphorylation (**A**), insulin receptor protein concentrations (**B**), insulin-stimulated IRS-1 tyrosine phosphorylation (**C**), IRS-1 protein concentrations (**D**), insulin-stimulated association between IRS-1 and p85 (**E**), insulin-stimulated Akt serine phosphorylation (**F**), Akt protein concentrations (**G**), insulin-stimulated eNOS serine phosphorylation (**H**) and eNOS protein concentrations (**I**). Values are shown as the means ± SEM and are expressed as a percentage of the insulin-stimulated control (100%). $^{**}p<0.0001$

Using monoclonal antibodies against Tyr-204 phosphorylated ERK1 and ERK2, the levels of ERK1/2 activation were examined in rat thoracic aortae in the basal state and after insulin stimulation in the in vivo experiments. Insulin was able to stimulate tyrosine phosphorylation of ERK1/2 differentially in aortae from 2- and 12-month-old Wistar rats (Fig. 4H). Of note, 12-month-old rats showed increased basal tyrosine phosphorylation of ERK1/2, 2.2-fold higher than those found in young rats (2m: 50±5% vs 12m: 110±6%, n=12, p<0.0001). After insulin injection into the cava vein, tyrosine phosphorylation of ERK1/2 was two times higher in aortae from obese middle-aged rats than in the control rats (2m: 100±8% vs 12m: 208±4%, n=12, p<0.0001). Next, whole tissue extracts obtained from aortae homogenates from young and middle-aged rats were resolved under reducing conditions in 10% SDS-PAGE, transferred to nitrocellulose membranes and incubated with polyclonal antibodies against ERK1/2 proteins to evaluate the tissue levels of these protein kinases. Obese middle-aged rats demonstrated 2.1 times higher levels of ERK1/2 in aortae than the younger control rats (2m: 100±4% vs 12m: 210±8%, n=12, p<0.0001; Fig. 4I).

Insulin signalling in skeletal muscle from SHR and WKY

Insulin-stimulated tyrosine phosphorylation of insulin receptor beta subunit was reduced in skeletal muscle from SHR, as compared to the control rats (WKY: 100±3% vs SHR: 47±8%, n=9, p<0.0001; Fig. 5A). The protein concentrations of IRβ did not change in muscle samples obtained from SHR and WKY (Fig. 5B).

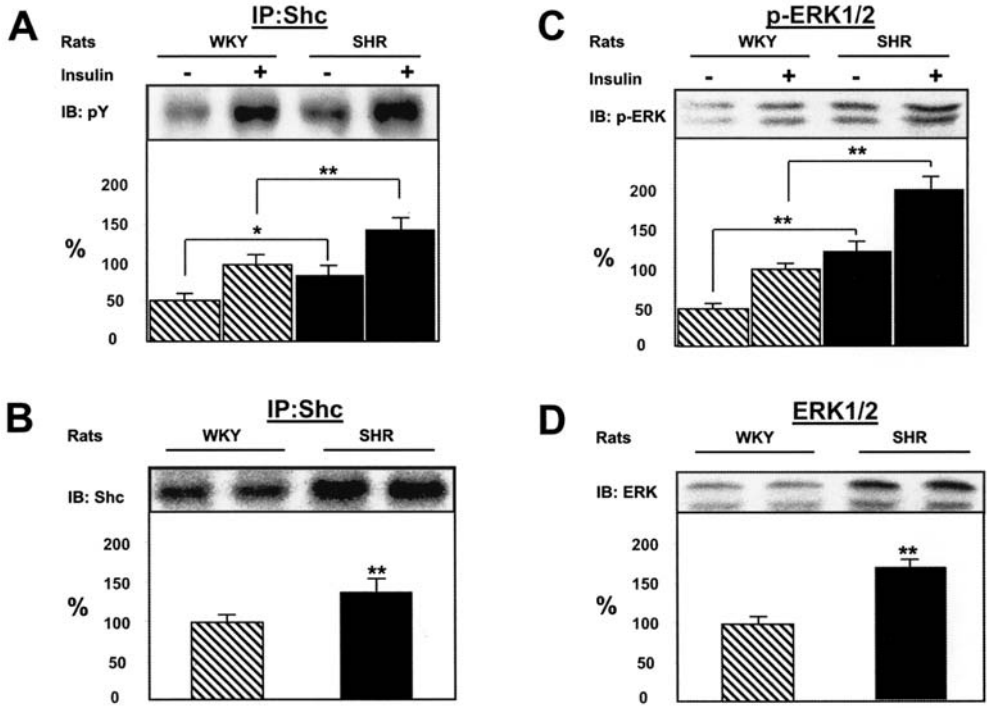

Fig.7A–D. Insulin signalling in thoracic aortae of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Insulin-stimulated Shc tyrosine phosphorylation (**A**), Shc protein concentrations (**B**), insulin-stimulated ERK1/2 tyrosine phosphorylation (**C**) and ERK1/2 protein concentrations (**D**). Values are shown as the means ± SEM and are expressed as a percentage of the insulin-stimulated control (100%). *, $p<0.05$; **, $p<0.0001$

The insulin-stimulated IRS-1 tyrosine phosphorylation showed a decrease in skeletal muscle from SHR as compared to WKY (WKY: 100±6% vs SHR: 30±8%, n=9, $p<0.0001$; Fig. 5C). IRS-1 protein concentrations suffered no changes in muscle from these two groups (Fig. 5D). The insulin-stimulated association between IRS-1 and the p85 subunit of PI 3-kinase was also reduced in muscle from SHR as compared to WKY (WKY: 100±4% vs SHR: 26±8%, n=9, $p<0.0001$; Fig. 5E).

Insulin was able to stimulate the serine phosphorylation of Akt in skeletal muscle from WKY and SHR groups of animals, but the latter showed reduced Akt activation after insulin infusion (WKY: 100±4% vs SHR: 60±6%, n=9, $p<0.0001$; Fig. 5F). A parallel decrease in Akt protein expression in skeletal muscle from SHR in comparison to WKY strain was observed (WKY: 100±8% vs SHR: 62±4%, n=9, $p<0.0001$; Fig. 5G).

Insulin-stimulated Shc tyrosine phosphorylation in skeletal muscle from SHR was not different from that observed in the WKY group (representative of six experiments, Fig. 5H), with no difference in protein concentrations (Fig. 5I).

Insulin infusion stimulated the tyrosine phosphorylation of ERK1/2 equally in skeletal muscle from WKY and SHR (WKY: 100±5% vs SHR: 97±4%, n=6; Fig. 5J). The immunoblotting was then conducted with anti-ERK1/2 antibodies (Fig. 5K) to show that protein concentrations of ERK1/2 in muscle did not change between these two groups.

Insulin signalling in aorta from SHR and WKY Insulin-stimulated IRβ tyrosine phosphorylation concentrations were similar in aortae from both groups of animals (WKY: 100±6% vs SHR: 95±6%, n=9; Fig. 6A). There was no difference in tissue levels of IRβ when the two groups were compared (Fig. 6B).

There was a reduction in insulin-stimulated IRS-1 tyrosine phosphorylation in aortae from SHR when compared to WKY control rats (WKY: 100±6% vs SHR: 60±4%, n=9, $p<0.0001$; Fig. 6C). However, no differences were found in IRS-1 protein concentrations in the aortae of WKY and SHR (Fig. 6D).

After insulin injection, the association between IRS-1 and PI 3-kinase (p85) was decreased in aortae from SHR in comparison to WKY (WKY: 100±6% vs SHR: 48±4%, n=9, $p<0.0001$; Fig. 6E).

The insulin-stimulated Akt serine phosphorylation was reduced in aortae from SHR when compared to WKY (WKY: 100±4% vs SHR: 62±3%, n=9, $p<0.0001$; Fig. 6F). There was no difference between the Akt protein concentrations in aortae from these two strains (Fig. 6G).

There was a reduction in insulin-stimulated serine phosphorylation of e-NOS in aortae from SHR compared to the control rats (WKY: 100±3% vs SHR: 53±6%, n=9, $p<0.0001$; Fig. 6H). In addition, protein concentrations of the endothelial isoform of NOS

were also reduced in aortae from SHR (WKY: 100±4% vs SHR: 33±7%, n=9, p<0.0001; Fig. 6I).

The basal Shc tyrosine phosphorylation in aortae from SHR was higher than in the WKY control rats (WKY: 50±4% vs SHR: 73±4%, n=9, p<0.05; Fig. 7A). The increase in Shc tyrosine phosphorylation in the vascular tissue after acute insulin infusion was greater in SHR when compared to the WKY group (WKY: 100±6% vs SHR: 140±6%, n=9, p<0.0001; Fig. 7A). This increased level of Shc phosphorylation was accompanied by an increase in protein concentrations found in aortae from SHR (WKY: 100±3% vs SHR: 137±6%, n=9, p<0.0001; Fig. 7B).

The levels of ERK1/2 activation were then examined in the vascular tissue both in the basal state and after acute insulin stimulation. Insulin was able to stimulate tyrosine phosphorylation of ERK1/2 differentially in aortae from SHR and WKY groups (Fig. 7C). Basal tyrosine phosphorylation of these two isoforms of MAP kinase in aortae from SHR was higher than in the WKY control rats (WKY: 50±2% vs SHR: 121±3%, n=9, p<0.0001; Fig. 7C). After insulin injection, there was also a greater increase in ERK1/2 tyrosine phosphorylation in aortae from SHR when compared to the WKY group (WKY: 100±1% vs SHR: 200±5%, n=9, p<0.0001; Fig. 7C). To further analyse the protein concentrations of ERK1/2 in this tissue, membranes were submitted to immunoblot with anti-ERK1/2 antibodies. SHR showed 1.6 times higher levels of ERK1/2 in aortae than in the controls (WKY: 100±7% vs SHR: 167±3%, n=9, p<0.0001; Fig. 7D).

Discussion

Reduced insulin sensitivity has been proposed as an important risk factor in the development of endothelial dysfunction and atherosclerosis and is also associated with hypertension [25]. Vascular diseases represent an important cause of the morbidity and mortality associated with diabetes and other insulin resistant states. A very large body of experimental work has sought to elucidate the cellular and molecular mechanisms that underlie this important pathophysiological process. We have characterized insulin signal transduction pathways in the vasculature of young (2-month-old) and obese middle-aged (12-month-old) Wistar male rats and also in SHR and in their normotensive, insulin-sensitive control strain, WKY. The 12-month-old rats are obese and have insulin resistance, normal fasting blood glucose, increased serum insulin and insulin resistance, characterized by a reduced glucose disappearance rate during the insulin tolerance test, but they develop neither hypertension nor atherosclerosis. In contrast, the SHR is a model characterized by hypertension and insulin resistance [26, 27], and this alteration in insulin sensitivity was confirmed in our study by the reduced glucose disappearance rate during the ITT.

The molecular mechanism of insulin resistance in obese middle-aged animals is probably related to abnormalities in insulin signalling pathways in skeletal muscle. The PI 3-kinase/Akt pathway is severely impaired in skeletal muscle from these rats, with reductions in insulin-stimulated phosphorylation of IRS-1 and Akt, as well as IRS-1 protein concentrations and in the association between IRS-1/PI 3-kinase, as described previously [28]. Since activation of PI 3-kinase pathway by insulin is linked to metabolic functions such as glucose transport and glycogen and protein synthesis [29, 30, 31, 32], alterations in insulin signalling pathways in muscle can contribute to the insulin resistance observed in these animals. In contrast, tyrosine phosphorylation of IRβ and IRS-1/2, insulin-stimulated association between IRS-1/2 and PI 3-kinase, and serine phosphorylation of Akt and eNOS are preserved in their aortae. The molecular mechanism of this tissue-specific regulation of insulin signalling (reduced in muscle and preserved in aorta) in the obese insulin-resistant animals could be related to the expression of proteins involved in early steps of insulin action, to the level of serine phosphorylation of IRβ and IRSs and to the activity of phosphotyrosine phosphatases. On the other hand, in SHR there were reductions in IR/IRS-1/PI 3-K/Akt pathway in muscle and in IRS-1/PI 3-K/Akt/eNOS pathway in aorta, which could be involved in insulin resistance and could be one factor that can contribute to the endothelial dysfunction in these rats, respectively.

Recently, insulin signalling in vascular tissues has been emphasized. It has been shown that activation of PI 3-kinase pathway could be involved in insulin's stimulatory effect on NO release in cultured vascular endothelial cells [3]. This effect of insulin on NO could be responsible for insulin's vasodilatory actions and in some insulin-resistant states, the vasodilation induced by insulin could be blunted as a result of the inhibition of PI 3-kinase pathway [8, 9]. Furthermore, it has been shown that insulin-stimulated tyrosine phosphorylation of IRβ and IRS proteins and PI 3-kinase activation were selectively impaired in vascular tissues of insulin-resistant obese Zucker rats [33]. Also, it has been reported that insulin's vasodilatory effect could be partially due to increases in eNOS gene expression via PI 3-kinase pathway [6]. Accordingly, in vascular endothelial cell insulin receptor knockout mice there is a reduction in eNOS expression [34] suggesting that insulin resistance in vessels is accompanied by a reduction in this enzyme. From these reports and our results showing blunted insulin signalling through the IRS/PI 3-kinase/Akt/eNOS pathway associated with a reduction in eNOS expression in aortae from SHR, we can suggest that these alterations might contribute to the loss of insulin's effect on NO production, which could contribute to endothe-

lial dysfunction and could also play a role in the hypertension presented by SHR. In contrast, the obese middle-aged rats could be protected from cardiovascular disease, even if confronted by severe insulin resistance, because PI 3-kinase/Akt/eNOS pathway was preserved in aorta.

In contrast to PI 3-kinase activation, much less is known about insulin's activation of ERK1/2 MAP kinase in insulin-resistant or diabetic conditions in vivo. Selective hyperexpression of insulin signalling on ERK1/2 MAP kinase pathway in the vascular tissues could be pathophysiologically important in the development of cardiovascular diseases. Pathway-selective insulin resistance could result in increased potentiation of VSMC proliferation and production of plasminogen activator inhibitor-1 (PAI-1) via the Ras/Raf/MEK/MAP kinase pathway [33]. Activation of the MAP kinase pathway by insulin is not reduced in Type 2 diabetes, perhaps allowing for some of the detrimental effects of chronic hyperinsulinemia on cellular growth in the vasculature [35]. The increased expression and activation of Shc and ERK1/2 in aortae from SHR could suggest a role of this pathway in vascular abnormalities of this rat. However, in the obese middle-aged rat model of insulin resistance, the increased activation and protein concentrations of MAP kinase in aorta were not associated with SMC proliferation, as shown by histological results, neither with hypertension nor atherosclerosis. This suggests that the isolated activation of this pathway, with preservation of the PI 3-kinase/Akt/eNOS pathway, might not be sufficient for the development of cardiovascular diseases in these rats.

In summary, this study shows that alterations in IRS/PI 3-kinase/Akt pathway in muscle from 12-month-old rats and SHR could have a role in the insulin resistance of these rats. The preservation of this pathway in aorta of 12-month-old rats could be one factor that contributes to explaining the absence of cardiovascular disease in this animal model. However, in aortae of SHR, the reduced insulin signalling through IRS/PI 3-kinase/Akt/eNOS pathway could contribute to the endothelial dysfunction of this rat.

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Effect of obesity on insulin signaling through JAK2 in rat aorta

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Abstract

Pathway specific resistance to insulin signaling through PI 3-kinase/Akt/eNOS associated with a normal or hyperactivated MAP kinase signaling in vascular tissues has recently been proposed as a candidate link between cardiovascular disease and insulin resistance. Growth stimulatory pathways other than ERK/MAP kinase, such as JAK/STAT have not yet been investigated in vessels of animal models of insulin resistance. Here we have examined whether insulin is able to activate JAK2/STAT pathway in rat aorta and also the regulation of this pathway in an animal model of obesity/insulin resistance. Our results demonstrate that insulin activates JAK2 tyrosine kinase activity in rat aorta in parallel with the activation of STAT3 and STAT5a/b. Moreover, it is shown that, in obese animals, JAK2/STAT and MAP kinase pathways are hyperactivated in response to insulin, which occurs in association with a reduced activation of PI 3-kinase/Akt pathway in aorta. The results of the present study suggest that, besides ERK/MAP kinase pathway, another potentially pro-atherogenic pathway, JAK2/STAT is hyperactivated in vessels in a state of insulin resistance and this phenomenon, in association with the inhibition of the PI 3-kinase/Akt pathway, may play an important role in the pathogenesis of cardiovascular diseases.

Keywords: Akt; Aorta; Atherosclerosis; Endothelial cell-nitric oxide synthase; Insulin resistance; Janus kinase; MAP kinase; Obesity; Phosphatidylinositol 3-kinase; Signal transducer and activator of transcription

1. Introduction

Insulin resistance has been proposed as an important risk factor for the development of cardiovascular disease (Howard et al., 1996; Reaven et al., 2004). The term "insulin resistance" underscores the inability of insulin to promote normal homeostasis of glucose. This reduced strength of insulin action demands the presence of compensatory elevated concentrations of insulin in order to maintain normoglycemia and normal utilization of glucose by insulin target tissues. However, insulin, as an anabolic hormone, exerts diverse effects on lipid and protein metabolism, ion and amino acid transport, cell cycle and proliferation, cell differentiation and nitric oxide (NO) synthesis. Nevertheless, insulin resistance may not affect all aspects of insulin action equally. Hence, diminished insulin action (as an effect of insulin resistance) may coexist with normal or even enhanced insulin action (as an effect of hyperinsulinemia) within the same tissue and within the same cell (Wang et al., 2004). Under physiological conditions, insulin regulates many vascular functions, such as amino acid transport, glycogen synthesis, DNA synthesis and gene expression (King et al., 1985; Obata et al., 1996). Specific vascular actions of insulin include the release of nitric oxide (NO) (Zeng and Quon, 1996) and the regulation of mRNA expression of matrix proteins (Anderson et al., 1996; Tamaroglio and Lo, 1994) and constitutive endothelial NO synthase (eNOS) (Kuboki et al., 2000; Papapetropoulos et al., 1999).

At the molecular level, binding of insulin to its cognate receptor (IR) results in the activation of IR's tyrosine kinase activity which, in turn, phosphorylates tyrosine residues of

Abbreviations: Akt; protein kinase B; α PY; antibody against phosphotyrosine; eNOS; endothelial cell-nitric oxide synthase; ERK; extracellular signal-related kinase; IR; insulin receptor; IRS; insulin receptor substrate; JAK2; Janus kinase 2; MAP; mitogen-activated protein; NO; nitric oxide; PI; phosphatidylinositol; STAT; signal transducer and activator of transcription; VSMC; vascular smooth muscle cells.

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insulin receptor substrates (IRSs). IRSs are adaptor proteins that transduce signals from IR to downstream signaling cascades including PI 3-kinase/Akt pathway (Saltiel and Kahn, 2001; Virkamaki et al., 1999). This pathway appears to be absolutely necessary for mediating metabolic effects of insulin (Shepherd et al., 1998; Shulman, 2000). In the vasculature, the activation of PI 3-kinase increases serine phosphorylation of Akt which, in turn, directly phosphorylates eNOS on serine 1177 and activates this enzyme, leading to increased NO production (Dimmeler et al., 1999; Fulton et al., 1999). In addition, a second postreceptor insulin signaling pathway involves the phosphorylation of Shc and activation of Ras, Raf, MEK and mitogen-activated protein (MAP) kinases (ERK 1 and 2), which is related to cellular growth (Sasaoka et al., 1996, 1994). Through the MAP kinase pathway, insulin activates two prenyltransferases (farnesyltransferase and geranylgeranyltransferase I) (Goalstone and Draznin, 1996), which promote prenylation of Ras and Rho proteins (Zhang and Casey, 1996) starting a strong proatherogenic signal. It has been demonstrated that a selective insulin resistance affecting the PI 3-kinase pathway in the vasculature of animal models of insulin resistance and cardiovascular disease may coexist with an intact or even hyperstimulated ERK/MAP kinase pathway (Jiang et al., 1999; Zecchin et al., 2003).

Insulin is also able to activate Janus kinase 2 (JAK2), a member of the Janus family of tyrosine kinases (Saad et al., 1996). In cytokine-responsive cells, stimulation of JAK activity leads to phosphorylation of STAT proteins (Shuai et al., 1993; Silvennoinen et al., 1993). Tyrosine-phosphorylated STATs are translocated to the nucleus where they bind to specific DNA sequences and activate early growth-response genes (Darnell et al., 1994; Darnell, 1997; Ihle, 2001). Also, the JAK-STAT pathway may play a similar role in the control of angiotensin II-mediated cell growth in vascular tissues (Banes et al., 2004; Marrero et al., 1995; Watanabe et al., 2004). However, the regulation of insulin-stimulated JAK/STAT pathway in aorta of an animal model of insulin resistance was not yet investigated. In this study, we evaluated the ability of insulin to activate JAK2/STAT and MAP kinase isoforms ERK1/2, as well as protein kinase B/Akt in aortae from normal rats and from an animal model of diet-induced obesity (DIO) and insulin resistance, the rat fed on a high-fat diet for 30 days (Prada et al., 2005).

2. Experimental/materials and methods

2.1. Experimental animals

Eight-week-old male Wistar rats were obtained from the University of Campinas Central Animal Breeding Center (Campinas, Sao Paulo, Brazil), divided into two groups with similar body weights (228±18 g) and assigned to receive two types of diet: a standard rodent chow (regular diet) or a high-fat diet for 30 days. The high-fat diet totalizes 5.4 kcal/g (carbohydrate, 38.5%; protein, 15%; fat, 46.5%) as opposed to the 3.8 kcal/g (carbohydrate, 70%; protein, 20%; fat, 10%) of the standard chow diet (Prada et al., 2005). Animals were allowed free access to diet and water ad libitum. Food was withdrawn 12 h before the experiments. Blood samples were taken for measuring plasma concentration of total cholesterol, high-density lipoprotein (HDL)-cholesterol, triacylglycerol (TG) and insulin. Lipids were measured in a routine diagnostic analyser (Modular, Roche Diagnostics, Mannheim, Germany) using enzymatic colorimetric assays (cholesterol, CHOD-PAP assay; triacylglycerol, GPO-PAP assay). Plasma glucose concentrations were measured by the glucose oxidase method, as described previously (Trinder, 1969). Serum insulin was determined by radioimmunoassay (Eizirik et al., 1994). All experiments involving animals were approved by the Ethics Committee at the University of Campinas.

2.2. Materials

Monoclonal antibodies against phosphotyrosine (PY20, sc-508) and phospho-ERK (Tyr 204, E-4, sc-7383), as well as polyclonal antibodies against Akt1 (C-20, sc-1618), ERK1 (C-16, sc-93: reactive with ERK1 p44 and, to a lesser extent, ERK2 p42), Janus kinase 2 (HR-758, sc-278), phospho-STAT3 (Tyr 705, sc-7993), STAT3 (K-15, sc-483), phospho-STAT5 (Tyr 694, sc-11761) and STAT5 (C-17, sc-835) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies against phospho-Akt (Ser 473) were obtained from New England Biolabs (Beverly, MA, USA). Human recombinant insulin was from Eli Lilly and Co. (Indianapolis, IN, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless specified elsewhere. [125 I]Protein A was obtained from Amersham (Amersham Biosciences Group, Little Chalfont, UK). Protein A-Sepharose 6 MB was obtained from Pharmacia Biotech (Uppsala, Sweden). Nitrocellulose (BA85; 0.2 μm) was purchased from Schleicher and Schuell (Keene, NH, USA). Molecular weight, Mr standards were myosin (194 kDa), α-galactosidade (116 kDa), BSA (85 kDa) and ovalbumin (49.5 kDa).

2.3. Fifteen-minute insulin tolerance test

Rats were fasted overnight and submitted to an intravenous insulin tolerance test (IVITT; 1 U/kg body weight of insulin, i.v.) and samples for blood glucose measurements were collected at 0 (basal), 4, 8, 12, and 16 min after injection. Rats were anesthetized with sodium amobarbital as described above, 40 μl of blood was collected from their tails and blood glucose concentration was measured by the glucose oxidase method. Thereafter, the rate constant for plasma glucose disappearance (Kitt) was calculated using the formula 0.693/(t1/2). The plasma glucose t1/2 was calculated from the slope of the least squares analysis of the plasma glucose concentrations during the linear phase of decay (Bonora et al., 1989).

2.4. Tissue extraction and immunoprecipitation

Rats were anesthetized with sodium thiopental and used 10–15 min later. As soon as anesthesia was assured by the loss

of pedal and corneal reflexes, the thoracic cavity was opened, thoracic aorta was isolated and kept for 45 min at 37 °C in Krebs-bicarbonate buffer equilibrated with 95% O2 : 5% CO2, pH 7.4 before insulin stimulation (2 μg) and then were frozen with liquid N2, powdered with a glass Dounce homogenizer on ice for at least 80 strokes and immediately homogenized in extraction buffer, as described elsewhere (Zecchin et al., 2003). Extracts were then centrifuged at 30,000 ×*g* and 4 °C for 45 min to remove insoluble material, and the supernatants were used for immunoprecipitation with α-JAK2 protein A-Sepharose 6MB. Protein concentration in the supernatants was determined by the Bradford method (Bradford, 1976).

2.5. Protein analysis by immunoblotting

The precipitated proteins and/or whole-tissue extracts were treated with Laemmli sample buffer (Laemmli, 1970) containing 100 mM dithiothreitol and heated in a boiling water bath for 5 min, after which they were subjected to SDS-PAGE in a Bio-Rad miniature slab gel apparatus (Mini-Protean). For total extracts, 150 μg of proteins were subjected to SDS-PAGE. Electrotransfer of proteins from the gel to nitrocellulose was performed for 120 min at 120 V in a Bio-Rad Mini-Protean transfer apparatus (Towbin et al., 1979). Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter for 2 h in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, 0.02% Tween 20). The nitrocellulose blot was incubated with specific antibodies overnight at 4 °C and then incubated with 125 I-labeled protein A. The results were visualized by autoradiography with preflashed Kodak XAR film. Band intensities were quantified by optical densitometry (Hoefer Scientific Instruments, San Francisco, CA; model GS300) of the developed autoradiographs.

2.6. JAK2 in vitro kinase activity assay

45 min after incubating thoracic aortae (obtained from fasted rats) with or without a very low concentration of insulin (0.2 μg), tissue extracts were obtained and immunoprecipitated with anti-JAK2 as described above. The resulting immune complexes were collected on Protein A/G Plus. The protein kinase activity of the immunoprecipitates was measured by incubating the immune complexes in 100 μL buffer containing 50 mM Tris (pH 7.5), 0.2 mM sodium vanadate, 0.1% Triton X-100, 3 mM MnCl2 and 15 μM cold ATP for 30 min at room temperature. The complexes were washed twice with cold buffer, then resuspended in Laemmli sample buffer and analyzed by SDS-PAGE. The incorporation of phosphate into the separated proteins was visualized by autoradiography using anti-phosphotyrosine immunoblots after transfer to nitrocellulose (Saad et al., 1996).

2.7. Statistical analysis

Experiments were always carried out by studying the groups of animals in parallel (control animals fed on regular diet vs. obese animals fed on high-fat diet). Data are means ± SEM accompanied by the indicated number of independent experiments. For comparisons, unpaired Student's *t* test was used. The significance level was set at *p* value of less than 0.05.

3. Results

3.1. Effect of insulin on tyrosine phosphorylation in the JAK/STAT pathway in rat aorta

To determine insulin-induced tyrosine phosphorylation of Janus kinase 2 (JAK2) we performed immunoprecipitation and Western blotting of thoracic aortae extracts with anti-JAK2 and anti-phosphotyrosine antibodies, respectively. The time course experiments were performed by incubating thoracic aortae with 2 μg insulin for different periods. As shown in Fig. 1A, JAK2 tyrosine phosphorylation was detectable 5 min after incubation with insulin and was maximal at 15 min (approximately 10-fold increase above basal state), decaying after 30 min (Fig. 1A, *upper panel*). The tissue levels of JAK2 were not modulated by the treatment (Fig. 1A, *middle panel*). To evaluate the association between JAK2 and IRS-1, nitrocellulose membranes of JAK2 immunoprecipitates were stripped and reblotted with antibodies against IRS-1. The association between JAK2 and IRS-1 revealed the same temporal pattern of that seen in insulin-stimulated JAK2 tyrosine phosphorylation (Fig. 1A, lower *panel*).

To test whether JAK2 kinase activity could be stimulated by insulin in rat aorta, we measured the enzyme autophosphorylation in vitro by immunoprecipitating aortae extracts (with or without a low concentration of insulin) with α JAK2 and performed an in vitro kinase assay using ATP, as described above. JAK2 kinase activity was increased significantly in aortae extracts after incubation with insulin for 15 min, as demonstrated by an increase in JAK2 autophosphorylation (Fig. 1B). Whole cell lysates obtained from thoracic aortae after incubation with insulin for different periods were submitted to immublotting with antibodies against phospho-STAT3 and STAT3 in order to evaluate, respectively, the activation and tissue levels of STAT3 by insulin in aorta. Insulin-stimulated STAT3 tyrosine phosphorylation was maximal 15 min after incubation with this hormone, without any significant change in STAT3 protein levels (Fig. 1C, *two upper panels*). Incubation with insulin led to an approximately 4-fold increase above basal state in STAT5 tyrosine phosphorylation at 15 min, without significant changes in STAT5 tissue levels (Fig. 1C, *two lower panels*). Hence, both STAT3 and STAT5 phosphorylation after insulin treatment paralleled the increase of JAK2 phosphorylation after the aortae were incubated with insulin.

3.2. Effect of obesity on insulin signaling pathways in rat aorta

In order to investigate the effect of obesity on insulin signaling pathways in aorta we induced obesity in male Wistar rats with a high-fat diet for 30 days. Animals fed on high-fat

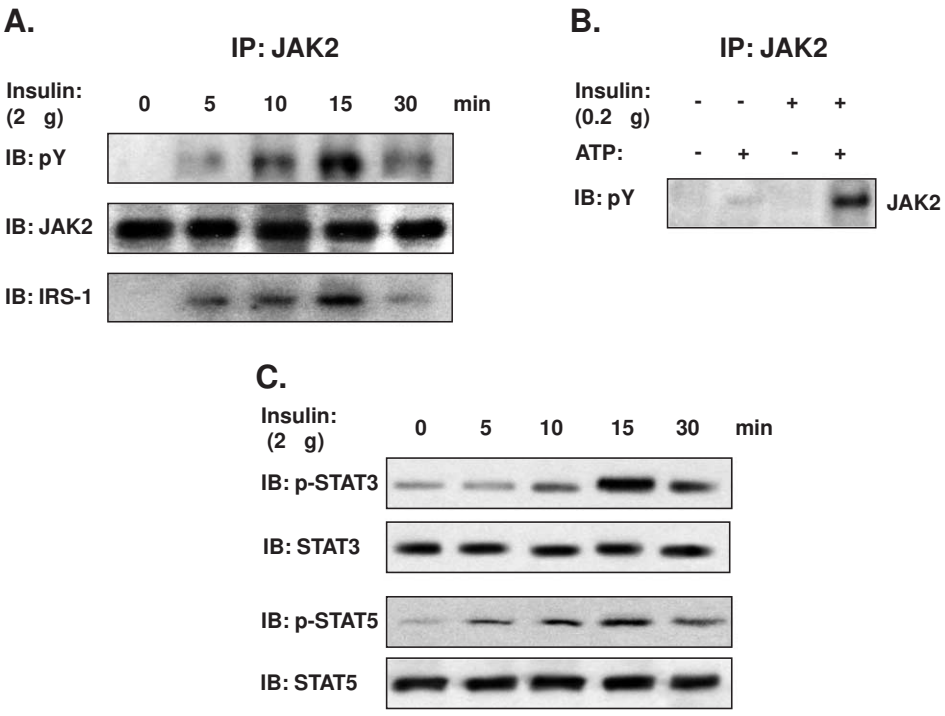

Fig. 1. Insulin signaling in aorta of control rats – time-course experiments. A, Immunoprecipitation (IP) with α-JAK2 and immunoblotting (IB) with α-phosphotyrosine (α PY) (*upper panel*); IP with α-JAK2 and IB with α-JAK2 (*middle panel*); IP with α-JAK2 and IB with α-IRS-1 (*lower panel*). Blots are representative of six independent experiments. B, JAK2 tyrosine activity measured by autophosphorylation in vitro. Thoracic aortae were incubated with (+) or without (−) a very low concentration of insulin (0.2 μg) and used 15 min later (maximum JAK2 tyrosine phosphorylation in aorta). To stimulate partial JAK2 autophosphorylation, JAK2 was immunoprecipitated (2 μg/mL) and allowed to autophosphorylate in vitro in the presence (+) or absence (−) of exogenous ATP. Tyrosine phosphorylation was measured by immunoblotting with α PY (1 μg/mL). These results are representative of four experiments. C, IB with α-phospho-STAT3 and IB with α-STAT3 (*two upper panels*); IB with α-phospho-STAT5 and IB with α-STAT5 (*two lower panels*). Blots are representative of six independent experiments.

diet for 30 days became obese and less insulin sensitive than their controls, as demonstrated by reduced insulin tolerance; higher levels of plasma insulin and triacylglycerol (TG); and lower levels of high-density lipoprotein (HDL)-cholesterol (Table 1). We first evaluated the ability of insulin to activate JAK2. Thoracic aortae were incubated with insulin for 15 min and analyzed by immunoblotting. JAK2 tyrosine phosphorylation was measured by immunoprecipitation and immunoblotting. There was no difference in insulin-stimulated JAK2 tyrosine phosphorylation in aortae from obese and control rats (Fig. 2A). The tissue levels of JAK2 did not change between the two groups (Fig. 2B). Next, we evaluated the ability of insulin to activate STAT3 and STAT5 in rat aorta. As shown in Fig. 2C, insulin was able to stimulate the STAT3 tyrosine phosphorylation in aortae from both obese and control rats. Basal levels of phospho-STAT3 were significantly increased (~3.8-fold) in aortae from obese rats, as well as insulin-stimulated STAT3 tyrosine phosphorylation (~2.5-fold; Fig. 2C) when compared with controls, but there were no difference in STAT3 protein levels (Fig. 2D). Basal contents of phospho-STAT5 were also increased (~2.2-fold) in vascular tissue from obese animals, as well as insulin-stimulated STAT5 tyrosine phosphorylation (~1.4-fold; Fig. 2E) when compared with controls, with no significant difference in STAT5 protein levels (Fig. 2F). Using monoclonal antibodies against Tyr-204 phosphorylated ERK1 and ERK2, the levels of ERK1/2 activation were examined in rat thoracic aortae in the basal state and after insulin stimulation. Insulin was able to stimulate tyrosine phosphorylation of ERK1/2 differentially in aortae from obese and control rats. Of note, obese rats also showed increased basal tyrosine phosphorylation of ERK1/2, 2.3-fold higher than those found in controls ($p<0.001$; Fig. 2G). After insulin incubation, tyrosine phosphorylation of ERK1/2 was 1.6 times higher in aortae from obese rats than in the control rats ($p<0.001$; Fig. 2G). Next, whole tissue extracts obtained from aortae homogenates from control and obese rats were resolved under reducing conditions in 10% SDS-PAGE, transferred to nitrocellulose membranes and incubated with polyclonal antibodies against ERK1/2 proteins to evaluate the tissue levels of these protein kinases. Obese rats demonstrated

Table 1
Metabolic and biochemical characteristics of the experimental animals

	Control	Obese
Body weight (g)	265±17 ($n=10$)	346±27 ($n=10$)[a]
Blood glucose (mg/dL)	100±7 ($n=10$)	106±4 ($n=10$)
Serum insulin (pmol/L)	11.2±1.8 ($n=10$)	25.0±4.7 ($n=10$)[a]
Kitt (%/min)	5.32±0.64 ($n=10$)	2.12±0.41 ($n=10$)[b]
Serum TG (mg/dL)	45.6±7.8 ($n=10$)	165.8±17.5 ($n=10$)[b]
Serum cholesterol (mg/dL)	64±2 ($n=10$)	68±4 ($n=10$)
Serum HDL (mg/dL)	48±7 ($n=10$)	43±5 ($n=10$)

Data are presented as means±SEM. p values of less than 0.05 were considered to be significantly different. [a] $p<0.05$ vs control; [b] $p<0.0001$ vs control.

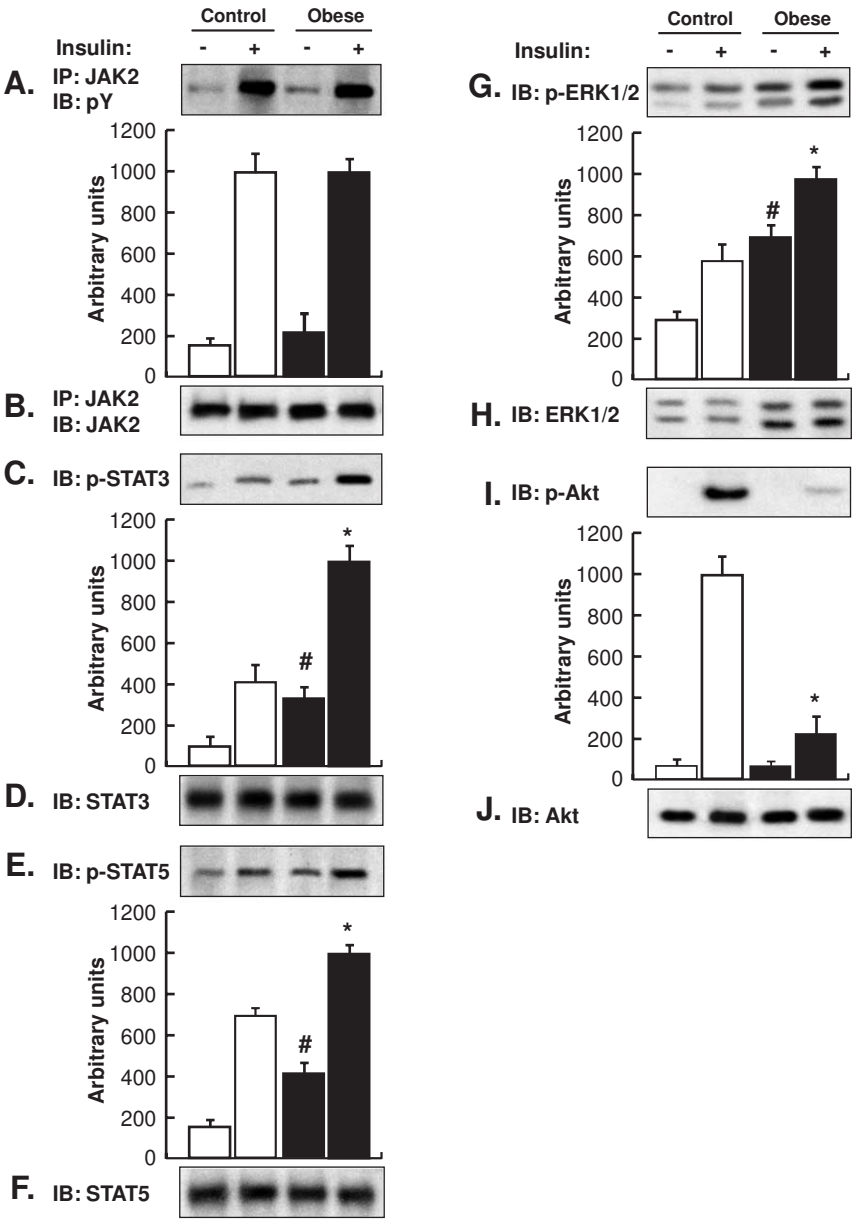

Fig. 2. Insulin signaling in aorta of control and obese rats. Tissue samples were incubated with medium with or without insulin (2 μg) and submitted to immunoprecipitation (IP) followed by immunoblotting (IB). A, IP with α-JAK2 and IB with α-phosphotyrosine (α PY). B, IP with α-JAK2 and IB with α-JAK2. C, IB with α-p- STAT3. D, IB with α-STAT3. E, IB with α-p-STAT5. F, IB with α-STAT5. G, IB with α-p-ERK1/2. H, IB with α-ERK1/2. I, IB with α-p-Akt. J, IB with α-Akt.Values are shown as the means ± SEM of six independent experiments. * $p<0.001$, insulin-20.stimulated obese vs. insulin-stimulated control; # $p<0.001$, basal (non-stimulated) obese vs. basal control.

~2.0 times higher levels of ERK1/2 in aortae than the control rats ($p<0.001$; Fig. 2H). Akt is activated in response to insulin and so becomes capable of phosphorylating endothelial cell-nitric oxide synthase in serine 1177 and 1179, activating the production of nitric oxide. To evaluate the activation of Akt in the vascular tissue, both before and after insulin stimulation, we conducted experiments in which whole cell lysates obtained from thoracic aortae incubated with or without insulin were submitted to immunoblotting with anti-phospho-Akt (Ser 473) antibodies. There was a significant decrease (~80%) in insulin-stimulated Akt activation in aorta from obese animals when compared with controls (Fig. 2I). After incubating the membranes with anti-Akt antibodies, we demonstrated that there was no difference between the protein concentration of Akt in aorta from these two groups (Fig. 2J).

4. Discussion

In the present study, we have shown that, in aorta of normal rats, activation of JAK/STAT pathway was induced by insulin by stimulating the phosphorylation of JAK2, STAT3 and STAT5a/b. In addition, we have also shown that this pathway is modulated in aorta of a classical animal model of insulin resistance, the diet-induced obese rat (Prada et al., 2005; Naderali et al., 2001).

Insulin-induced JAK2 tyrosine phosphorylation was accompanied by an increase in JAK2 tyrosine kinase activity in aorta, in accordance with previous data in other rat tissues (Saad et al., 1996). Activation of STAT proteins by different growth factors and hormones, including platelet-derived growth factor (Marrero et al., 1997), epidermal growth factor (Ruff-Jamison et al., 1994, 1995), vascular endothelial growth factor (Bartoli et al., 2000), ANG II (Marrero et al., 1995, 1997) and, as described here, by insulin, may play a mechanistic role in the exaggerated growth seen in many pathophysiological situations including diabetic glomerulosclerosis (Andersen et al., 2000a,b) and also atherosclerosis (Watanabe et al., 2004; Madamanchi et al., 2005). Activation of JAK/STAT pathway in kidney glomerular mesangial cells was essential for ANG II and/or hyperglycemia-induced collagen IV, TGF α and fibronectin production (Banes et al., 2004). Therefore, it appears that the activation of JAK2 and STAT proteins might play an important role in both promoting cell proliferation and synthesis of extracellular matrix molecules. Recently, the JAK/STAT pathway was shown to partially mediate IL-6-induced MCP-1 production and DNA synthesis in rat VSMC, implicating a role for the JAK/STAT pathway in the development of vascular disease and atherosclerosis (Watanabe et al., 2004). As previously reported, insulin has the ability to activate STAT proteins through the phosphorylation and activation of JAK2 (Saad et al., 1996; Giorgetti-Peraldi et al., 1995; Grimley et al., 1999; Welham et al., 1995). However, our data adds a new information showing that the activation process, measured as the phosphorylation levels, occurs in aorta of an in vivo model of metabolic syndrome, characterized by insulin resistance, central fat depot, increase in TG levels and a decrease in HDL-cholesterol levels. This animal model also presents endothelial dysfunction (data not shown), as previously described (Naderali et al., 2001).

Insulin resistance has been proposed as an important risk factor in the development of endothelial dysfunction and atherosclerosis and is also associated with hypertension (Howard et al., 1996). In the presence of insulin resistance, endothelial cells present decreased expression and activity of the endothelial cell nitric oxide synthase, leading to the reduction of antiatherogenic second messenger NO production and loss of its protective effects on vasculature, including impaiment of endothelium-dependent vasodilation. The expression of eNOS is apparently regulated by insulin receptor-mediated PI 3-kinase signaling pathway through Akt, which also induces the activation of eNOS (Kuboki et al., 2000). Selective inhibition of insulin actions on PI 3-kinase/Akt pathway in vasculature in insulin resistant states, with preservation of Ras/Raf/MEK/Erk pathway has been proposed as an important cause of NO production downregulation leading to an imbalance that could lead to the development of atherosclerosis (Jiang et al., 1999; Zecchin et al., 2003). Compensatory hyperinsulinemia that accompanies insulin resistance generates a higher-than-normal initial signal at the level of the insulin receptor. Progression of this signal downstream is impaired along the PI 3-kinase pathway. In contrast, the signal is allowed to proceed normally or with greater strength along the Shc/Ras/MAP kinase and JAK/STAT pathways, eliciting greater responses of the downstream targets of this pathway. Although insulin may have antiatherogenic and anti-inflammatory actions (Dandona et al., 2002), hyperinsulinemia, in the presence of insulin resistance (impaired PI 3-kinase signaling) may exert a detrimental influence on the arterial wall and could potentiate the development of atherosclerosis. Thus, amelioration of the metabolic insulin resistance through an improvement of the insulin signaling via PI 3-kinase pathway might be expected to remove excessive stimulation of the ERK/MAP kinase and JAK/STAT signaling pathways and thereby improve antiatherogenic properties of hyperinsulinemia in insulin resistance (Wang et al., 2004).

In summary, we have documented here a selective insulin resistance in PI 3-kinase pathway associated with hyperactivation/hyperexpression of insulin signaling in ERK1/2 MAP kinase and JAK2/STAT pathways in vascular tissue of obese insulin-resistant rats. Pathway-selective insulin resistance could result in increased potentiation of VSMC proliferation and production of plasminogen activator inhibitor-1 (PAI-1) via the Ras/Raf/MEK/MAP kinase pathway (Jiang et al., 1999). Activation of the MAP kinase pathway by insulin is not reduced in type 2 diabetes, perhaps allowing for some of the detrimental effects of chronic hyperinsulinemia on cellular growth in the vasculature (Cusi et al., 2000). The increased activation of potentially pro-atherogenic pathways (MAP kinase and JAK/STAT) associated with a reduced stimulation through anti-atherogenic PI 3- kinase/Akt/eNOS pathway may contribute to explain the development of cardiovascular disease associated with states of insulin resistance.

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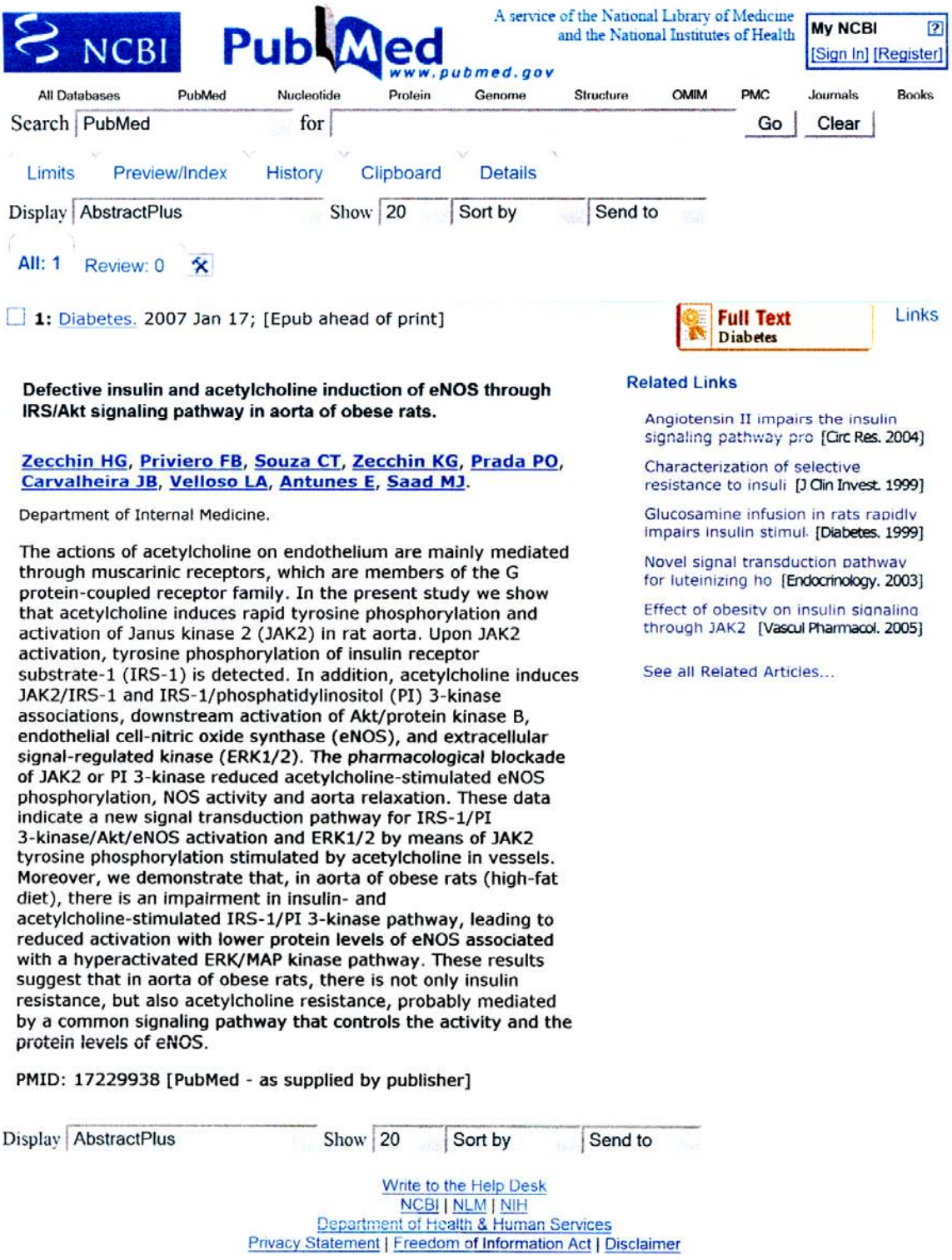

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Defective insulin and acetylcholine induction of eNOS through IRS/Akt signaling pathway in aorta of obese rats.
Zecchin HG, Priviero FB, Souza CT, Zecchin KG, Prada PO, Carvalheira JB, Velloso LA, Antunes E, Saad MJ.
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The actions of acetylcholine on endothelium are mainly mediated through muscarinic receptors, which are members of the G protein-coupled receptor family. In the present study we show that acetylcholine induces rapid tyrosine phosphorylation and activation of Janus kinase 2 (JAK2) in rat aorta. Upon JAK2 activation, tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) is detected. In addition, acetylcholine induces JAK2/IRS-1 and IRS-1/phosphatidylinositol (PI) 3-kinase associations, downstream activation of Akt/protein kinase B, endothelial cell-nitric oxide synthase (eNOS), and extracellular signal-regulated kinase (ERK1/2). The pharmacological blockade of JAK2 or PI 3-kinase reduced acetylcholine-stimulated eNOS phosphorylation, NOS activity and aorta relaxation. These data indicate a new signal transduction pathway for IRS-1/PI 3-kinase/Akt/eNOS activation and ERK1/2 by means of JAK2 tyrosine phosphorylation stimulated by acetylcholine in vessels. Moreover, we demonstrate that, in aorta of obese rats (high-fat diet), there is an impairment in insulin- and acetylcholine-stimulated IRS-1/PI 3-kinase pathway, leading to reduced activation with lower protein levels of eNOS associated with a hyperactivated ERK/MAP kinase pathway. These results suggest that in aorta of obese rats, there is not only insulin resistance, but also acetylcholine resistance, probably mediated by a common signaling pathway that controls the activity and the protein levels of eNOS.
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Abstract:

The actions of acetylcholine on endothelium are mainly mediated through muscarinic receptors, which are members of the G protein-coupled receptor family. In the present study we show that acetylcholine induces rapid tyrosine phosphorylation and activation of Janus kinase 2 (JAK2) in rat aorta. Upon JAK2 activation, tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) is detected. In addition, acetylcholine induces JAK2/IRS-1 and IRS-1/phosphatidylinositol (PI) 3-kinase associations, downstream activation of Akt/protein kinase B, endothelial cell-nitric oxide synthase (eNOS), and extracellular signal-regulated kinase (ERK1/2). The pharmacological blockade of JAK2 or PI 3-kinase reduced acetylcholine-stimulated eNOS phosphorylation, NOS activity and aorta relaxation. These data indicate a new signal transduction pathway for IRS-1/PI 3-kinase/Akt/eNOS activation and ERK1/2 by means of JAK2 tyrosine phosphorylation stimulated by acetylcholine in vessels. Moreover, we demonstrate that, in aorta of obese rats (high-fat diet), there is an impairment in insulin- and acetylcholine-stimulated IRS-1/PI 3-kinase pathway, leading to reduced activation with lower protein levels of eNOS associated with a hyperactivated ERK/MAP kinase pathway. These results suggest that in aorta of obese rats, there is not only insulin resistance, but also acetylcholine resistance, probably mediated by a common signaling pathway that controls the activity and the protein levels of eNOS.

Keywords: insulin resistance, acetylcholine, insulin receptor substrate-1, Janus Kinase, endothelial cell-nitric oxide synthase.

Abbreviations: ACh, acetylcholine; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; GTN, gryceryl trinitrate; IR, insulin receptor; IRS, insulin receptor substrate; JAK2, Janus kinase 2; MAP kinase, mitogen-activated protein kinase.

Under physiological conditions, insulin regulates many vascular functions (1;2) including the release of nitric oxide (NO) (3), and the regulation of mRNA expression of matrix proteins (4;5) and constitutive endothelial cell-nitric oxide synthase (eNOS) (6;7).

At the molecular level, binding of insulin to its cognate receptor (IR) results in activation of IR's tyrosine kinase activity which, in turn, phosphorylates tyrosine residues of insulin receptor substrates (IRSs). IRSs are adaptor proteins that transduce signals from IR to downstream signaling cascades including phosphatidylinositol (PI) 3-kinase/Akt pathway (8;9). In the vasculature, the activation of PI 3-kinase increases serine phosphorylation of Akt which, in turn, directly phosphorylates eNOS on ser1177 and activates the enzyme, leading to increased NO production (10;11). In addition, a second postreceptor insulin signaling pathway involves the activation of Ras, Raf, MEK and mitogen-activated protein (MAP) kinases (ERK1/2), which is related to cellular growth (12;13). Recent findings suggest that impaired IRS-1/PI 3-kinase/Akt/eNOS signal transduction may play a mechanistic role in endothelial dysfunction, and in the development of cardiovascular diseases in situations of insulin resistance (14;15).

Although insulin is well known to induce vascular relaxation, the physiological significance of circulating insulin in vascular control is uncertain. Activation of eNOS is generally a calcium-dependent process (16). The vasorelaxation effect of acetylcholine is mediated via muscarinic receptors that promote the release of calcium from intracellular stores. This raises the intracellular free calcium concentration, enabling calcium-calmodulin to bind to eNOS, displacing caveolin-1 and activating eNOS (17).

Recently, it was demonstrated that inhibition of PI 3-kinase by wortmannin attenuated acetylcholine-induced dilation of the basilar artery *in vivo* (18), raising the hypothesis that cholinergic agonists may also signal through classical tyrosine kinase pathways. However, acetylcholine acts through a G protein-coupled receptor (GPCR), which does not have intrinsic tyrosine kinase activity. Previous reports demonstrated that other hormones that act through GPCRs, including angiotensin (ANG) II, vasopressin, and LH can induce tyrosine phosphorylation of cytoplasmic proteins, including insulin receptor substrates (19 22). ANG II activates JAK2, a member of the JAK family, and probably uses this kinase to induce several intracellular protein tyrosine phosphorylations.

In this study we evaluated the ability of acetylcholine to activate JAK2 and to induce the tyrosine phosphorylation of IRS-1, as well as IRS-1/PI 3-kinase association and the phosphorylation of Akt and eNOS in rat aorta. We also compared the activation of IRS-1/PI 3-kinase/Akt/eNOS pathway in response to both acetylcholine and insulin in aorta from normal rats, and from an animal model of diet-induced obesity and insulin resistance i.e. rat fed on a high-fat diet for 60 days.

Research Design and Methods:

Experimental animals and cells: Eight-week-old male Wistar rats were obtained from the State University of Campinas Central Animal Breeding Center (Campinas, SP, Brazil), divided into two groups with similar body weights (230±25g) and assigned to receive two types of diet: a standard rodent chow or a high-fat diet for 60 days. The high-fat diet totals 5.4kcal/g (carbohydrate,38.5%; protein,15%; fat,46.5%) as opposed to the 3.8kcal/g (carbohydrate,70%; protein,20%; fat,10%) of the standard chow diet (23). Animals were allowed free access to diet and water *ad libitum*. At the end of the diet period, body weight, epididymal, perirenal and mesenteric fat were weighed. Food was withdrawn 12h before the experiments. Blood samples were taken for measuring plasma concentration of total cholesterol, high-density lipoprotein (HDL)-cholesterol, triacylglycerol (TG), insulin, and plasma glucose (24;25). Rabbit aortic endothelial cells (26) were maintained in F12 medium (Cultilab, Campinas, SP, Brazil) containing 1.2 g/L sodium bicarbonate, 2.5 mmol/L Lglutamine, 15 mmol/L HEPES and 0.5 mmol/L sodium pyruvate and upplemented with 10% fetal bovine serum (FBS, Cultilab), 100 µg/ml gentamycin (Cultilab), 100 IU/ml penicillin (Cultilab), and 100 mg/ml streptomycin (Cultilab) at 37 °C in a humidified atmosphere of 5% CO_2. All experiments involving animals were approved by the Ethics Committee at the State University of Campinas.

Materials: All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), except anti-phospho-Akt_{ser473}, which was obtained from New England Biolabs (Beverly, MA, USA). Human recombinant insulin was from Eli Lilly and Co. (Indianapolis, IN, USA).

Routine reagents were from Sigma Chemical Co. (St. Louis, MO, USA) unless specified elsewhere. [125I]Protein A was from Amersham (Little Chalfont, UK). Protein A-Sepharose MB was from Pharmacia Biotech (Uppsala, Sweden). Nitrocellulose (BA85;0.2µm) was from Schleicher&Schuell (Keene, NH, USA). The PI 3-kinase inhibitor LY294002 (Calbiochem, San Diego, CA, USA) was dissolved in dimethyl sulfoxide and further diluted in saline (dimethyl sulfoxide 0.1%). The JAK2 inhibitor tyrphostin AG490, and the calcium ion chelator EGTA were from Calbiochem and Sigma Chemical Co., respectively. L-[^{3}H]arginine (1mCi) was obtained form PerkinElmer Life Sciences and cGMP assay kits were from Cayman (Ann Arbor, MI).

Tissue extraction and immunoprecipitation: Rats were anesthetized with sodium thiopental (100mg/kg bw) and used 10–15min later. As soon as anesthesia was assured by the loss of pedal and corneal reflexes, the thoracic cavity was open, thoracic aorta was isolated and kept for 45min at 37°C in Krebs-bicarbonate buffer equilibrated with 95%O_2:5%CO_2, pH7.4, before being stimulated with insulin or acetylcholine. It was then frozen with liquid N_2, powdered with a glass Dounce homogenizer on ice for at least 80 strokes, and immediately homogenized in extraction buffer, as described elsewhere (15). Extracts were centrifuged at 30,000 x g and 4°C for 45min to remove insoluble material.

Protein analysis by immunoblotting: The precipitated proteins and/or whole-tissue extracts were treated with Laemmli sample buffer and then subjected to SDS-PAGE in a Bio-Rad miniature slab gel apparatus (Mini-Protean), as previously described (15;27).

JAK2 in vitro kinase activity assay: The protein kinase activity of the immunoprecipitates was measured as previously described (28).

Hyperinsulinemic-euglycemic clamp: After 5h fasting, 6 animals from each group were anesthetized *ip* with sodium pentobarbital (50mg/kg bw), catheters were then inserted into the left jugular vein (for glucose and insulin infusion) and carotid artery (for blood sampling), as previously described (29).

Blood pressure measurement. Blood pressure measurements in conscious rats were conducted by a tail-cuff system (MKIV, Narco BioSystems, Austin, TX, USA) (30).

Vascular reactivity studies: In these studies, control and obese rats were anesthetized with halothane (Cristália Laboratories, Itapira, Brazil) and killed by decapitation. The chest of each rat was opened and the entire descending thoracic aorta was excised quickly. Tissues were prepared as previously described (31). After 1h of stabilization period, the rings were precontracted with phenylephrine (1µmol/L). Cumulative concentration-response curves to insulin, acetylcholine (ACh) or glyceryl trinitrate (GTN) were obtained in intact aortic rings. Concentration-response curves to GTN were also constructed in endothelium-denuded aortic rings. In these rings, the endothelium was removed mechanically by rubbing the lumen of the aorta with a closed pair of fine-tipped forceps. The absence of the endothelium was confirmed by the loss of a relaxant response to acetylcholine at the start of the experiments. Relaxations were plotted as percentages of the contraction induced by phenylephrine. In another experiment, aortic rings were incubated for 30 min with specific inhibitors AG490 (10 umol/L) or LY294002 (50 umol/L) before being stimulated with Ach in order to study the effects of blockade of JAK2 or PI 3-kinase, respectively, over Ach induced vascular relaxation.

L-[^{3}H]arginine to L-[^{3}H] citrulline conversion assay

Determination of eNOS catalytic activity in intact cells was performed by measurement of the conversion of L-[^{3}H]arginine to to L-[3H]citrulline as previously described (32). The cells were treated with acetylcholine (10^{-5}M) or insulin (10^{-7}M) for 15 min, and in selected experiments cells were pre-incubated with LY294002 or AG490 for 30 minutes. Under all conditions employed, the L-[^{3}H]citruline generation was fully inhibited by 2 mM nitro-L-arginine methyl ester (L-NAME). In individual experiments, a minimum of three wells was used for each treatment group. All findings were confirmed in at least six independent studies.

Estimation of Endothelium-derived NO

Confluent rabbit aortic endothelial cells in six well plates were equilibrated for 30 min in PSS supplemented with 250 µM 3-isobutyl-1-methylxanthine (IBMX) and 250 µM l-

arginine in the absence of presence of L-NAME or the inhibitor of interest (LY294002 or AG490). Equilibrated cells were then exposed to acetylcholine(10^{-5}M) or insulin(10^{-7}M) for 30 minutes before the cells were lysed by the addition of 6% ice-cold trichloroacetic acid. Cell lysates were then subjected to centrifugation at 12,000 X g for 10 min, and determination of cGMP in supernatants was performed as described (33) in accordance with the kit assay.

Statistical analysis: Experiments were carried out by studying the groups of animals in parallel (animals fed on standard diet *vs.* animals fed on high-fat diet). Data are mean±SEM accompanied by the indicated number of independent experiments. For comparisons, ANOVA was used. The significance level was set at *p* value of less than 0.05. A statistics software was used (Instat v3.05, GraphPAD, U.S.A.).

Results:

Insulin signaling pathway in aorta of control animals: The presence of phosphorylated IRS-1 was detectable 5min after incubation with insulin and was maximal at 15min, and 30min after incubation, IRS-1 tyrosine phosphorylation was nearly undetectable (Fig.1A, *upper panel*). Nitrocellulose membranes of IRS-1 immunoprecipitates were stripped and reblotted with antibodies against p85 (PI 3-kinase) showing that the maximum level of association between these proteins occurred at 15min (Fig.1A, *middle panel*). Insulin was also able to stimulate JAK2 tyrosine phosphorylation 5min after incubation with the hormone, reaching maximal levels at 15min (Fig.1B, *upper panel*). The association between JAK2 and IRS-1 was also investigated (Fig.1B, *middle panel*), revealing the same temporal pattern of insulin-induced JAK2 tyrosine phosphorylation. Insulin treatment did not modulate the expression of IRS-1 and JAK2 (Fig.1A/1B, *lower panels*). Insulin-stimulated Akt serine phosphorylation was maximal 15min after incubation with this hormone (Fig.1C, *upper panel*), without any significant change in Akt protein levels (Fig.1C, *lower panel*). Insulin led to an approximately 4-fold increase above basal state in eNOS serine phosphorylation at 15min (Fig.1D, *upper panel*), without significant changes in eNOS tissue levels (Fig.1D, *lower panel*). It was also observed that insulin was able to stimulate the phosphorylation of ERK1/2 isoforms of MAP kinase, leading them to a

maximum activation by 15-30min (Fig.1E, *upper panel*), without any significant change in protein levels (Fig.1E, *lower panel*). Insulin-stimulated IRS-1 tyrosine phosphorylation was dose-dependent, as well as its association with p85 subunit: they were both detectable after incubation with 10^{-8} mol/L insulin. Maximal effect occurred at 10^{-7} mol/L (Fig.1F).

Acetylcholine signaling pathway in aorta of control animals: Acetylcholine induces JAK2 tyrosine phosphorylation 5min after incubation with this hormone, reaching maximum levels by 15min (Fig.2A, *upper panel*). Next, the association between JAK2 and IRS-1 was evaluated, being maximal at 15min (Fig.2A, *middle panel*). The tissue levels of JAK2 did not change among the samples (Fig.2A, *lower panel*). Acetylcholine was also able to stimulate the tyrosine phosphorylation of IRS-1, as well as its association with PI 3-kinase, in a time-dependent manner (Fig.2B, *upper/middle panels*), without changes in IRS-1 protein expression (Fig.2C, *lower panel*). To test whether JAK2 kinase activity could be stimulated by acetylcholine in rat aorta, we measured the enzyme autophosphorylation *in vitro* by immunoprecipitating aortae extracts (with or without a low concentration of acetylcholine) with α-JAK2, and performed an *in vitro* kinase assay using ATP, as described above. JAK2 kinase activity was increased significantly in aortae extracts after incubation with acetylcholine for 15min, as demonstrated by an increase in JAK2 autophosphorylation (Fig.2C). Acetylcholine also stimulated Akt_{ser473} phosphorylation maximally after 15min (Fig.2D, *upper panel*), without any change in Akt protein levels (Fig.2D, *lower panel*). Acetylcholine resulted in an approximately 5-fold increase in eNOS serine phosphorylation above basal at 15min (Fig.2E, *upper panel*), without changes in eNOS tissue levels (Fig.2E, *lower panel*). Acetylcholine also stimulated the phosphorylation of ERK1/2, leading them to a maximum activation by 15- 30min (Fig.2F, *upper panel*), without any modulation in their protein levels (Fig.2F, *lower panel*). Acetylcholine stimulated IRS-1 tyrosine phosphorylation was dose-dependent, as well as its association with p85 subunit: they were both detectable after the incubation with 10^{-5} mol/L and were maximal with 10^{-4} mol/L, decreasing to about 60% of the maximum with 10^{-3} mol/L acetylcholine (Fig.2G). Similar results were obtained in tissue extracts immunoprecipitated with anti-JAK2 followed by immunoblotting with anti-phosphotyrosine antibodies (Fig.2G, *lower panel*).

Effects of blockade of proximal activators of eNOS: In order to evaluate the importance of JAK-2 or PI 3-kinase over the activation of eNOS, we conducted controlled experiments in aortic tissue with the aid of specific pharmacological inhibitors, respectively AG490 or LY294002 (Fig.3A). ACh-stimulated IRS-1 tyrosine phosphorylation was not affected by the presence of LY294002, but it was almost completely abolished when aortae were pre-incubated with AG490 (Fig.3A, *upper panel*). The presence of AG490, as expected, completely inhibited ACh-stimulated tyrosine phosphorylation of JAK2, whereas LY294002 had no effect on this event (Fig.3A, *next panel*). Treatment of aortae with AG490 or LY294002 abolished acetylcholine-induced Akt and ERK1/2 phosphorylation but only reduced eNOS phosphorylation, suggesting that eNOS can be partially activated by a pathway independent of JAK2/IRS-1/Akt (Fig.3A, *lower panels*).

The endothelial function was then evaluated by the endothelial-dependent vasorelaxation in response to acetylcholine in the presence or absence of AG490 or LY294002 (Fig.3B). The potency of acetylcholine (pEC_{50}: 6.85± 0.01, n=5) was significantly reduced in the presence of AG490 (pEC_{50}: 6.36 ± 0.09, n=7, $p<0.01$) or LY294002 (pEC_{50}: 5.97 ± 0.20, n=6, $p<0.01$). Similarly, the maximal responses produced by acetylcholine (86 ± 2%, n=5) were significantly inhibited after incubation with AG490 (64 ± 6%, n=7, $p<0.05$) or LY294002 (44 ± 3%, n= 6, $p<0.001$).

Since more than one cell type comprises the isolated aorta, we next conducted experiments in cultured aortic endothelial cells. Also, in order to evaluate the relative participation of calcium-dependent and calcium-independent pathways by which ACh can activate eNOS, we incubated endothelial cells with EGTA (a specific intracellular calcium ion chelator) or LY294002 (Fig.3C). The ACh-stimulated tyrosine phosphorylation of IRS-1 was not affected by any of these two inhibitors (Fig.3C, *upper panel*). Treatment of endothelial cells with any of these inhibitors, however, reduced the activation of eNOS by different degrees: LY294002 reduced ACh-stimulation of eNOS serine phosphorylation to ~60% of controls and a greater inhibition (to ~30% of controls) of eNOS was observed when cells were treated with EGTA before being stimulated with ACh (Fig.3C, *lower panel*).

We also investigated in endothelial cells the effects of LY294002 and aG490 on Ach or insulin-induced NOS activity, by the L-[3H]arginine conversion to L-[3H]citrulline, and on

endothelial NO production by the increase in cellular cGMP content. The results showed that acetylcholine had the stronger ability to induce NOS activation and cGMP accumulation, and this effect was reduced by ~50% when the cells are pretreated with LY or AG490 (Fig. 3D and 3E). Insulin also increased NOS activity and cGMP accumulation, but this effect was weaker than acetylcholine. Treatment of cells for 30 minutes with LY294002 completely blocked insulin's effect on cGMP accumulation. However treatment of these cells with AG490 for 30 minutes had no effect on the ability of insulin to activate NOS and induce NO production (Fig. 3D and 3E).

PI 3-kinase and MAP kinase pathways in aorta of obese animals - effect of insulin and acetylcholine: The effect of obesity on acetylcholine-induced IRS-1/PI 3-kinase/Akt/eNOS activation as well as on ERK1/2 MAP kinase isoforms was studied in male Wistar rats fed on a high-fat diet for 60 days. Characteristics of these animals are detailed in Table 1. Animals fed on a high-fat diet for 60 days were significantly heavier than those fed on a standard rodent diet and presented higher contents of visceral adipose tissue in different sites, were also more insulin resistant, and had higher fasting insulin and TG levels than controls. Systolic blood pressure was normal and similar in both groups of animals.

Obese rats presented endothelial dysfunction, with significant reductions in endothelial dependent vasorelaxation in response to both acetylcholine and insulin (Fig.4) when compared to their age-matched controls. Acetylcholine produced a concentration dependent relaxation in endothelium-intact preparations with a pEC_{50} values of 6.85±0.02, whereas in obese rats, there was a significant reduction in the pEC_{50} values (6.59±0.01, $p<0.001$). Furthermore, acetylcholine-induced maximum relaxation was attenuated by 44% in obese rats (86±2% and 48±5%, control *vs.* obese, respectively, $p<0.001$) whereas the relaxations elicited by insulin were reduced by approximately 66% at all concentrations assayed. The potency of the endothelium-independent relaxation produced by GTN was also reduced in intact aortic rings of obese rats (pEC_{50}:7.45±0.04 *vs.* pEC_{50}:7.06±0.04, control *vs.* obese, respectively, $p<0.001$). However, after endothelium denudation, the potency of GTN was potentiated in both groups (pEC_{50}:7.75±0.01 and 7.82±0.01, control *vs.* obese, respectively; data not shown). It has been reported that the endothelium-independent relaxations induced by NO-donors are potentiated in eNOS knockout mice (34) or not affected after

endothelium denudation in rats (35).Our results demonstrate a significant decrease in the potency of GTN in endothelium-intact aorta of obese rats. Nevertheless, it does not involve changes in the VSMC since the relaxation produced by GTN was potentiated in both control and obese groups after mechanic endothelium denudation. Taken together, these data suggest that the impaired relaxations observed herein are related to an endothelial dysfunction.

Insulin-stimulated JAK2 tyrosine phosphorylation was significantly reduced (~60%) in aortae from obese animals when compared to controls, but there was no difference in JAK2 phosphorylation when tissues were incubated with acetylcholine (Fig.5A). Moreover, the association between JAK2 and IRS-1 in aortae from obese rats was also significantly reduced when vessels were incubated with insulin, but not with acetylcholine (Fig.5B). Tissue levels of JAK2 did not vary between the two groups of animals (Fig.5C). There was a great decrease (~70%) in insulin-stimulated IRS-1 tyrosine phosphorylation in aorta from obese animals when compared to controls (Fig.5D). Acetylcholine-stimulated IRS-1 tyrosine phosphorylation was also reduced (~30%) in aortae from obese animals compared to controls (Fig.5D). The insulin- and acetylcholine-stimulated association between IRS-1 and the p85 regulatory subunit of PI 3-kinase were both diminished in aortae from obese rats compared to their controls (Fig.5E). There was no significant difference between the protein concentrations of IRS-1 in aortae from both groups of animals (Fig.5F). Obese animals presented significantly higher basal contents of IRS-1$_{ser307}$ phosphorylation (about 2.5-fold higher) in their aortae when compared to control animals (Fig.5G). Obese rats showed reduced Akt activation after insulin and acetylcholine stimulus (~80%) compared to controls (Fig.6A), with no differences between the protein concentrations of Akt in aortae from these two groups (Fig.6B). The serine phosphorylation of eNOS in aortae from obese rats was significantly reduced both in response to insulin (by ~70%) and also to acetylcholine (by ~60%) (Fig.6C) when compared to controls. eNOS tissue levels in aortae from obese rats was significantly reduced by ~30% compared to controls (Fig.6D). In addition, the ratio of phosphorylation per protein of eNOS was also reduced in obese rats after stimulation with insulin (by ~60%) and ACh (by ~40%). Obese rats showed increased basal tyrosine phosphorylation of ERK1/2, 2.3- fold higher than those found in controls (p<0.001; Fig.6E). After incubation with insulin or acetylcholine, tyrosine phosphorylation

of ERK1/2 was respectively 1.7 and 1.6 times higher in aortae from obese rats than in the control rats ($p<0.001$; Fig.6E). However, obese rats demonstrated ~2.0 times higher protein levels of ERK1/2 in aortae than the control rats ($p<0.001$; Fig.6F), suggesting that increased ERK1/2 tyrosine phosphorylation was due to increased levels of these kinases found in aortae of obese animals.

Discussion:

Here we show that acetylcholine can activate eNOS through IRS-1/PI 3-kinase/Akt pathway in rat aorta and in endothelial cells. Also, we show that, in an animal model of insulin resistance and obesity i.e. rat fed on a high-fat diet for 60 days, acetylcholine and insulin signaling through this pathway in aorta is blunted. This study provides data to the hypothesis that the endothelial dysfunction in aorta of obese rats is related to insulin and acetylcholine resistance.

The results of the present study show that acetylcholine activates JAK2/IRS-1 signaling pathway, as described by others GPCR (36,37), and by inducing IRS-1 tyrosine phosphorylation, is able to activate PI 3- kinase/Akt/eNOS pathway (Figure 7), suggesting another pathway by which acetylcholine can induce eNOS activation. The pharmacological blockade of JAK2 or PI 3-kinase was able to reduce acetylcholine-stimulated eNOS activation/phosphorylation and cGMP accumulation by ~50%, suggesting that this pathway may have an important role in acetylcholine-induced eNOS activity, but certainly is not the only pathway that accounts for this Ach effect. Although insulin also activated JAK2 tyrosine phosphorylation, the pharmacological blockade of JAK2 did not change insulin-induced IRS-1 tyrosine phosphorylation or Akt phosphorylation neither insulin effect on eNOS activity and aorta relaxation, suggesting that JAK2 is not necessary for the insulin action in aorta. Thus, the requirement for insulin to activate IRS system via JAK2 seems redundant and it is corroborated by a recent study in transfected L6 myotubes with siRNA against JAK2 (38). As expected, the effect of acetylcholine in causing eNOS activation, cGMP accumulation and endothelial-dependent vasorelaxation is stronger than insulin.

Tyrosine-phosphorylated IRS-1 can also proceed through the Grb2/SOS and Ras pathway, leading to the activation of ERK1 and 2 (39). The present study demonstrates that acetylcholine induces ERK/MAP kinase activation and that JAK2 has an important role in

this activation. Furthermore, the present results are in accordance with recent evidence that acetylcholine activates ERK/MAP kinase (40;41).

Our results showed impaired relaxation of the aorta induced by insulin or acetylcholine in obese rats as a consequence of endothelial dysfunction. These animals represent a very adequate model of metabolic syndrome, characterized by insulin resistance, central fat depot, increase in TG levels and also by cardiovascular disease demonstrated by endothelial dysfunction. We did not observe hypertension in these animals. Hypertension is not always uniformly observed in diet-induced obesity or in other animal models of insulin resistance with endothelial dysfunction (42;43). It is possible that the rat strain, and mainly the duration of the diet may influence the development of hypertension. Recently, it was demonstrated that insulin resistance and endothelial dysfunction precedes the development of hypertension and in some strains it might take one year or more of high-fat diet to induce hypertension (43;44).

The expression of eNOS is apparently regulated by insulin receptor-mediated PI 3-kinase signaling pathway (6). Selective inhibition of insulin actions on PI 3-kinase pathway in vasculature in insulin-resistant states, with preservation of Ras/Raf/MEK/Erk pathway, has been proposed as an important cause of NO production downregulation leading to an imbalance that could lead to the development of atherosclerosis (14;15). Although there was a reduction in insulin- and acetylcholine-induced IRS-1 tyrosine phosphorylation, the activation of ERK1/2 by both hormones was increased in aorta of obese rats, both due to the increase in ERK1/2 protein expression, and probably also because other substrates of IR and JAK2, such as Shc, may compensate IRS-1 on ERK1/2 activation, as we demonstrated in aorta of another animal model of insulin resistance, the spontaneously hypertensive rat (15).

It has been reported that, in states of insulin resistance and obesity, activation of some serine kinases induces ser307 phosphorylation of IRS-1 (45;46), leading to a decrease in insulin-stimulated PI 3-kinase activity. The reduction in insulin-stimulated IRS-1 tyrosine phosphorylation was associated with an increase in IRS-1$_{ser307}$ phosphorylation in aorta of obese animals. The negative modulation of IRS-1 could make this substrate more refractory to the activation by insulin and also by acetylcholine. The acetylcholine-stimulated activation of JAK2 is not altered in obese rats when compared to controls.

However, the activation of eNOS was reduced both in response to insulin and acetylcholine in obese rats. The reduced activation of eNOS in aorta of obese rats could be due to reduced stimulus through IRS-1/PI 3-kinase/Akt pathway. Furthermore, this pathway may also have a role in the control of eNOS expression, and reduced signaling through IRS-1/PI 3-kinase/Akt may contribute to explain the lower eNOS levels in aorta of obese rats. Our data suggest that reduced eNOS expression and/or activation may have an important role in endothelial dysfunction of this obese animal. An important evidence that IRS-1/PI 3-kinase pathway may be relevant for acetylcholine signaling comes from mice lacking IRS-1 (47). In these animals, the relaxation induced by acetylcholine was significantly reduced in aortic strips of IRS-1 knockout mice compared to controls. Taken together, these previous results and data presented herein, it can be suggested that the inhibition of IRS-1 function could not only represent an unifying mechanistic link between different factors involved in insulin resistance, but also in endothelial dysfunction.

In summary, we have documented here a novel pathway of acetylcholine-stimulated activation of eNOS, involving JAK2/IRS-1/PI 3-kinase/Akt pathway. Besides a normal activation of JAK2 in response to acetylcholine in aorta of obese insulin-resistant rats, the increased IRS-1$_{ser307}$ content could inhibit the ability of IRS-1 to be activated by both insulin and acetylcholine, leading to a reduction in the PI 3-kinase pathway activation, and to a reduced expression of eNOS, producing endothelial dysfunction. These results suggest that in aorta of obese rats there is not only insulin resistance, but also acetylcholine resistance, probably mediated by a common signaling pathway that controls the activity and the protein levels of eNOS.

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Table 1: Metabolic and biochemical characteristics of the experimental animals

	Control	Obese
Body weight (g)	270 ± 20 (*n*=10)	350 ± 30 (*n*=10)*
Bilateral epididymal fat (g/100g)	2.21 ± 0.20 (*n*=10)	3.68 ± 0.32 (*n*=10)*
Bilateral perirenal fat (g/100g)	2.34 ± 0.09 (*n*=10)	2.98 ± 0.15 (*n*=10)*
Mesenteric fat (g/100g)	3.08 ± 0.40 (*n*=10)	5.50 ± 0.50 (*n*=10)*
Blood glucose (mmol/L)	5.72 ± 0.9 (*n*=10)	6.00 ± 0.4 (*n*=10)
Serum insulin (pmol/L)	10.2 ± 1.1 (*n*=10)	21.5 ± 2.6 (*n*=10)*
Serum TG (mmol/L)	0.56 ± 0.09 (*n*=10)	1.61 ± 0.13 (*n*=10)†
Serum cholesterol (mmol/L)	1.55 ± 0.13 (*n*=10)	1.65 ± 0.10 (*n*=10)
Serum HDL (mg/dL)	1.16 ± 0.11 (*n*=10)	1.08 ± 0.07 (*n*=10)
GIR (mg/kg•min^{-1})	23 ± 4 (*n*=6)	8 ± 2 (*n*=6)*
Systolic Blood Pressure (mmHg)	110 ± 2 (*n*=10)	114 ± 4 (*n*=10)

Data are mean±SEM. GIR: steady-state glucose infusion rates obtained from averaged rates of 90-120 min of 10% glucose infusion during hyperinsulinemic-euglycemic clamp procedures. *p<0.01 vs control; †p<0.0001 vs control.

Legends:

Figure 1. Insulin signaling in aorta of control rats - time and dose-response. Aortae were incubated with medium with or without insulin, frozen and powdered as described in Methods section, and extracts were submitted to immunoprecipitation (IP) followed by immunoblotting (IB). A, IP with α-IRS-1 and IB with α-pY (*upper panel*); IP with α-IRS-1 and IB with α-p85 (*middle panel*); IP with α-IRS-1 and IB with α-IRS-1 (*lower panel*). B, IP with α-JAK2 and IB with α-pY (*upper panel*); IP with α-JAK2 and IB with α-IRS-1 (*middle panel*); IP with α- JAK2 and IB with α-JAK2 (*lower panel*). C, IB with α-p-Akt (*upper panel*); IB with α-Akt (lower *panel*). D, IB with α-p-eNOS (*upper panel*); IB with α-eNOS (lower *panel*). E, IB with α-p-ERK1/2 (*upper panel*); IB with α-ERK1/2 (*lower panel*); F, IP with α-IRS-1 and IB with α-pY (*upper panel*); IP with α-IRS-1 and IB with α p85 (*middle panel*); IP with α- IRS-1 and IB with α-IRS-1 (*lower panel*). Blots are representative of six independent experiments each.

Figure 2. Acetylcholine (ACh) signaling in aorta of control rats - time and dose-response. Aortae were incubated with medium with or without acetylcholine, frozen and powdered as described in Methods section, and extracts were submitted to immunoprecipitation (IP) followed by immunoblotting (IB). A, IP with α-JAK2 and IB with α-pY (*upper panel*); IP with α-JAK2 and IB with α-IRS-1 (*middle panel*); IP with α-JAK2 and IB with α-JAK2 (*lower panel*). B, IP with α-IRS-1 and IB with α-pY (*upper panel*); IP with α-IRS-1 and IB with α-p85 (*middle panel*); IP with α-IRS-1 and IB with α-IRS-1 (*lower panel*) C. JAK2 tyrosine activity measured by autophosphorylation *in vitro*. Thoracic aortae were incubated with (+) or without (-) a very low concentration of acetylcholine (10^{-5}M) and used 15 min later (maximum JAK2 tyrosine phosphorylation in aorta), to stimulate partial JAK2 autophosphorylation. JAK2 was immunoprecipitated (2 μg/mL) and allowed to autophosphorylate *in vitro* in the presence (+) or absence (-) of exogenous ATP. Tyrosine phosphorylation was measured by immunoblotting with αPY (1 μg/mL). D, IB with α-p Akt (*upper panel*); IB with α- Akt (lower *panel*). E, IB with α-p-eNOS (*upper panel*); IB with α-eNOS (lower *panel*). F, IB with α-p-ERK1/2 (*upper panel*); IB with α-ERK1/2 (*lower panel*). G, IP with α-IRS-1 and IB with α-pY (*upper panel*); IP with α-IRS-1 and IB with α-p85 (*middle panel*); IP with α- IRS-1 and IB with α-IRS-1 (*lower panel*); IP with α

JAK2 and IB with α-pY (*lowest panel*). Blots are representative of six independent experiments each.

Figure 3. Effects of pharmacological inhibitors on Ach signaling and vascular reactivity in aortae, and on Ach-stimulated eNOS phosphorylation and NOS actitivy in endothelial cells. A, fresh aortae were incubated with medium with or without acetylcholine (10^{-4}mol/L) and specific pharmacological inhibitors (LY294002 50 µmol/L or AG490 10 µmol/L) frozen and powdered as described in Methods section, and extracts were submitted to immunoprecipitation (IP) followed by immunoblotting (IB). IP with α IRS-1 and IB with α- pY (*upper panel*); IP with α-JAK2 and IB with α-pY (*middle panel*); IB with α-p-Akt (*middle panel*); IB with α-p-eNOS (*middle panel*) and. IB with α-p-ERK1/2 (*lower panel*) B. Vascular reactivity in the aortic rings of control rats pre-incubated with LY294002 50 µmol/L or AG490 µmol/L. Experimental values were calculated relative to the maximal changes from the contraction produced by phenylephrine in each tissue, which was taken as 100%. The pEC_{50} values for acetylcholine (ACh) were determined as -log of the molar concentration to produce 50% of the maximal relaxation in phenylephrine-contracted tissues. C, rabbit endothelial cells (REC) were incubated with medium with or without acetylcholine (10^{-4} mol/L) and specific pharmacological inhibitors (LY294002 50 µmol/L or EGTA 5 µmol/L), homogenized and extracts were submitted to immunoprecipitation (IP) followed by immunoblotting (IB). IP with α-IRS-1 and IB with α-pY (*upper panel*); IB with α-p-eNOS (*lower panel*). Intact cells (REC) were pretreated with vehicle, LY294002 or AG490 for 30 minutes and then stimulated with insulin or Ach for 15 minutes and the L-[3H]arginine conversion to L-[3H]citrulline was assessed (D). REC were pretreated with the same blockers and then incubated with insulin or Ach for 30 minutes, and cGMP accumulation was determined (E).

Data are shown as the percentage of relaxation of six independent experiments, expressed as mean±SEM. *$p<0.001$ treated *vs.* control, #$p<0.05$ treated *vs.* control, § $p<0.05$ Ach *vs* Ach plus inhibitors

Figure 4. Vascular reactivity in the aortic rings. Experimental values were calculated relative to the maximal changes from the contraction produced by phenylephrine in each

tissue, which was taken as 100%. The pEC_{50} values for acetylcholine (ACh) and glyceryl trinitrate (GTN) were determined as -log of the molar concentration to produce 50% of the maximal relaxation in phenylephrine-contracted tissues. Data are shown as the percentage of relaxation of six independent experiments, expressed as mean±SEM. *p< 0.001, #p<0.01, §p<0.05, obese *vs*. control.

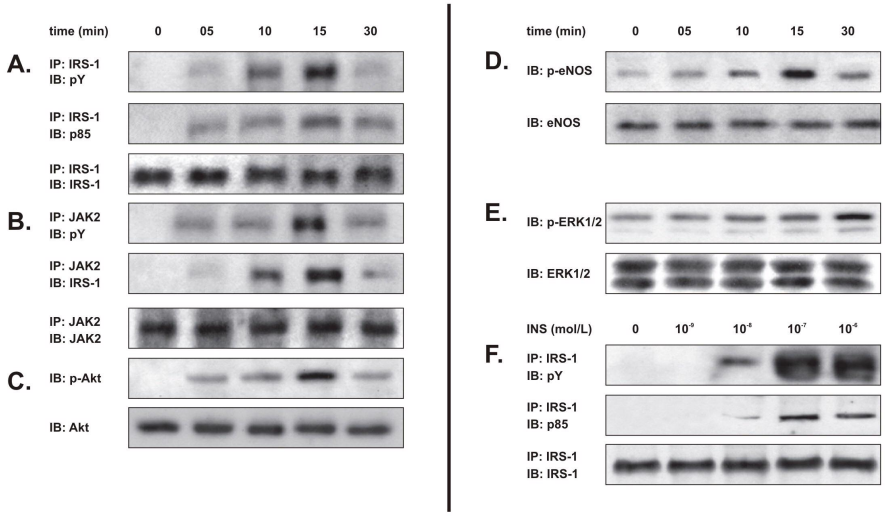
Figure 5. Insulin signaling in aorta of control and obese rats. Aortae were incubated with medium with or without insulin (10^{-7}mol/L) or acetylcholine (10^{-4} mol/L), frozen and powdered as described in Methods section, and extracts were submitted to immunoprecipitation (IP) followed by immunoblotting (IB). A, IP with α-JAK2 and IB with α-pY. B, IP with α-JAK2 and IB with α-IRS-1. C, IP with α-JAK2 and IB with α JAK2. D, IP with α-IRS-1 and IB with α-pY. E, IP with α-IRS-1 and IB with α-p85. F, IP with α-IRS- 1 and IB with α-IRS-1. G, basal IRS-1_{ser307} phosphorylation (IB with α- IRS 1_{ser307}). The results of scanning densitometry are shown and indicate arbitrary units relative to phosphorylation (or association) / protein level. Values are mean±SEM of six independent experiments each. *p< 0.001, insulin-stimulated obese *vs*. insulin-stimulated control; #p<0.001, acetylcholine-stimulated obese *vs*. acetylcholine-stimulated control.

Figure 6. Insulin signaling in aortae of control and obese rats. Aortae were incubated with medium with or without insulin (10^{-7}mol/L) or acetylcholine (10^{-4}mol/L), frozen and powdered as described in Methods section, and extracts were submitted to immunoprecipitation (IP) followed by immunoblotting (IB). A, IB with α-p-Akt. B, IB with α-Akt. C, IB with α-p-eNOS. D, IB with α-eNOS. E, IB with α-p-ERK1/2; F, IB with α-ERK1/2. The results of scanning densitometry are shown and indicate arbitrary units relative to phosphorylation / protein level. Values are mean±SEM of six independent experiments each. *p< 0.001, insulin-stimulated obese *vs*. insulin-stimulated control; #p<0.001, acetylcholine-stimulated obese *vs*. acetylcholine-stimulated control.

Figure 7. Alternative pathway trough JAK2/IRS-1/PI3K/AKT by which acetylcholine can induce eNOS activation

1

Insulin



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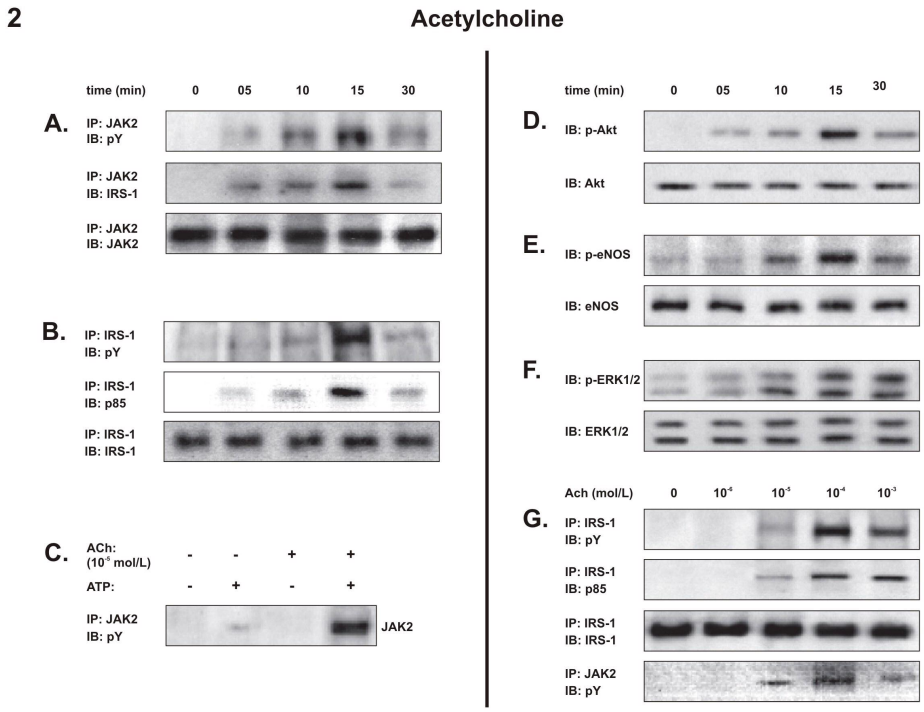
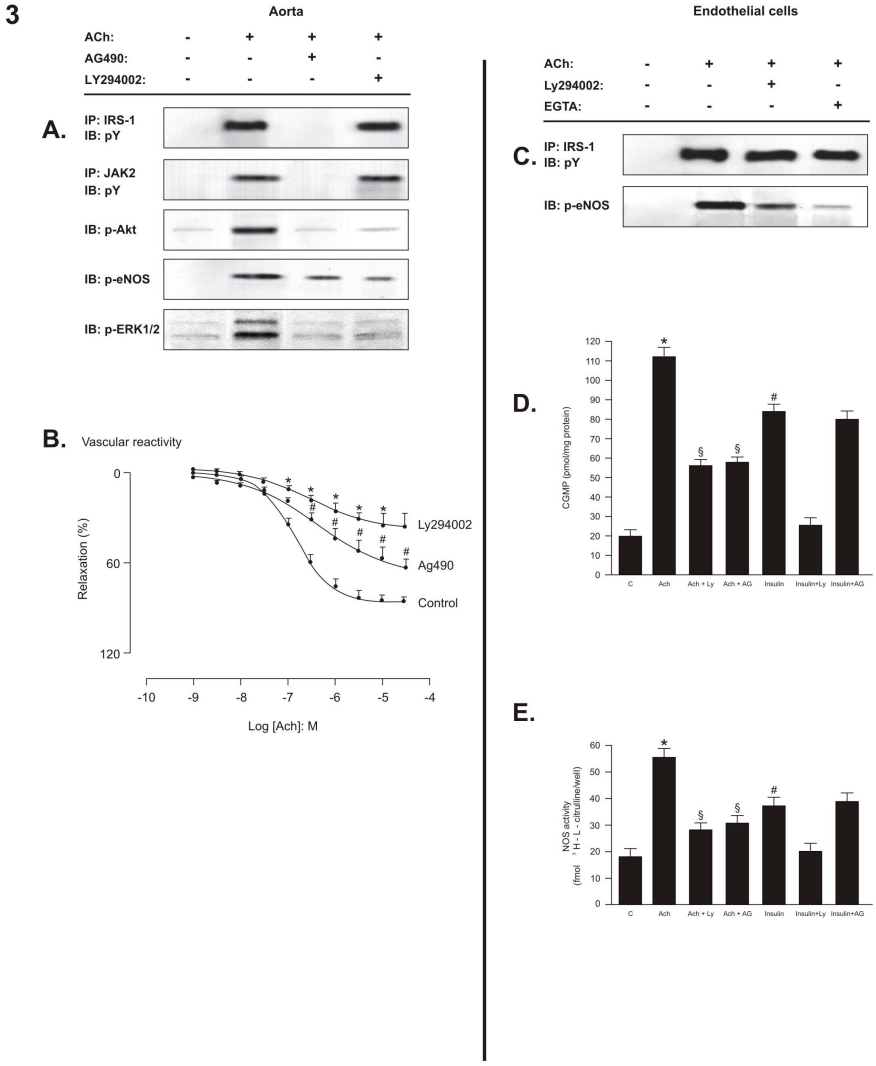
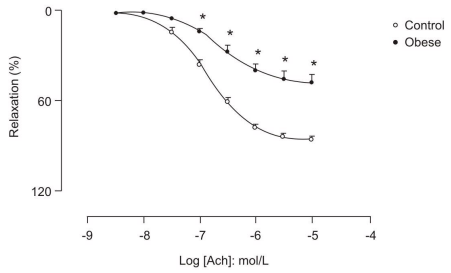
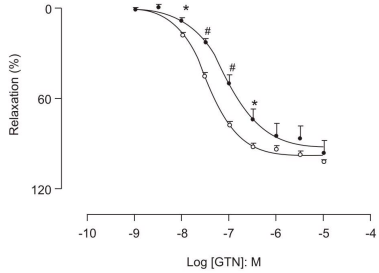

Acetylcholine
time (min) 0 05 10 15 30
A.
IP: JAK2
IB: pY
IP: JAK2
IB: IRS-1
IP: JAK2
IB: JAK2
B.
IP: IRS-1
IB: pY
IP: IRS-1
IB: p85
IP: IRS-1
IB: IRS-1
C.
ACh:
(10-5 mol/L) - - + +
ATP: - + - +
IP: JAK2
IB: pY
JAK2
time (min) 0 05 10 15 30
D.
IB: p-Akt
IB: Akt
E.
IB: p-eNOS
IB: eNOS
F.
IB: p-ERK1/2
IB: ERK1/2
Ach (mol/L) 0 10-6 10-5 10-4 10-3
G.
IP: IRS-1
IB: pY
IP: IRS-1
IB: p85
IP: IRS-1
IB: IRS-1
IP: JAK2
IB: pY

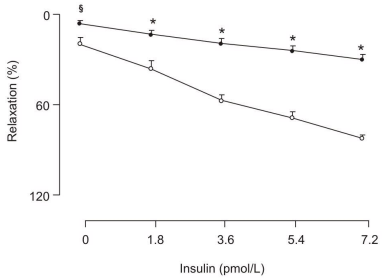
3


Aorta
ACh: - + + +
AG490: - - + -
LY294002: - - - +
A.
IP: IRS-1
IB: pY
IP: JAK2
IB: pY
IB: p-Akt
IB: p-eNOS
IB: p-ERK1/2
B.
Vascular reactivity
Relaxation (%)
0
60
120
Ly294002
Ag490
Control
-10 -9 -8 -7 -6 -5 -4
Log [Ach]: M
Endothelial cells
ACh: - + + +
Ly294002: - - + -
EGTA: - - - +
C.
IP: IRS-1
IB: pY
IB: p-eNOS
D.
CGMP (pmol/mg protein)
C Ach Ach + Ly Ach + AG Insulin Insulin+Ly Insulin+AG
E.
NOS activity
(fmol ³H-L-citrulline/well)
C Ach Ach + Ly Ach + AG Insulin Insulin+Ly Insulin+AG

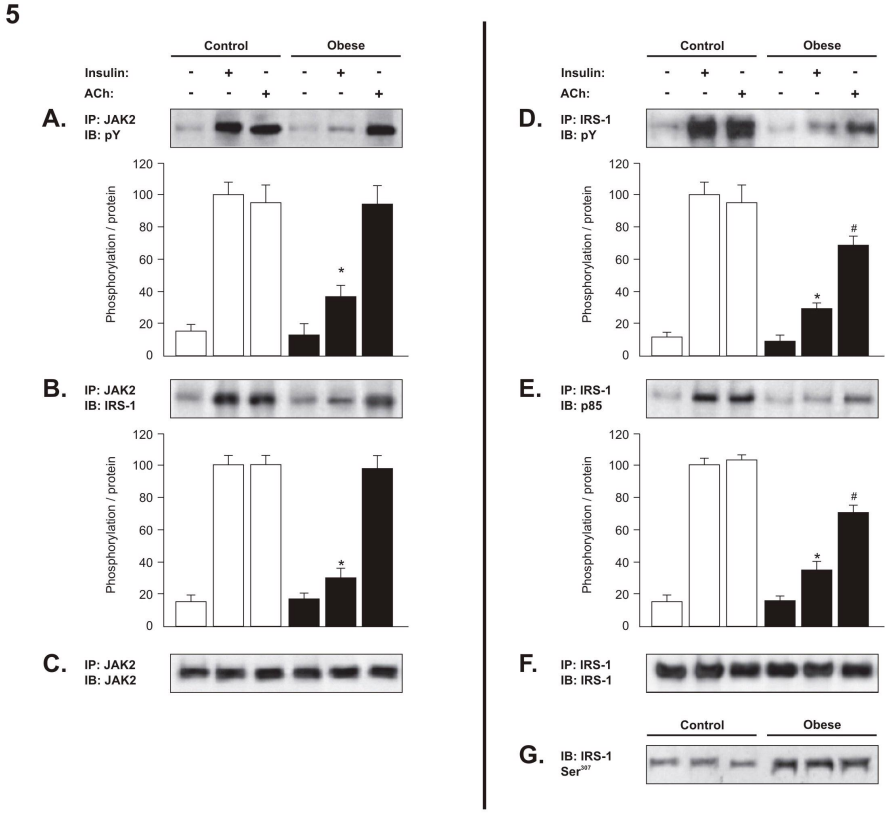
4


Control
Obese
Relaxation (%)
0
60
120
-9
-8
-7
-6
-5
-4
Log [Ach]: mol/L

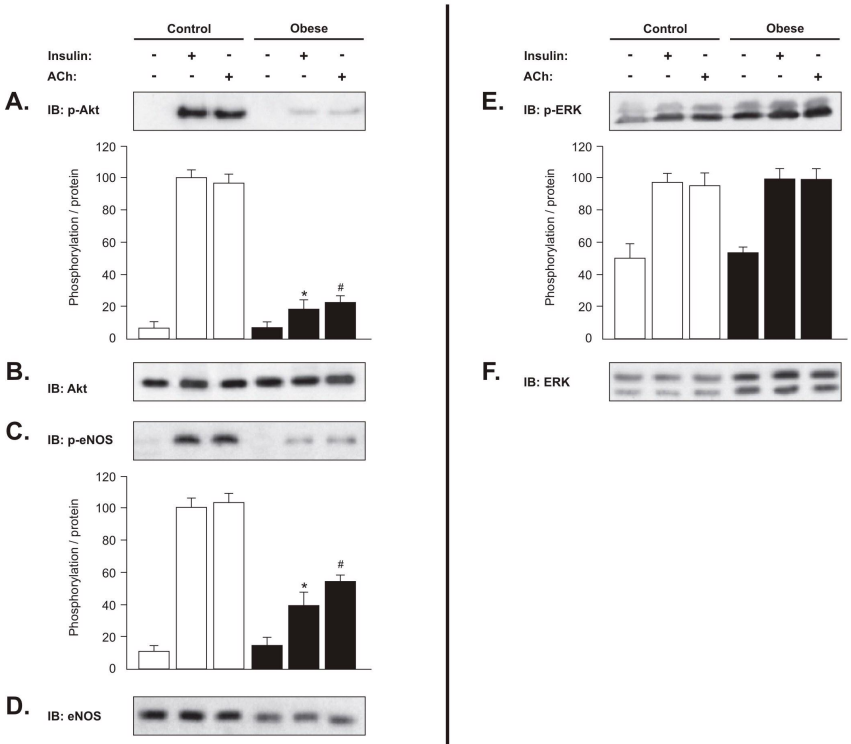

Relaxation (%)
0
60
120
-10
-9
-8
-7
-6
-5
-4
Log [GTN]: M


Relaxation (%)
0
60
120
0
1.8
3.6
5.4
7.2
Insulin (pmol/L)

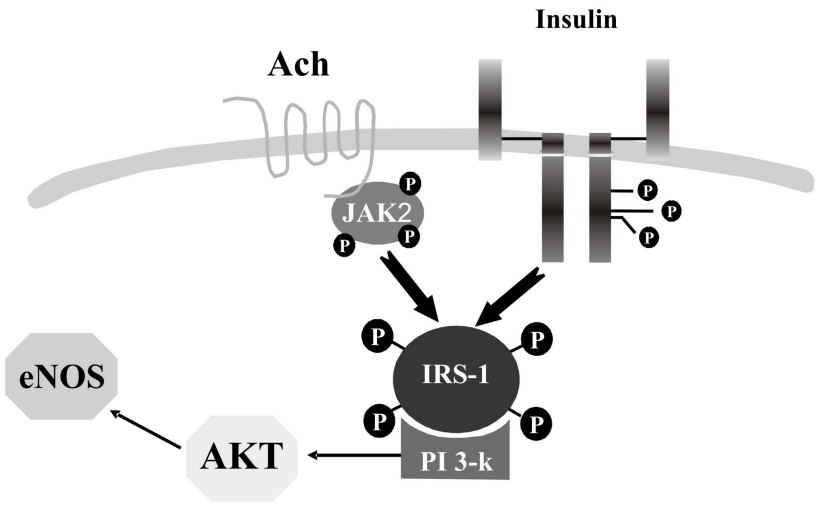
5



6



7



4- DISCUSSÃO

A insulina desencadeia seus processos metabólicos e de crescimento celular inicialmente através de um receptor de membrana específico (Cuatrecasas, 1972; Freychet et al., 1971; Kahn, 1985). A ligação da insulina à subunidade α extracelular implementa a atividade tirosina-quinase do receptor contra ele mesmo (Patti & Kahn, 1998), gerando resíduos de fosfotirosina na subunidade β do receptor, os quais atuam como sítios de reconhecimento para substratos intracelulares, como as quatro proteínas da família IRS (White, 1998) e outros substratos, incluindo Gab-1, $p60^{dok}$, Cbl, APS e isoformas da Shc (Pessin et al., 2000). Essas proteínas são fosforiladas em resíduos de tirosina pelo receptor de insulina, podendo então estabelecer interações não-covalentes com proteínas adaptadoras com domínios SH2, os quais apresentam alta afinidade pela fosfotirosina (Backer et al., 1992; Folli et al., 1993; Kuhne, Pawson, Lienhard, & Feng, 1993; Pawson AJ, 1998), como a subunidade regulatória p85 da enzima fosfatidilinositol (PI) 3-quinase e uma proteína adaptadora intracelular conhecida por Grb-2. A ligação dos substratos do receptor a essas proteínas adaptadoras com domínios SH2 pode regular suas atividades ou, em alguns casos, sua localização subcelular.

A PI 3-quinase tem papel fundamental nas ações metabólicas e mitogênicas da insulina (Shepherd et al., 1998). A inibição da PI 3-quinase bloqueia a maior parte das ações metabólicas da insulina, incluindo a estimulação do transporte de glicose e a síntese de glicogênio e lipídios. Essa enzima catalisa a fosforilação de fosfoinositídeos na posição 3 para produzir fosfatidilinositol-3-fosfatos, especialmente $PtdIns(3,4,5)P_3$, os quais promovem o direcionamento da Akt para a membrana celular para que esta possa ser alvo da enzima PDK1, uma das serina quinases que fosforilam e ativam a serina/treonina quinase Akt (Alessi et al., 1997). Tem sido sugerido que a ativação da Akt tenha papel importante nas ações da insulina sobre o crescimento celular e sobre o metabolismo de glicose e proteínas.

Foi demonstrado, em células endoteliais de veia umbilical humana (HUVEC), que a Akt pode fosforilar e ativar diretamente a eNOS em dois resíduos de serina (1177 e 1179), levando ao aumento da produção endotelial de óxido nítrico através da conversão de L-arginina em L-citrulina e NO (Dimmeler et al., 1999; Fulton et al., 1999). O óxido nítrico é um determinante fundamental da homeostase cadiovascular, participando da regulação da

pressão arterial sistêmica, da remodelação vascular, da angiogênese e da agregação plaquetária (Moncada et al., 1993; Murohara et al., 1998; Rudic et al., 1998). A redução na biodisponibilidade do óxido nítrico é uma característica de pacientes com doença coronariana (Zeiher, 1996) e está associada ao desenvolvimento de lesões ateroscleróticas (Moroi et al., 1998).

Como ocorre com outros fatores de crescimento, a insulina também é capaz de estimular uma via que leva à modulação de uma família de serina/treonina quinases conhecida por MAP quinase, componentes principais de vias controladoras da embriogênese e da diferenciação, proliferação e morte celulares (Pearson et al., 2001). A ativação de vias de sinalização intracelulares por fatores de crescimento, hormônios e neurotransmissores é mediada através de duas MAP quinases muito relacionadas, p44 e p42, denominadas ERK1 e ERK2, respectivamente. Esta via se inicia com a fosforilação em tirosina de proteínas IRS e/ou Shc, as quais interagem com a proteína adaptadora Grb2, recrutando a proteína SOS (*Son-of-sevenless*) para a membrana plasmática para ativação da Ras. A ativação da Ras também requer a estimulação da tirosina fosfatase SHP2, através da interação com substratos do receptor, como Gab-1 ou IRS-1/2. Uma vez ativada, a Ras opera estimulando uma cascata de serina quinases com a ativação seqüencial da Raf, MEK e ERK. As proteínas ERK1/2 são reguladas por dupla fosforilação em resíduos específicos de tirosina e treonina dentro de uma seqüência característica *Thr-Glu-Tyr* (TEY). Em resposta à ativação, estas duas MAP quinases podem fosforilar outras proteínas em serina e treonina. A ERK ativada também pode se translocar para o núcleo, onde catalisa a fosforilação de fatores de transcrição como $p62^{TCF}$, iniciando uma série de eventos que culminam com a proliferação ou diferenciação celulares (Boulton et al., 1991). O bloqueio desta via com mutantes dominantes negativos ou inibidores farmacológicos previne o crescimento celular estimulado pela insulina, mas não tem repercussão sobre as ações metabólicas do hormônio (Lazar et al., 1995). Embora modelos de animais que não expressam alguns componentes desta via (como Raf-1, B-Raf e MEK1) apresentem um desenvolvimento embrionário anormal do sistema vascular, incompatível com a vida, camundongos que não expressam o gene ERK1 são normais sob vários aspectos fenotípicos, sem alterações na angiogênese ou no desenvolvimento placentário (Giroux et al., 1999; Pages et al., 1999; Wojnowski et al., 1997; Wojnowski et al., 1998).

Assim, após estímulo com insulina, o receptor de insulina pode ativar duas vias intracelulares no endotélio: uma via (PI 3-quinase / Akt / eNOS) que leva ao aumento da produção vascular de óxido nítrico e, portanto, apresenta potencial anti-aterogênico, e outra via (Ras / Raf / MAP quinase), associada ao crescimento de células vasculares e à expressão de proteínas da matriz extracelular (Xi, Graf, Goetze, Hsueh, & Law, 1997), com potencial pró-aterogênico.

Apesar das vias de transmissão do sinal de insulina terem sido muito estudadas no fígado, músculo e tecido adiposo, estas vias foram pouco estudadas no tecido vascular. O estudo da sinalização de insulina no vaso se justifica pela grande associação existente entre os estados de resistência à insulina, hipertensão e aterosclerose. Os protocolos apresentados neste trabalho permitiram caracterizar as vias de transmissão do sinal de insulina na aorta torácica *in vivo* e *in vitro* após estímulo agudo com este hormônio e imunoprecipitação e *immunoblotting* de extratos teciduais com anticorpos específicos.

O primeiro modelo animal de resistência à insulina escolhido foi o **rato Wistar com 12 meses de idade**, o qual foi estudado paralelamente ao rato com 2 meses de idade. Estes animais apresentaram, com o envelhecimento, ganho de peso e maior resistência à insulina, caracterizada por hiperinsulinemia e por uma menor velocidade de desaparecimento de glicose após teste de tolerância à insulina curto. Os animais com 2 e 12 meses de idade mantiveram-se normoglicêmicos e normotensos, com medidas não-invasivas de pressão arterial sistólica basal iguais às dos animais jovens. Para avaliar a presença ou não de aterosclerose nos animais envelhecidos, foi realizado o processamento e análise morfológica de cortes seriados de suas aortas torácicas e não foram observadas hipertrofia e hiperplasia de células musculares lisas e nem alterações estruturais compatíveis com aterosclerose nas aortas de animais com 12 meses. Assim, nesse modelo animal, a resistência à insulina não foi acompanhada de hipertensão arterial sistêmica ou aterosclerose.

Os mecanismos moleculares da resistência à insulina no fígado e músculo de ratos envelhecidos já foram estudados (Carvalho et al., 1996). Os animais envelhecidos apresentaram, em relação aos animais jovens, redução na fosforilação insulino-estimulada do IRS-1 em resíduos de tirosina no músculo, assim como redução na fosforilação da Akt

em serina; redução dos níveis protéicos de IRS-1 no músculo; redução na associação insulino-estimulada entre IRS-1 e PI 3-quinase no músculo. Como a ativação da via da PI 3-quinase pela insulina está ligada a funções metabólicas, como transporte de glicose no músculo e tecido adiposo (Cheatham et al., 1994; Haruta et al., 1995) e à síntese de glicogênio e proteínas no fígado e músculo (Mendez, Myers, Jr., White, & Rhoads, 1996; Shepherd, Nave, & Siddle, 1995), as alterações demonstradas nas vias de sinalização de insulina no músculo podem ter contribuído para a resistência à insulina observada nesses animais.

Neste trabalho foi demonstrado que a insulina tem efeito direto no tecido vascular usando as vias de sinalização comumente descritas em outros tecidos. Os graus de fosforilação insulino-estimulados do receptor de insulina em resíduos de tirosina não diferiram significativamente entre as aortas dos animais com 2 e 12 meses de idade. Os níveis teciduais da subunidade β do receptor também não variaram entre os dois grupos. A fosforilação em tirosina do IRS-1 e a sua associação com a subunidade p85 da PI 3-quinase, após estímulo agudo com insulina, foram idênticas quando comparadas as aortas dos animais jovens e envelhecidos. A quantidade de IRS-1 não variou entre as aortas dos dois grupos de animais. Também não foram observadas diferenças significativas nos graus de fosforilação do IRS-2 em tirosina e na sua associação com a p85, após estímulo com insulina, quando foram comparadas as aortas dos dois grupos de animais; os níveis teciduais de IRS-2 também não variaram com o envelhecimento e obesidade.

Os graus de fosforilação da Akt em serina, tanto na ausência de estímulo quanto após estímulo com insulina, foram idênticos nas aortas de animais com 2 e 12 meses de idade, bem como seus níveis teciduais. A associação insulino-estimulada entre Akt e eNOS não diferiu significativamente entre as aortas destes dois grupos de animais estudados. Provavelmente em conseqüência dessa associação normal, a fosforilação basal e insulino-estimulada da eNOS em serina também foi idêntica nas aortas dos animais jovens e envelhecidos. Os níveis protéicos de eNOS também não variaram com o envelhecimento e obesidade nesse modelo animal de resistência à insulina.

A seguir, foram avaliados os efeitos do envelhecimento e da obesidade sobre a ativação e quantidade das proteínas ERK1 e ERK2 nas aortas dos animais com 2 e 12 meses de idade. Observou-se que animais com 12 meses apresentam um aumento da

fosforilação basal (sem estímulo insulínico) dessas duas isoformas da MAP quinase da ordem de 2,2 vezes o valor basal avaliado em animais com 2 meses de idade. Após estímulo com insulina, as proteínas ERK1/2 apresentaram maiores graus de fosforilação em tirosina nas aortas dos animais envelhecidos. A variação da fosforilação da ERK1/2, ou seja, antes e após o estímulo com insulina, acompanhou o aumento dos níveis teciduais de ERK1/2 na aorta dos animais envelhecidos, aproximadamente 2 vezes a quantidade de proteína encontrada nas aortas dos animais jovens.

Estes dados estão parcialmente de acordo com Jiang e colaboradores (Jiang et al., 1999a), os quais demonstraram que, em cultura de microvasos extraídos de epidídimo de ratos Zucker obesos, há aumento da fosforilação basal de ERK1/2 (2 vezes); porém, após estímulo com insulina, o grau de fosforilação encontrado em ratos magros e obesos era idêntico. Durante *clamp* euglicêmico, entretanto, o autor não observou fosforilação em tirosina da MAP quinase na aorta e fígado destes animais, atribuindo a falta de resultados positivos nestes tecidos à dificuldade de isolar o *pool* da MAP quinase especificamente ativado pela insulina nos estudos de *clamp*.

A inativação ou atenuação do estímulo na MAP quinase é mediado por uma classe de proteínas fosfatases que inclui a MKP-1 (*MAP quinase fosfatase-1*), também conhecida por CL100, Erp e hVH-1 (Sun, Tonks, & Bar-Sagi, 1994). Em culturas primárias de células musculares lisas vasculares de ratos espontaneamente hipertensos (SHR), Begum et al (Begum, Ragolia, Rienzie, McCarthy, & Duddy, 1998) observaram indução deficiente na MKP-1 atribuída a um bloqueio na via da PI 3-quinase/NOS após estímulo com insulina, levando a uma ativação sustentada da MAP quinase e crescimento celular acelerado. Nos ratos Wistar aqui estudados, uma redução na atividade da MKP-1 no vaso poderia contribuir para o aumento da fosforilação da MAP quinase, embora isso não possa ser atribuído a um bloqueio da via da PI 3-quinase.

Ao contrário da ativação da PI 3-quinase, pouco se conhece sobre a ativação da ERK1/2 induzida pela insulina em condições de resistência à insulina *in vivo*. Os dados aqui apresentados são os primeiros na literatura a demonstrar que a insulina estimula o complexo MAP quinase na aorta de ratos *in vivo* e que estas proteínas estão hiperativadas nos animais adultos em relação aos jovens, tanto na ausência quanto na presença de

estímulo, possivelmente devido ao aumento de seus níveis teciduais associados ao envelhecimento e obesidade.

A ativação da via da MAP quinase pela insulina não está reduzida em diabéticos tipo 2, possivelmente permitindo que a hiperinsulinemia crônica exerça efeitos deletérios sobre o crescimento das células musculares lisas dos vasos (Cusi et al., 2000). No entanto, no modelo animal de resistência à insulina aqui apresentado, o aumento da ativação e dos níveis teciduais da MAP quinase na aorta não foi associado à aterosclerose e hipertensão, sugerindo que provavelmente a preservação da via da PI 3-quinase / Akt / eNOS no vaso contribuiu para evitar o desenvolvimento de doença cardiovascular nestes animais.

Nas artérias de indivíduos diabéticos, a disfunção endotelial parece envolver tanto uma resistência à insulina específica na via da PI 3-quinase quanto a hiperglicemia (Hsueh & Law, 1998; Jiang et al., 1999b). A resistência seletiva na via da PI 3-quinase resultaria em redução na produção endotelial da molécula anti-aterogênica de óxido nítrico. Por outro lado, a manutenção ou aumento da atividade da via da MAP quinase poderia levar a uma maior proliferação das células musculares lisas vasculares e maior produção de PAI-1 (Hsueh et al., 1998).

Os mecanismos moleculares da regulação tecido-específica da sinalização de insulina no rato Wistar com 12 meses de idade, o qual apresenta resistência à insulina mas não desenvolve doença cardiovascular, podem estar relacionados à expressão de proteínas envolvidas nas etapas iniciais da ação da insulina, aos graus de fosforilação em serina do IRβ e IRSs e à atividade de fosfotirosina-fosfatases. Desta forma, a redução nos níveis teciduais do IRS-1 poderia contribuir para explicar a reduzida transmissão do sinal de insulina através da via da PI 3-quinase no músculo destes animais.

Recentemente foi demonstrado que a fosforilação insulino-estimulada do IRβ e IRS-1/2, bem como a ativação da PI 3-quinase, estão seletivamente reduzidas no tecido vascular de ratos Zucker obesos resistentes à insulina (Jiang et al., 1999a). Como o rato Zucker obeso é um modelo experimental que associa obesidade e *diabetes* à doença cardiovascular, como a hipertensão arterial sistêmica (Carlson, Shelton, White, & Wyss,

2000), poder-se-ia supor que doenças cardiovasculares associadas à resistência à insulina poderiam ser parcialmente explicadas por anormalidades na via da PI 3-quinase nos tecidos vasculares, e que os ratos Wistar com 12 meses de idade não apresentaram doença cardiovascular, mesmo na presença de resistência à insulina acentuada, porque a via da PI 3-quinase / Akt / eNOS está preservada na aorta destes animais.

Para avaliar esta hipótese, estudamos as vias de sinalização de insulina na aorta de um modelo animal de resistência à insulina e doença cardiovascular, o **rato espontaneamente hipertenso (SHR)**. Nestes animais, a via IRS-1 / PI 3-quinase / Akt apresentou-se menos ativada no músculo esquelético e na aorta em comparação ao observado em seus controles (WKY), o que pode ter contribuído, respectivamente, para a resistência à insulina e a disfunção endotelial nestes animais. Em alguns estados de resistência à insulina, a vasodilatação desencadeada pela insulina poderia estar reduzida como conseqüência da menor ativação da via da PI 3-quinase (Scherrer, Randin, Vollenweider, Vollenweider, & Nicod, 1994; Steinberg et al., 1994). Além disso, foi demonstrado que a fosforilação em tirosina do receptor de insulina e do IRS-1, bem como a ativação da PI 3-quinase, estão seletivamente reduzidas no vasos de ratos Zucker obesos e resistentes à insulina (Jiang et al., 1999a). Ainda, o camundongo que não expressa o receptor de insulina no endotélio (VENIRKO) apresenta expressão reduzida de eNOS (Kondo et al., 2003; Vicent et al., 2003), sugerindo que a resistência à insulina nos vasos está acompanhada da redução nos níveis teciduais desta enzima.

O modelo SHR apresentou, além da menor ativação da via da PI 3-quinase no vaso, maior expressão e ativação das proteínas da MAP quinase, semelhante ao observado nos animais adultos com 12 meses. No entanto, no rato com 12 meses, esse achado não foi acompanhado de doença cardiovascular, sugerindo que a ativação isolada desta via de crescimento, com a preservação da via da PI 3-quinase / Akt / eNOS, pode não ser suficiente para o desenvolvimento de doença cardiovascular nestes animais. Mais ainda, a preservação da via da PI 3-quinase no vaso pode ser fundamental para a ativação normal da eNOS e da produção de NO e, conseqüentemente, para a proteção vascular.

No segundo artigo, foi estudada outra via promotora do crescimento celular e potencialmente pró-aterogênica, a via da JAK2/STAT, na aorta de outro modelo animal de resistência à insulina e doença cardiovascular, o **rato com obesidade induzida por dieta**

hiperlipídica e hipercalórica (DIO), um modelo de síndrome metabólica. A ativação das proteínas STAT por diferentes fatores de crescimento e hormônios, incluindo PDGF, EGF, VEGF e ANG II (Bartoli et al., 2000; Marrero et al., 1995; Marrero et al., 1997; Ruff-Jamison et al., 1994; Ruff-Jamison, Chen, & Cohen, 1995) pode ser importante na etiopatogenia do crescimento celular acelerado observado na glomerulosclerose diabética (Andersen et al., 2000; Andersen, Tarnow, Rossing, Hansen, & Parving, 2000) e na aterosclerose (Madamanchi et al., 2005; Watanabe et al., 2004). Foi demonstrado que a insulina estimula a ativação da JAK2 e posteriormente a fosforilação da STAT3 e STAT5a/b na aorta de animais controles e obesos. No entanto, na aorta dos animais obesos, tanto a via da MAP quinase quanto a via JAK2/STAT encontraram-se hiperativadas, principalmente devido à maior expressão destas proteínas, em comparação à menor ativação da via da PI 3-quinase /Akt / eNOS. O desequilíbrio gerado entre as vias anti- e pró-aterogênicas estimuladas pela insulina, novamente, pode ser um fator relevante na patogênese da doença cardiovascular associada à resistência à insulina.

No **terceiro trabalho** foi demonstrada a capacidade da acetilcolina (ACh) de ativar a via da PI 3-quinase / Akt / eNOS na aorta de ratos e em cultura de células endoteliais. Também foi demonstrado que, no rato com obesidade induzida por 60 dias de dieta hipercalórica e hiperlipídica, a sinalização nesta via está reduzida tanto após estímulo agudo com insulina quanto com acetilcolina. Os resultados deste estudo demonstraram que a ACh, de forma semelhante a outros estímulos ativadores de receptores acoplados a proteínas G (GPCR) (Carvalho et al., 2003; Thirone, Carvalho, & Saad, 1999), ativa a via de sinalização JAK2/IRS-1, e desta forma é capaz de promover a fosforilação em tirosina do IRS-1, ativando a via PI 3-quinase / Akt / eNOS. Esta é uma nova via de sinalização, cálcio-independente, através da qual a ACh pode ativar a eNOS. O bloqueio com inibidores farmacológicos específicos da JAK2 (AG490) ou da PI 3-quinase (LY294002) foi capaz de reduzir a ativação/fosforilação da eNOS e o acúmulo de GMP cíclio após estímulo com ACh em ~50%, sugerindo que esta via tem importância na ativação da eNOS estimulada pela ACh, embora não seja exclusiva, ocorrendo em paralelo com a ativação clássica dependente de cálcio/calmodulina. Embora a insulina também ative a JAK2, o bloqueio farmacológico da JAK2 não alterou a fosforilação em tirosina do IRS-1 ou a fosforilação da Akt, nem mesmo o relaxamento vascular em resposta à insulina, sugerindo que a sua

ativação é redundante e desnecessária para a ação da insulina na aorta. Este efeito é corroborado por um estudo recente em miotúbulos L6 transfectados com siRNA contra a JAK2 (Thirone, JeBailey, Bilan, & Klip, 2006).

A fosforilação em tirosina do IRS-1 também ativa a via Grb2/SOS/Ras, ativando por fim as isoformas ERK1 e 2 da MAP quinase (Pelicci et al., 1992). Neste estudo demonstrou-se que a ACh ativa a MAP quinase, de acordo com estudos recentes (Guo, Kumahara, & Saffen, 2001; Tsuneki et al., 2004) e que a JAK2 participa desta ativação.

Os resultados apresentados também demonstraram menor relaxamento de anéis de aorta torácica de ratos obesos após estímulo com insulina ou ACh. Além da disfunção endotelial, estes animais apresentaram resistência à insulina, maior adiposidade visceral medida em diferentes locais no abdômen e elevação de triglicérides, embora não sejam hipertensos. Isto torna este modelo adequado para o estudo da síndrome metabólica. A presença de pressão arterial normal também ocorre em outros modelos de resistência à insulina e disfunção endotelial (Kondo, Vincent, Ilany, King, & Kahn, 2002; Roberts et al., 2005). Outros fatores podem influenciar o desenvolvimento da hipertensão arterial, como a cepa do animal e a duração da dieta.

A expressão da eNOS é aparentemente regulada, de forma contínua, pelo estímulo na via da PI 3-quinase desencadeado pela insulina (Kuboki et al., 2000). O modelo SHR também apresentou, em paralelo à redução na ativação da via IRS-1 / PI 3-quinase / Akt / eNOS na aorta, redução acentuada nos níveis teciduais de eNOS. A inibição seletiva da via da PI 3-quinase no vaso do rato obeso, acompanhada do aumento da expressão de proteínas da via de crescimento MAP quinase, provavelmente contribuiu para a ocorrência de doença cardiovascular no modelo DIO, assim como foi observado no SHR.

Em alguns estados de resistência à insulina e obesidade, a ativação de algumas serina-quinases pode promover a fosforilação do IRS-1 em ser307 (Aguirre, Uchida, Yenush, Davis, & White, 2000; Aguirre et al., 2002; Prada et al., 2005). A fosforilação do IRS-1 em serina é inibitória, reduzindo a sua capacidade de ser fosforilado em tirosina e conseqüentemente a sua capacidade de se associar à PI 3-quinase. Na aorta do rato obeso, a

redução na fosforilação do IRS-1 em tirosina estimulada por insulina foi acompanhada por aumento na fosforilação do IRS-1 em ser307, reduzindo a sua capacidade de ser ativado pela insulina e ACh. A ativação da JAK2 em resposta à ACh na aorta de animais obesos não foi diferente da observada em animais normais. No entanto, a ativação da eNOS foi significativamente reduzida nos ratos obesos em resposta tanto à insulina quanto à ACh. A menor ativação da eNOS na aorta dos animais obesos pode ser decorrente do menor estímulo através da via IRS-1 / PI 3-quinase / Akt pois, como já mencionado, esta via pode ser importante para controlar a expressão da eNOS. A redução da ativação e dos níveis teciduais da eNOS na aorta do rato obeso pode contribuir para explicar a disfunção endotelial deste animal. Camundongos que não expressam IRS-1 também apresentam menor relaxamento vascular em resposta à ACh que seus controles (Abe et al., 1998b). Assim, a inibição da função do IRS-1 pode representar um mecanismo que unifica diferentes fatores envolvidos tanto na resistência à insulina quanto na disfunção endotelial.

Os dados apresentados sugerem que a disfunção endotelial na aorta de ratos obesos está relacionada à resistência à insulina e à acetilcolina no endotélio.

5- CONCLUSÕES

- A insulina pode ativar diferencialmente as vias da PI 3-quinase e da MAP quinase no músculo esquelético e no vaso *in vivo*.

- Ratos Wistar machos com 12 meses são obesos, resistentes à insulina, normoglicêmicos e normotensos.

- As alterações na transmissão do sinal de insulina no músculo (redução na ativação da via da PI 3-quinase / Akt) podem contribuir para explicar a resistência à insulina neste modelo animal.

- O aumento da expressão de proteínas do complexo MAP quinase não resultou em hipertrofia e hiperplasia, nem em alterações estruturais na aorta torácica de ratos Wistar com 12 meses.

- A preservação da via IRS / PI 3-quinase / Akt / eNOS provavelmente contribuiu para evitar o desenvolvimento de doença cardiovascular no animal adulto obeso e resistente à insulina.

- Ratos espontaneamente hipertensos (SHR) apresentam resistência à insulina associada à hipertensão arterial e disfunção endotelial, na ausência de dislipidemia.

- A menor ativação da via IRS / PI 3-quinase / Akt / eNOS na aorta dos SHR em face à maior ativação da via da MAP quinase provavelmente contribuiu para o desenvolvimento de doença cardiovascular neste modelo.

- Ratos com obesidade induzida por dieta hiperlipídica e hipercalórica apresentam resistência à insulina e disfunção endotelial.

- Em ratos com obesidade induzida por dieta, além da maior ativação da via da MAP quinase, outra via potencialmente pró-aterogênica, a via da JAK2 / STAT, também está hiperativada, concomitantemente à redução da ativação da via IRS / PI 3-quinase / Akt / eNOS.

- A acetilcolina ativa a via IRS-1 / PI 3-quinase / Akt / eNOS através da JAK2 na aorta de ratos normais e obesos.

- Há redução da ativação das proteínas da via IRS-1 / PI 3-quinase / Akt / eNOS em resposta ao estímulo colinérgico e à insulina na aorta do rato com obesidade induzida por dieta.

- A redução da ativação da via da PI 3-quinase no vaso de obesos pode ser decorrência da maior fosforilação do IRS-1 em resíduos de serina associada à redução dos níveis teciduais de eNOS.

- A menor ativação da via Akt / eNOS pode favorecer o desenvolvimento de disfunção endotelial no rato com obesidade induzida por dieta.

- Em animais obesos (DIO), resistentes à insulina e que apresentam disfunção endotelial, ocorre não apenas resistência à insulina na via da PI 3-quinase no vaso, como também resistência à acetilcolina.

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