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MECANISMO MOLECULAR DE AÇÃO DO HORMÔNIO DO CRESCIMENTO (GH) NO ANIMAL INTACTO: EFEITOS DO ENVELHECIMENTO, NO CRESCIMENTO RENAL E DO ANTAGONISTA DO GH, G120K-PEG

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DEDICATÓRIA

Aos meus pais, Celso e Rita, ao meu marido André Luiz e ao meu filho João Arthur

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**LISTA DE ABBREVIATURAS**

- Akt: serina/treonina quinase
- ATP: trifosfato de adenosina
- CHO: células de ovário de hamster
- c-fos: proto-oncogene
- c-jun: proto-oncogene
- CRC: crescimento renal compensatório
- DAG: diacilglicerol
- DNA: ácido desoxirribonucleico
- EGF: fator de crescimento epidérmico
- ERKs: quinasas reguladoras da sinalização extracelular
- G120K-PEG: antagonista do GH ligado a polietileno-glicol
- GDP: difosfato de guanosina
- GH: hormônio do crescimento
- GHBP: proteína ligante do GH
- GHR: receptor do hormônio do crescimento
- GLUT4: proteína transportadora de glicose
- GRB2: proteína ligante do receptor para fator de crescimento
- GTP: trifosfato de guanosina
- IGF-1: fator de crescimento semelhante à insulina
- IGF-BP: proteína ligante de IGF
- IL: interleucina
- IR: receptor de insulina
- IRS-1: substrato-1 do receptor de insulina
- IRS-2: substrato-2 do receptor de insulina
- JAK1 e 2: quinasas da família Janus
KDa  quilo Daltons
MAPK  quinase ativadora da atividade mitogênica
NO  óxido nítrico
INOS  sintetase de óxido nítrico
PDGF  fator de crescimento derivado de plaquetas
PEG  polietileno-glicol
PI3K  fosfatidilinositol 3-quinase
PKC  proteína quinase C
Raf  serina quinase citoplasmática ativada pelo Ras e ativadora das MAPKs
Ras  proteína originalmente identificada como um oncogene, tem participação na regulação do metabolismo e crescimento celular
RIE  radioimunoensaio
RNA  ácido ribonucléico
SDS-PAGE  gel de poliacrilamida para eleetroforese dodecil sulfato de sódio
SHC  molécula adaptadora e substrato do receptor de insulina
SH2  segunda homologia ao Src
SH3  terceira homologia ao Src
SHP2  fosfotirosina fosfatase ativada pelo IRS-1 fosforilado
SOS  fator ativador do Ras, apresenta homologia à Drosophila son-of-sevenless
Src  oncogene originalmente definido como produto da sarcoma vírus Rous
STAT  sinalizador transmissor/ativador da transcrição
STZ  estreptozotocina
Unx  uninefrectomia
RESUMO
O hormônio do crescimento (GH) exerce ações somatotróficas e também interfere em muitos aspectos do metabolismo. As ações somatotróficas são desempenhadas pelo próprio GH e pela geração de um outro fator de crescimento, a IGF-1 (insulin-like growth factor-1). Estudos mostram que o GH exerce uma ação inicial no metabolismo de carboidratos semelhante à insulina (“insulina-like”). A fim de exercer seus efeitos biológicos, o GH deve ser reconhecido e especificamente ligado ao seu receptor de membrana (GHR) das células-alvo. O GHR não contém um sítio tirosina quinase intrínseco, entretanto a ligação do GH a seu receptor determina estimulação da atividade tirosina quinase do JAK-2, resultando em fosforilação do receptor de GH e do próprio JAK-2. Demonstramos in vivo que, as proteínas IRSs e SHC servem como moléculas sinalizadoras para o GH em tecidos de ratos em jejum e que ocorre ativação da atividade tirosina quinase do JAK2 em direção ao IRS-1 após estímulo com GH, sugerindo que o IRS-1 possa interagir primariamente com JAK2, o qual seria mediator da fosforilação em tirosina deste substrato.

Verificamos também que G120K-PEG pode modular a transmissão do sinal do GH em camundongos in vivo através da sua capacidade de diminuir as fosforilações em tirosina do JAK2, IRS-1, IRS-2 e SHC estimuladas por este hormônio, sugerindo que este antagonista do GH possa ser uma droga potencial na terapêutica de doenças associadas com os efeitos indesejáveis do GH.

A fosforilações em tirosina induzidas por GH das proteínas JAK2, IRS-1, IRS-2 e SHC nos rins de ratos submetidos a uninefrectomia foi semelhante àquela dos animais-controle. Estes dados sugerem que nenhuma alteração na transmissão do sinal do GH é necessária para a hipertrofia renal compensatória à nefrectomia unilateral.

As fosforilações das proteínas JAK2, IRS-1, SHC, MAPK e Akt e as associações IRS-1/PI 3 quinase, IRS-1/Grb2 e SHC/Grb2 induzidas por GH estão aumentadas em rins de ratos diabéticos por STZ. Estes dados sugerem que diabetes é caracterizado por aumento na sinalização do GH que pode contribuir para o desenvolvimento da doença renal diabética. Também, administração de G120K-PEG em camundongos diabéticos teve efeitos inibitórios no crescimento renal e no aumento da via de sinalização do GH observada em animais diabéticos. Encontramos alterações nas etapas iniciais da
transmissão do sinal do GH em rins de ratos e camundongos diabéticos e sugerimos que um bloqueador específico do GHR pode prevenir estas alterações e representar um novo conceito no tratamento da doença renal diabética.

As fosforilações estimuladas por GH das proteínas JAK2, IRS-1 e IRS-2 estão diminuídas em tecido adiposo de ratos de 18 meses (senis) quando comparadas às fosforilações destas proteínas em ratos de 6 semanas, enquanto a fosforilação em tirosina da proteína SHC em tecido adiposo de ratos senis após estímulo com GH é semelhante à dos controles. Por outro lado, as fosforilações em tirosina do JAK2, IRS-1, IRS-2 e SHC estimuladas por GH no fígado de ratos senis após estímulo com GH é semelhante à dos controles. Assim, nossos dados sugerem que envelhecimento é caracterizado por distúrbios na sinalização do GH em tecido adiposo que podem contribuir para as alterações neste tecido associadas com envelhecimento normal.
1. INTRODUÇÃO
1.1. TRANSMISSÃO DO SINAL DO HORMÔNIO DO CRESCIMENTO (GH).

O hormônio do crescimento (GH) é necessário para o crescimento linear normal, um fato que está condicionado à sua descoberta, ao seu nome e às principais linhas de pesquisas envolvendo este hormônio conduzidas nos últimos trinta anos após o isolamento do GH (CASANUEVA, 1992). Além dessa ação no crescimento, o GH também interfere em muitos aspectos do metabolismo tendo sido descrito como um hormônio que exerce efeitos anabólicos, lipóliticos e diabetogênicos (DAVIDSON, 1987).

O hormônio do crescimento é constituído por uma cadeia peptídica única de 191 aminoácidos com duas pontes dissulfeto intramoleculares apresentando peso molecular de 22 kDa. O gene humano para o GH está presente no braço longo do cromossomo 17. Com o desenvolvimento das técnicas de radioimunoensaio para dosagem de GH foi possível a mensuração acurada dos níveis séricos deste hormônio durante o transcorrer do dia. Identificou-se então que o hormônio do crescimento não é secretado em uma velocidade constante, mas de forma episódica com picos de secreção intercalados por períodos de nenhuma ou baixa secreção, sendo que os picos secretórios estão claramente relacionados com as fases 3 e 4 do sono (DAUGHADAY, 1992).

O GH é essencial para o crescimento corporal, mas considerando que o crescimento normal acontece por um período relativamente curto da vida, enquanto o GH permanece sendo secretado durante a vida adulta, este hormônio parece estar implicado em outras funções (SALOMON & SONKSEN, 1987). As ações somatotróficas são desempenhadas pelo próprio GH e pela geração de um outro fator de crescimento, a “insulin-like growth factor-1” (IGF-1). Tradicionalmente, o figado foi tido como única fonte de produção de IGF-1 mediada pelo GH, mas posteriormente demonstrou-se que a maioria dos tecidos é capaz de sintetizar IGF-1 (D’ÉRCOLE, STILES, UNDERWOOD, 1984). IGF-1 circulante encontra-se ligado a seis proteínas específicas (IGF-BPs), tendo estas proteínas carreadoras o papel de modular a ação da IGF-1 (CASANUEVA, 1992).

Subsequentemente à demonstração de seus efeitos de crescimento, o GH foi associado a alterações no metabolismo de lípides e carboidratos e na composição corporal. São clássicos os estudos sobre a ação diabetogênica do GH tanto em animais experimentais...
quanto em humanos (HOUSSAY & BIOSATTI, 1932; YOUNG, 1937). Estudos mostram que o GH exerce duas ações antagônicas no metabolismo de carboidratos, isto é, uma ação inicial semelhante à insulina (“insulina-like”) seguida por uma ação diabetogênica de duração bem mais prolongada (DAVIDSON, 1987). As ações “insulina-like” têm uma duração de cerca de duas horas, não são mediadas pela insulina e envolvem aumento no transporte de aminoácidos, aumento no transporte de glicose e lipogênese (DAVIDSON, 1987). A ação diabetogênica pode ser demonstrada pelo menos três horas após a administração de GH e cursa com relevante hiperglicemia a despeito de níveis elevados de insulina (CASANUEVA, 1992).

A fim de exercer seus efeitos biológicos, o GH deve ser reconhecido e especificamente ligado ao seu receptor de membrana (GHR) das células-alvo, uma proteína com baixa capacidade, mas com altas afinidade e especificidade para o hormônio circulante. Os efeitos do hormônio do crescimento nas células responsivas são transmitidos pelo GHR (LEUNG et al., 1987; DE VOS, ULTSCH, KOSSIAKOFF, 1992), uma glicoproteína transmembrânica de cadeia única com 620 aminoácidos que se expressa na superfície de células hepáticas, adiposas, renais, cardíacas, intestinais, pulmonares e musculares (MATHEWS, ENBERG, NORSTEDT, 1989; TIONG & HERINGTON, 1991). O receptor de hormônio do crescimento é composto por três porções: uma porção extracelular constituída por 246 aminoácidos que se liga e é dimerizada por uma única molécula de GH, contém 5 sitos potenciais de “glicosilação” e 7 resíduos-cisteína que permitem o estabelecimento de 3 pontes dissulfeto dentro da cadeia, formando pequenas alças de 10-15 aminoácidos; um segmento transmembrânico curto e uma porção intracelular de 350 aminoácidos que é requerida para a transmissão do eventos intracelulares de sinalização estimulados pelo GH (LEUNG et al., 1987; CUNNINGHAM et al., 1991; COLOSI et al., 1993).

Sabe-se que GHR pertence à bem definida classe de receptores, a superfamília de receptores GH-prolactina-citoquinas (KELLY et al., 1993). Especificamente os domínios extracelulares dos receptores de GH e prolactina estão relacionados com os receptores para interleucina (IL)-2, IL-3, IL-4, IL-5, IL-6 e IL-7, eritropoeitina, fatores estimuladores de colônias de macrófagos-granulócitos, proteína gp 130 e, de forma mais
distante, com receptores de interferon (KELLY, DJIANE, POSTEL-VINAY, 1991). A regulação da expressão do gene do receptor de GH é complexa. Em geral, o nível de RNAm correlaciona-se bem com os sítios relatados de ligação de GH ou imunorreatividade do receptor de GH; o figado e o tecido adiposo contém a mais alta expressão do RNAm para receptor de GH (HUGHES & FRIESEN, 1985). A expressão do receptor é variável dependendo do tecido examinado, e estudo desenvolvido em ratos mostrou que em tecidos hepático e adiposo há alta expressão do receptor de GH, enquanto a expressão é mediana em linfócitos, intestino, coração, rim, pulmão, pâncreas, cartilagem e músculo esquelético; a expressão é baixa em cérebro, glândula mamária e ovário e não detectável no testículo, timo e baço (TIONG & HERINGTON, 1991).

Diferente dos receptores de insulina e IGF-1, o GHR não contém um domínio tirosina quinase intrínseco (LEUNG et al., 1987). Entretanto a ligação do GH a seu receptor determina rápido aumento de fosforilação em tirosina de múltiplas proteínas celulares (CAMPBELL, CHRISTIAN, CARTER-SU, 1993). Vários receptores, dentre eles o da insulina (SAAD et al., 1996), são capazes de se ligar e ativar a proteína JAK-2, que é um membro da família Janus de tirosinas quinases (HARPUR et al., 1992; SILVENNOINEN et al., 1993). Em cultura de células, está bem demonstrado que em reposta à ligação do GH a seu receptor dimerizado também ocorre fosforilação e ativação da proteína JAK-2 (ARGETSINGER et al., 1993; SILVA et al., 1994). A estimulação da atividade tirosina quinase do JAK-2 resulta em fosforilação do receptor de GH e do próprio JAK-2 (WANG et al., 1993; CAMPBELL, CHRISTIAN, CARTER-SU, 1993). Assim, a fosforilação em tirosina de outros substratos celulares é esperada em resposta à ativação do receptor de GH. Desde a identificação do JAK-2 como uma tirosina quinase associada ao receptor de GH, muitas moléculas em adição ao próprio JAK-2 e ao receptor de GH têm sido sugeridas como substratos para o JAK-2. Em particular, membros da família STAT (sinalizador transmissor/ativador da transcrição) de proteínas citoplasmáticas que funcionam como fatores latentes de transcrição têm sido mostrado serem ativadas em reposta ao GH. STATs 1, 3 e 5 são fosforiladas em tirosina após tratamento com GH em cultura de células (MEYER et al., 1994; CAMPBELL et al., 1995; WOOD et al., 1995). As proteínas STAT fosforiladas translocam para o núcleo, se ligam ao DNA e podem ativar a transcrição de genes específicos (WAXMAN et al., 1995).
A existência dos efeitos “insulina-like” do GH sugere que este hormônio possa utilizar algumas moléculas sinalizadoras utilizadas pela insulina. Consistente com isto estão os trabalhos que demonstraram que o GH é capaz de estimular a fosforilação em tirosina do substrato-1 do receptor de insulina (IRS-1) em cultura de células de adipócitos de ratos (SOUZA et al., 1994; RIDDERSTRALE, DEGERMAN, TORNQVIST, 1995) e em fibroblastos 3T3-F442A (ARGETSINGER et al., 1995). Demonstrou-se também que em cultura de fibroblastos 3T3-F442A, o GH estimula a fosforilação em tirosina de outra proteína relacionada a esta primeira, o substrato-2 do receptor de insulina (IRS-2) (ARGETSINGER et al., 1996). As fosforilações em tirosina do IRS-1 e do IRS-2 em resposta à insulina proporcionam sítios de ligação para proteínas específicas que contém um domínio SH2 (homologia 2 ao Src), incluindo a subunidade regulatória de 85 kDa da PI 3-quinase (BACKER et al., 1992; SUN et al., 1993; SUN et al., 1995), SHP2 (KUHNE et al., 1993) e GRB2 (MYERS et al., 1994; CHEATAM & KAHN, 1995). De forma similar, em cultura de células, o GH promove a ligação da subunidade regulatória de 85 kDa da PI 3-quinase ao IRS-1 e IRS-2 fosforilados (RIDDERSTRALE, DEGERMAN, TORNQVIST, 1995; ARGETSINGER et al., 1995; ARGETSINGER et al. 1996). A jusante ao estimulo da PI 3-quinase, ocorre a ativação de uma serina-treonina quinase, Akt, e sua fosforilação parece ser um mecanismo primário através do qual a atividade enzimática é regulada (BURGERING & COFFER, 1995). A ativação da PI 3-quinase parece ser importante para o transporte de glicose estimulado pela insulina (OKADA et al., 1994) e esta enzima é capaz também de ativar o complexo Ras (YAMAUCHI, HOLT, PESSIN, 1993). Ras é uma proteína intracelular com importante papel no controle do crescimento celular e metabolismo. O Ras ativado estimula a fosforilação e ativação da Raf1-quinase que tem sido implicada como estimuladora da atividade das MAPK (proteína quinase ativadora da mitogênese). Assim a PI 3-quinase é mediadora da sinalização que leva à atividade transcripcional de c-fos (YAMAUCHI, HOLT, PESSIN, 1993). Em cultura de fibroblastos 3T3-F442A, o GH rapidamente aumenta a expressão dos proto-oncogenes c-fos e c-jun (GURLAND et al., 1990), e posteriormente, estudos utilizando esta mesma linhagem celular demonstram que o GH estimula a atividade da MAP quinase (CAMPBELL et al., 1992).
Outra via pelo qual as MAP quinases podem ser ativadas pelo GH é através da fosforilação em tirosina de outro substrato proteico, o SHC (homologia ao Src e α-collágeno) (CHEATAM & KAHN, 1995). Esta via de ativação anteriormente descrita para outros receptores de membrana, pode ser também observada para o GHR, já que estudos em cultura de células demonstram que a proteína SHC interage com o complexo GHR-JAK2 através do domínio SH2 do SHC e esta proteína é fosforilada em tirosina pelo JAK2. O SHC fosforilado em tirosina, por sua vez, liga-se à GRB2 tendo como efeito final estimulação das MAP quinases (VANDERKURR et al., 1995). Estes achados indicam que a sinalização pelo GH pode, pelo menos em parte, utilizar a mesma cascata quinase semelhante àquela que tem sido proposta para outros receptores de membrana que apresentam atividade tirosina quinase.

Está bem caracterizado que não são necessárias tirosinas específicas no receptor de GH (GHR) para a fosforilação do IRS-1, IRS-2 e SHC (ARGETSINGER et al., 1995; ARGETSINGER et al., 1996; VANDERKURR et al., 1995) e a natureza da interação do GH estimulado com o IRS-1 parece diferir daquela da interação deste substrato com insulina, IGF-1 e IL-4. Estudos utilizando GHR truncados e mutados expressos em células CHO sugerem que a fosforilação em tirosina do IRS-1, IRS-2 e SHC são dependentes da ativação do JAK-2 (ARGETSINGER et al., 1995; ARGETSINGER et al., 1996; VANDERKURR et al., 1995).

Estudos em cultura de células têm fornecido importantes informações dos mecanismos intracelulares de sinalização do GH, entretanto os achados neste sistema experimental podem não refletir as reações de sinalização que ocorrem em resposta ao GH em tecidos animais intactos, quando os níveis de GH basais não estão suprimidos. Embora um estudo anterior tenha demonstrado que o GH não pode induzir a fosforilação em tirosina do IRS-1 e SHC em tecidos animais in vivo (CHOW et al., 1996), esta falta de resposta pode representar um efeito da hiperinsulinemia, já que estes experimentos foram realizados em animais alimentados, e a fosforilação basal dessas proteínas já se encontravam elevadas antes do estímulo com GH. Assim, não se sabe se o GH induz a fosforilação em tirosina das proteínas IRSs e SHC em tecidos de animais em jejum e se estas proteínas fosforiladas podem se associar com outras proteínas que contêm um
domínio SH2. Este aspecto é interessante porque poderá indicar condições fisiológicas nas quais estas vias possam ser utilizadas e poderá também facilitar o estudo da regulação das reações de sinalização em estados patológicos caracterizados por resistência ou tratamento crônico com GH (THIRONE et al., 1997; THIRONE et al., 1998).

Tendo em vista todas as considerações acima, surge-nos o objetivo primeiro deste estudo que é avaliar se a exposição aguda ao GH estimula a atividade tirosina quinase do JAK2 e também identificar os efeitos deste hormônio na fosforilação em tirosina das proteínas IRS-1, IRS-2, SHC e JAK2 em fígado, coração, músculo, rim e tecido adiposo de ratos in vivo.

1.2. G120K-PEG, UMA ANTAGONISTA DO GH.

O GH que circula no sangue se liga ao seu receptor, uma proteína transmembrânica, pertencente à superfamília de receptores GH-prolactina-citoquinas (KELLY et al., 1993) e que não possui atividade tirosina quinase intrínseca (LEUNG et al., 1987). A estrutura cristalizada do complexo ligante (DE VOS, ULTSCH, KOSSIAKOFF, 1992) mostra que o GH liga-se a duas subunidades idênticas de receptor (hGHBP) para induzir a dimerização que ocorre sequencialmente, isto é, o GH liga-se ao primeiro GHR através do sítio 1 do GH e subsequentemente liga-se ao segundo GHR através do sítio 2 do GH, estabelecendo um complexo GH(GHBP)_2 (CUNNINGHAM et al., 1991).

Assim, após a ligação ao receptor e dimerização do GHR, ocorrem múltiplos eventos de sinalização que podem mediar as ações do GH, incluindo a fosforilação em tirosina de vários polipeptídeos celulares, tornando a produção do complexo ativo GH(GHBP)_2 um passo crítico na mediação dos efeitos biológicos do GH. Demonstrou-se claramente que a terceira hélice-α da molécula de GH é crítica para dimerização do GHR (CUNNINGHAM et al., 1991; CHEN et al., 1991a; CHEN et al., 1991b; FUH et al., 1992; OKADA et al., 1992). A dimerização poderá ser evitada determinando-se mutações na porção da terceira hélice-α correspondente ao sítio 2 que impede o evento de ligação GH:GHR, criando-se assim um antagonista do receptor de GH, chamado G120K-PEG
(CHEN et al., 1995b). G120K-PEG pode também ser conjugado com polímeros de polietileno-glicol (PEG), o que aumenta o volume hidrodinâmico da molécula, retardando o seu clearence sistêmico (ZALIPSKY & LEE, 1992).

Assim, seria interessante verificar os efeitos do G120K-PEG na fosforilação em tirosina de proteínas envolvidas na transmissão do sinal do GH, o que colaboraria para avaliação deste composto como possível droga na terapia de doenças causadas por excesso de GH. Então, o segundo objetivo deste estudo é investigar a capacidade do G120K-PEG em inibir a fosforilação em tirosina do JAK2, IRS-1, IRS-2 e SHC estimulada por GH em fígado de animais in vivo.

1.3. REGULAÇÃO DA TRANSMISSÃO DO SINAL DO GH EM TECIDO RENAL.

1.3.1. Nefrectomia.

O rim é um órgão alvo para um grande número de fatores de crescimento. Vários estudos indicam estes polipeptídeos como causadores de aumento renal e alterações nas funções renais que ocorrem em condições como hipersomatropismo, hipertrofia compensatória e diabetes mellitus. Acredita-se que os fatores de crescimento sejam mediadores das adaptações nas funções renais que ocorrem nas reduções crônicas do tecido funcionante renal e promovam regeneração após uma lesão renal aguda. Embora as adaptações induzidas por estes fatores de crescimento sejam inicialmente salutares, os efeitos do crescimento renal contínuo podem ser mal adaptativos (HAMMERMAN, O’SHEA, MILLER, 1993).

Em animais experimentais, a redução do tecido renal funcionante leva a um crescimento renal compensatório (CRC), bem como um aumento adaptativo nas funções glomerulares e tubulares. Há evidências que sugerem uma ligação causal entre CRC e desenvolvimento de lesões cicatriciais renais (FOGO & ICHIKAWA, 1989), e a caracterização dos mediadores envolvidos na resposta inicial de crescimento renal pode permitir a manipulação e prevenção das subseqüentes lesões cicatriciais. Estudos anteriores sugeriram um papel para o GH e IGF-1 na patogênese do crescimento renal experimental e lesões cicatriciais (STILES, SOSENKO, D’ERCOLE, 1985; DOI, STRIKER, Quaife, 1988).
A nefrectomia unilateral é acompanhada por CRC, com crescimento rápido do rim que se manifesta ao fim de 24-48 horas e envolve hipertrofia e hiperplasia (SEYER-HANSEN, 1978). Considerando que estudos recentes têm sugerido que o CRC pode ser dependente de GH e com conhecimento do mecanismo celular através do qual o GH desencadeia seus efeitos, o terceiro objetivo deste trabalho é avaliar os efeitos da uninefrectomia (Unx) na fosforilação em tirosina induzida por GH das proteínas JAK-2, IRS-1, IRS-2 e SHC em rim contratralateral de ratos in vivo.

1.3.2. Diabetes Mellitus / G120K-PEG


Os efeitos do octreotide, um análogo de longa duração da somatostatina, que é um potente inibidor de GH e IGF-1, têm sido investigados em diabetes experimental e sua administração durante os primeiro 7 dias depois da indução de diabetes eliminou tanto o acúmulo renal inicial de IGF-1, quanto hipertrofias renal e glomerular (FLYVBJERG, et al., 1989). Por outro lado, embora sejam frequentemente observados altos níveis circulantes de GH em pacientes com diabetes mellitus insulino-dependentes (JOHANSEN & HANSEN, 1971; VIGNERI et al., 1976; HAYFORD et al., 1980; DUNGER et al., 1991),

Considerando que o GH possa ter papel na nefropatia diabética, o quarto objetivo deste estudo é dimensionar os efeitos de diabetes induzido por estreptozotocina nas fosforilações induzidas por GH das proteínas JAK-2, IRS-1, SHC, MAPK e Akt e nas associações IRS-1/PI3-quinase, IRS-1/Grb2 e SHC/Grb2 em rins de ratos diabéticos.

A dimerização do receptor de GH é um passo crítico na mediação da atividade biológica do GH. O composto G120K-PEG, conforme anteriormente descrito, é um antagonista do GH, sendo um análogo do GH desenvolvido através de engenharia genética produzido por uma mutação na terceira hélice-α que bloqueia a ação do GH impedindo a dimerização do receptor de GH. Assim, a investigação dos efeitos do G120K-PEG no crescimento e na transmissão do sinal do GH em animais diabéticos complementa o quarto objetivo deste estudo.

1.4. REGULAÇÃO DA TRANSMISSÃO DO SINAL DO GH EM TECIDOS HEPÁTICO E ADIPOSO: EFEITO DO ENVELHECIMENTO.

O envelhecimento biológico está associado a um declínio na síntese protética, função tecidual e aumento na susceptibilidade a doenças (RUDMAN et al., 1990). Estudos prévios indicam que este estado catabólico, característico de animais e homens senis, está associado a uma redução na alta amplitude de secreção de GH, com um concomitante declínio na concentração plasmática de IGF-1 (FLORINI et al., 1981; SONNTAG et al., 1980; RUDMAN et al., 1981; BRESEE, INGRAM, SONNTAG, 1991; KAHLER et al., 1986). Embora o declínio da concentração plasmática de IGF-1 relacionado à idade tenha sido documentado em várias espécies de roedores, primatas não-humanos e humanos,
investigações a respeito do mecanismo responsável pela diminuição da IGF-1 têm sido direcionadas somente para a redução da secreção do GH com a idade. Sabe-se que a secreção de IGF-1 é regulada por pulsos de alta amplitude de GH secretado pela hipófise anterior (TANNENBAUM & MARTIN, 1976; TANNENBAUM & LING, 1984). Estudos anteriores forneceram importantes evidências de que a diminuição na amplitude dos episódios secretórios de GH é o principal fator contribuinte no declínio de IGF-1 e, subseqüentemente, na síntese protéica com o aumento da idade (RUDMAN et al., 1990; SONNTAG, HYLKA, MEITES, 1984; JOHANSON & BIZZARD, 1981). Embora um declínio na secreção de GH seja um importante aspecto no declínio da IGF-1, outros fatos tornam-se relevantes.

Considerando-se que o figado é a maior fonte de IGF-1, estudos em ratos Long-Evans senis demonstraram um grande aumento no número de receptores de GH em tecido hepático comparado àquele de ratos jovens (TAKAHASHI & MEITES, 1987) e, entretanto, o nível plasmático de IGF-1 permaneceu 40 a 50% menor nestes animais idosos. Tem sido levantada a hipótese de que redução na transmissão do sinal através do GHR seja fator contribuinte para um aumento na resistência tecidual ao GH com a idade (XU et al., 1995). Demonstrou-se que a despeito de um aumento na densidade de GHR e da ligação ao GHR no figado de camundongos senis, IGF-1 decresceu dramaticamente com a idade (XU et al., 1995; XU & SONNTAG, 1996). Também se observou um declínio na fosforilação do GHR e do JAK-2 in vitro em figado de camundongos de 31 meses de idade (XU et al., 1995; XU & SONNTAG, 1996). Entretanto, a transmissão do sinal do GH em animais senis in vivo não está ainda caracterizada. Assim, surge-nos o quinto e último objetivo deste estudo que é investigar a existência de possíveis alterações na transmissão do sinal do GH pós-receptor com o evoluir da idade, avaliando a fosforilação em tirosina induzida por GH das proteínas JAK2, IRS-1, IRS-2 e Shc em figado e tecido adiposo de ratos senis in vivo.
2. OBJETIVOS
1. Avaliar se a exposição aguda ao GH estimula a atividade tirosina quinase do JAK2 e também identificar os efeitos deste hormônio na fosforilação em tirosina das proteínas IRS-1, IRS-2, SHC e JAK2 em figado, coração, músculo, rim e tecido adiposo de ratos in vivo.

2. Investigar a capacidade do G120K-PEG em inibir a fosforilação em tirosina do JAK2, IRS-1, IRS-2 e SHC estimulada por GH em figado de animais in vivo.

3. Avaliar os efeitos da uninefrectomia na fosforilação em tirosina induzida por GH das proteínas JAK2, IRS-1, IRS-2 e SHC em rim contralateral de ratos in vivo.

4. Dimensionar as fosforilações induzidas por GH das proteínas JAK-2, IRS-1, SHC, MAPK e Akt e as associações IRS-1/PI3-quinase, IRS-1/Grb2 e SHC/Grb2 em rins de ratos diabéticos por tratamento com estreptozotocina. Caracterizar os efeitos do G120K-PEG no crescimento e na transmissão do sinal do GH também em animais diabéticos.

5. Avaliar a fosforilação em tirosina induzida por GH das proteínas JAK2, IRS-1, IRS-2 and She em figado e tecido adiposo de ratos senis in vivo.
3. TRABALHOS PUBLICADOS/A PUBLICAR
Growth Hormone Stimulates the Tyrosine Kinase Activity of JAK2 and Induces Tyrosine Phosphorylation of Insulin Receptor Substrates and Shc in Rat Tissues

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ABSTRACT
GH stimulates the tyrosine phosphorylation of various cellular polypeptides, including the GH receptor itself, in an early part of the intracellular response. Some of these phosphorylations are catalyzed by a GH receptor-associated kinase identified as JAK2, a member of the Janus family of tyrosine kinases. In cultured cells, GH stimulates the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), IRS-2, and Shc. This study investigated whether GH could cause the tyrosine phosphorylation of IRS-1 and Shc proteins in fasted rat tissues in vivo. GH was administered to fasted Wistar rats via a portal vein, and extracts of different tissues were immunoprecipitated with specific antibodies. GH increased the tyrosine phosphorylation of IRS-1, IRS-2, JAK2, and Shc proteins in the liver, heart, kidney, muscle, and adipose tissue of rats. The roles of these substrates as signaling molecules for GH were further demonstrated by the finding that GH stimulated the association of IRS-1/2 with phosphatidylinositol 3-kinase (PI3K), Grb2, and phosphotyrosine phosphatase and of Shc with Grb2. The correlation between JAK2 tyrosyl phosphorylation and IRS-1 tyrosyl phosphorylation in response to GH together with the results of the in vitro tyrosine kinase assay are consistent with the hypothesis that JAK2 may mediate GH-induced phosphorylation of IRS-1. (Endocrinology 140: 55–62, 1999)

GH regulates important physiological processes, including somatic growth and development, carbohydrate and lipid metabolism, and liver metabolic functions (1). Some of these effects of GH are indirect and are mediated by insulin-like growth factor I (IGF-I) that is produced in the liver in response to GH stimulation, whereas others result from the direct action of GH acting through the GH receptor (GHR) on responsive cells (2, 3). GHR, a transmembrane protein expressed on the surface of liver, adipose, kidney, heart, intestine, lung, and muscle cells (4, 5), is a member of the cytokine/hematopoietin receptor superfamily (6). The GHR lacks intrinsic tyrosine kinase activity (2), but after receptor binding, multiple signaling events occur that may mediate the actions of GH, including the tyrosine phosphorylation of multiple cellular polypeptides and GHR itself (7). In cultured cells, JAK2, a member of the Janus family of tyrosine kinases, is activated after its association with a dimerized GHR in response to hormone binding (8). As a consequence of the kinase activation, GH-stimulated tyrosine phosphorylation of a number of intracellular signaling molecules occurs.

GH is known to have short-term effects that mimic the actions of insulin in tissues that have been deprived of GH, including increased amino acid transport, glucose transport, and lipogenesis (9). The insulin-like effects of GH suggest that GH may use some of the same signaling molecules that are used by insulin. Consistent with this, GH has been shown to stimulate the tyrosyl phosphorylation of insulin receptor substrate-1 (IRS-1) in primary cultures of rat adipocytes (10, 11) and in 3T3-F442A fibroblasts (12), and of a related protein, IRS-2, in 3T3-F442A fibroblasts (13). Tyrosyl phosphorylation of IRS-1 and IRS-2 in response to insulin provides binding sites for specific proteins containing SH2 domains, including the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (14, 15), tyrosine phosphatase SHP2 (16), and Grb2 (17). Similarly, GH promotes the binding of the 85-kDa regulatory subunit of PI3K to IRS-1 and IRS-2 in cultured cells (11–13). In 3T3-F442A fibroblasts and CHO cells expressing rat GHR, GH promotes rapid tyrosyl phosphorylation of other insulin signaling molecules, the 45-, 52-, and 66-kDa splice variants of Shc, as well as the subsequent Grb2 association with Shc proteins (18).

Specific tyrosines in GHR are not required for IRS-1, IRS-2 or Shc phosphorylation (12, 13, 18) and the nature of the GH-stimulated interaction with IRS-1 is likely to differ from the interaction with the receptors for insulin, IGF-I, and IL-1. Studies using truncated and mutated GHRs expressed in CHO cells suggest that tyrosyl phosphorylation of IRS-1, IRS-2, and Shc is dependent on JAK2 activation (12, 13, 18).

Although a recent study has shown that GH cannot induce the tyrosine phosphorylation of IRS-1 or Shc in animal tissues in vivo (19), this lack of response may represent an effect of hyperinsulinemia, because the experiments were performed with fed animals, and the basal phosphorylation of these proteins was already high before GH stimulation. It is not known whether GH induces the tyrosine phosphorylation of IRS-1 and Shc proteins in the tissues of fasted animals and whether these phosphorylated proteins can associate with other proteins containing SH2 domains. This aspect is interesting, because it could indicate physiological conditions...
under which these pathways may be used and may also facilitate the study of the regulation of these signaling reactions in pathological states characterized by GH resistance or chronic GH treatment (20, 21). In this study we examined whether acute exposure to GH could stimulate the tyrosine kinase activity of Jak2 and also assessed the effects of this hormone on IRS-1, IRS-2, Shc, and Jak2 tyrosyl phosphorylation in the liver, heart, muscle, kidney, and adipose tissue of rats in vivo.

Materials and Methods

The reagents for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonylfluoride, aprotinin, diethiothreitol, Triton X-100, Tween-20, and glycerol were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium amoborital was purchased from Eho Lilly & Co. (Indianapolis, IN). Human biobionic GH (Nordotropin) was purchased from Novo Nordisk A/S (Bagvaard, Denmark). [125I]Protein A was obtained from Amersham (Aylesbury, UK), and protein A-Sepharose 4 MB was obtained from Pharmacia Biotech (Uppsala, Sweden). Nitrocellulose (BAE: 0.2 μm) was purchased from Schleicher & Schuell, Inc. (Keene, NH). Male Wistar rats were obtained from the UNICAMP Central Animal Breeding Center (Campinas SP, Brazil). Monoclonal antiphosphorysine antibody (αPY, 4G10) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-IRS-1 (aIRS-1, C-20), anti-IRS-2 (aIRS-2-A-19), anti-Shc (aShc, C-20), and anti-Jak2 (aJak2, H-258), anti-Phospho [α-PY, pS473, 2-8], anti-Shp2 (aShp2, C-18), and anti-Gerb (aGerb, C-23) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animals

Six-week-old male Wistar rats were provided with standard rodent chow and water ad libitum. Food was withdrawn 12-14 h before the experiments.

Methods

Rats were anesthetized with sodium amoborital (15 mg/kg BW, ip) and were used 10-15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein was exposed, and 0.2 ml normal saline (zero time) or GH was injected at a dose of 1.8 mg/kg BW. At 1, 3, 15, 30, and 60 min after GH injection, liver, heart, kidney, muscle, and adipose tissue were removed, minced separately, and homogenized in ice-cold extraction buffer (150 mM Tris-HCl, 100, and 100 mM Tris [pH 7.4] containing 100 mM sodium pyrophosphate, 10 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonylfluoride, and 0.1 μg aprotinin/ml) at 4°C with a Pelletron PTA 20 generator (model PT 10/35, Brinkmann Instruments, Inc., Westbury, NY) operated at maximum speed for 30 sec. The extracts were centrifuged at 30,000 x g for 4°C in a Beckman Coulter, Inc. 70.1 Ti rotor (Faro Alto, CA) for 4 min to remove insoluble material. The supernatant of these tissues was used for immunoprecipitation with aPhospho [α-PY, pS473, 2-8], aJak2, and protein A-Sepharose 4 MB or Protein A/G Plus (Santa Cruz Biotechnology, Inc.).

Protein analysis by immunoblotting

The precipitated proteins were treated with Laemmli sample buffer (22) containing 100 mM dithiothreitol and heated in boiling water bath for 4 min, after which they were subjected to SDS-PAGE (6% biacrylamide) in a Bio-Rad mini-protean slab gel apparatus (Mini-Protein, Bio-Rad Laboratories, Inc., Richmond, CA).

Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protein) as described by Towbin et al. (23) except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high molecular mass proteins. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4°C in blocking buffer (5% nonfat dry milk, 10 mM Tris, 130 mM NaCl, and 0.02% Tween-20). The nitrocellulose blot was incubated with antiphosphosine, anti-PI3K, anti-HSP2, or anti-Gerb antibody; diluted in blocking buffer (0.3% BSA instead of nonfat dry milk) overnight at 4°C and then washed for 60 min with blocking buffer without milk. The blots were subsequently incubated with 2 μCi [125I]Protein A (30 μCi/μl) in 10 ml blocking buffer for 1 h at room temperature and then washed again for 30 min as described above. [125I]Protein A bound to the antiphosphosine and antiphosphotyrosine antibodies was detected by autoradiography using prefiltered Kodak XAR film (Eastman Kodak Co., Rochester, NY) with Cronex Lightning Plus intensifying screens at 80°C for 12-48 h. Band intensities were quantitated by optical densitometry (model GS 500, Hoefer Scientific, San Francisco, CA). The developed autoradiographs were scanned with a commercial software program.

Results

GH stimulates tyrosyl phosphorylation of IRS-1 and the association of this substrate with PI3K, SHP2, and Grb2

GH can induce tyrosyl phosphorylation of IRS-1 in adipocytes (10, 11) and 3T3-F442A fibroblasts (12). To investigate whether IRS-1 was tyrosyl phosphorylated after stimulation by GH, we infused GH into the portal vein of fasted rats and then removed and homogenized the liver and immunoprecipitated the proteins with αIRS-1. These immunoprecipitates were analyzed for tyrosyl phosphorylation by immunoblotting with αPY. The presence of phosphorylated IRS-1 was detectable 1 min after GH infusion and was maximal (12-fold above basal) 3 min after GH injection. By 15 min post-GH, the phosphorylation was 50% of the maximum and remained elevated (3-fold above basal) for up to 60 min after administration of the hormone (Fig. 1A). This increased tyrosyl phosphorylation was also observed 2 h after GH administration (20, 21).

There is a relatively high affinity interaction between IRS-1 and the 85-kDa regulatory subunit of PI3K after insulin stimulation (14), and previous studies demonstrated that in primary cultures of rat adipocytes (11) and in 3T3-F442A fibroblasts (12), GH promotes the binding of PI3K to IRS-1, such that both proteins are coprecipitated by antibodies to either protein. When blots previously immunoprecipitated with αIRS-1 antibody were subsequently incubated with antibodies against the 85-kDa subunit of PI3K, we observed that the intensity of the bands increased after GH stimulation. This increased parallel that in IRS-1 phosphorylation and was consistent with a stable association of IRS-1 and PI3K (Fig. 1B). The association was maximal (8-fold above basal) 5 min after GH infusion and remained elevated (1.2-fold above basal) for up to 60 min after GH administration. SHP2 is an SH2 domain-containing tyrosine phosphatase.
FIG. 1. Time course of GH-stimulated tyrosine phosphorylation of IRS-1 and IRS-2, protein levels, and the association of these substrates with SH2 domain-containing proteins in the liver of normal fasted rats. Liver extracts from rats injected with saline (−; 0 min) or GH (+; 1, 5, 15, 30, and 60 min) were prepared as described in Materials and Methods. Tissue extracts were immunoprecipitated with αIRS-1 (2 μg/ml) and immunoblotted with αPY (1 μg/ml; A). The same blot was incubated with αPI3K (1 μg/ml; B), αGrb2 (1 μg/ml; C), αSHP2 (1 μg/ml; D), and αIRS-1 (1 μg/ml; E). Liver extracts were also immunoprecipitated with αIRS-2 (2 μg/ml) and immunoblotted with αPY (1 μg/ml; F). This same membrane was incubated with αPI3K (1 μg/ml; G), αGrb2 (1 μg/ml; H), αSHP2 (1 μg/ml; I), and αIRS-2 (1 μg/ml; J). These results are representative of six independent experiments.

that associates with the carboxy-terminal tyrosine phosphorylation sites of IRS-1 after insulin stimulation (16). To determine whether SHP2 could interact with IRS-1 in a similar manner after stimulation by GH, the same blots were incubated with antibody against SHP2. There was a clear increase in the association between IRS-1 and SHP2 that reached a maximum (15-fold above basal) 5 min after GH injection (Fig. 1C). This association gradually declined over the next 60 min.

Tyrosyl phosphorylation of IRS-1 in response to insulin provides binding sites for Grb2, a protein linked to mitogenic pathways (17). Similarly, there was a GH-dependent increase in the amount of Grb2 that coprecipitated with IRS-1. This association was maximal (6-fold above basal) 5 min after GH administration and declined to 50% of the maximum after 60 min (Fig. 1D).

To determine the expression of IRS-1 in liver during the time course, we studied the IRS-1 protein levels before and after GH injection. Figure 1E shows that there was no change in protein levels after administration of the hormone.

GH stimulates tyrosyl phosphorylation of IRS-2 and the association of this substrate with PI3K, SHP2, and Grb2.

IRS-2 has substantial structural similarity to IRS-1, including multiple potential tyrosyl phosphorylation sites that can be tyrosyl phosphorylated after stimulation of 3T3-F442A fibroblasts with GH (18). To determine whether IRS-2 was tyrosyl phosphorylated in response to GH, rats received GH via the portal vein, and liver extracts were prepared and immunoprecipitated with αIRS-2 antibody and immunoblotted with αPY. Increased tyrosyl phosphorylation of a protein with a Mr appropriate for IRS-2 (180,000–190,000) was detected within 1 min post-GH, with maximum stimulation...
(10-fold above basal) detected at 3 min (Fig. 1F). IRS-2 tyrosyl phosphorylation subsequently declined to 20% of the maximum after 30 min.

Conserved sites between IRS-1 and IRS-2 include those previously seen to bind PI3K, Grb2, and SHP2. PI3K is known to bind to IRS-2 and is activated after insulin stimulation (15). GH also promotes the association of IRS-2 with both PI3K and SHP2 in 3T3-F442A fibroblasts (13). To examine whether GH could induce the association of PI3K, SHP2, and Grb2 with IRS-2, solubilized proteins from liver stimulated with GH were immunoprecipitated with αIRS-2 antibody and subsequently incubated for different times with αPI3K, αSHP2, or αGrb2. The results showed that GH promoted the association of IRS-2 with PI3K (Fig. 1C), SHP2 (Fig. 1H), and Grb2 (Fig. 1I) and that this response paralleled the increase in IRS-2 phosphorylation.

As with IRS-1, there was no change in the levels of IRS-2 protein in liver after GH stimulation during the time-course experiments (Fig. 1J).

### GH stimulates tyrosyl phosphorylation of Shc in rat liver

As the involvement of Shc in tyrosine kinase signaling pathways appears to require its phosphorylation, and considering that GH has been demonstrated to promote the tyrosyl phosphorylation of Shc proteins in 3T3-F442A fibroblasts (18), we examined whether GH could induce Shc tyrosine phosphorylation in rat liver. Liver extracts were removed and homogenized after portal vein infusion of GH. The solubilized proteins were immunoprecipitated with αShc, and the presence of phosphorylated tyrosines was assessed by Western blotting with αP (Fig. 2A). We have recently demonstrated that the 52-kDa Shc isoform has a higher level of tyrosine phosphorylation than the 46-kDa species when stimulated by insulin (25), probably as a consequence of the higher amounts of the former compared with those of other Shc isoforms in rat tissues. We observed similar results when the tyrosyl phosphorylation of this substrate was induced by GH. Increased tyrosyl phosphorylation of a protein migrating at a M, of approximately 52,000 (appropriate for Shc) was observed within 1 min and was maximal (8-fold above basal) 15 min after the infusion of GH. Shc tyrosine phosphorylation decreased within 30 min and was not different from the basal level at the end of the first hour. There was no detectable tyrosyl phosphorylation in the 46- and 66-kDa Shc isoforms. When the same blot was reprobed with αShc antibody, p52 was the predominant band, and p46 and p66 were barely detected.

Mitogen-activated protein (MAP) kinase activation appears to require the recruitment of Grb2, which has been shown to bind phosphorylated tyrosines in Shc (26). In 3T3-F442A fibroblasts, GH promotes the association of Shc with Grb2 (18). To investigate whether the same effect of GH could be seen in vivo, Shc and associated proteins were immunoprecipitated with αShc and Western blotted with αGrb2. A protein recognized by αGrb2 in Western blots and migrating with the appropriate size for Grb2 (M, 23,000) was precipitated by αShc in a GH-dependent fashion (Fig. 2B). The association of Shc/Grb2 was maximal (12-fold above basal)

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**Fig. 2.** Time course of GH-stimulated tyrosine phosphorylation of Shc, protein levels, and association of this substrate with Grb2 in the liver of normal fasted rats. Liver extracts from rats injected with saline (−, 0 min) or GH (+, 1, 5, 15, 30 and 60 min) were prepared as described in Materials and Methods. Tissue extracts were immunoprecipitated with αShc (2 μg/ml) and immunoblotted with αP (1 μg/ml). A 13 μg/ml; B and αShc (1 μg/ml). C). Those results are representative of four independent experiments.

13 min after GH stimulation and had almost vanished at the end of the first hour.

The effect of GH on Shc protein levels in liver was examined by immunoprecipitation and immunoblotting techniques, using anti-Shc antibody. As shown in Fig. 2C, there was no change in the level of this protein after acute GH stimulation.

**GH stimulates tyrosyl phosphorylation of JAK2 and stimulates JAK2 kinase activity toward IRS-1 in rat liver**

To determine whether GH stimulates the tyrosine phosphorylation of JAK2 in vivo, we performed a time-course experiment after the administration of GH via the portal vein. As shown in Fig. 3A, solubilized proteins from rat liver were immunoprecipitated with αJAK2 and immunoblotted with αP. By 1 min after exposure to GH there was an increase in the phosphorylation of a protein with a M, of 130,000 (appropriate for JAK2). Liver JAK2 tyrosine phos-
GH STIMULATES IRSs AND Shc PHOSPHORYLATION IN VIVO

Fig. 3. A. Time course of GH-stimulated tyrosine phosphorylation of JAK2 in the liver of normal fasted rats. Liver extracts from rats injected with saline (−) or GH (−; 1, 3, 15, 30, and 60 min) were prepared as described in Materials and Methods. Tissue extracts were immunoprecipitated with aJAK2 (2 μg/ml) and immunoblotted with αPY (1 μg/ml). B. Protein levels of JAK2 in liver. The same blots were incubated with aJAK2 (1 μg/ml). C. The association of JAK2 with IRS-1 in rat liver. Rat liver extracts were immunoprecipitated with aJAK2 (2 μg/ml), and the membrane was immunoblotted with aIRS-1 (1 μg/ml). D. JAK2 tyrosine activity measured by autophosphorylation in vitro. Saline (−) or a very low dose of GH (1.8 μg/kg BW; −) was injected into the portal vein of the rat, and the liver was excised 5 min later (maximum JAK2 tyrosine phosphorylation in liver). 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 aPY (1 μg/ml). There was an upper band that was phosphorylated after GH infusion in vitro and addition of ATP in vitro. Reprobing with aIRS-1 (1 μg/ml) data not shown) showed that this band co-migrated with IRS-1. These results are representative of four experiments.

Phosphorylation was maximal (16-fold above basal) 5 min after GH injection, although the level of phosphorylation decreased after 15 min and remained 2-fold above basal at 60 min. Immunoblotting the same membranes with aJAK2 showed that there was no change in the level of JAK2 protein during the time-course experiments (Fig. 3B).

When liver extracts were immunoprecipitated with aJAK2 and blotted with αIRS-1, a band corresponding to IRS-1 was detected in the basal state. After stimulation with GH, there was a 2.3-fold increase in the intensity of this band (Fig. 3C), showing that JAK2 interacts with IRS-1 and forms stable complexes after exposure to GH.

To test whether JAK2 kinase activity could be stimulated by GH, we measured enzyme autophosphorylation in vitro by immunoprecipitating liver extracts (with or without a low dose of GH) with aJAK2 and performed an in vitro kinase assay using ATP, as described above. JAK2 kinase activity was increased significantly in liver extracts after a portal infusion of GH, as demonstrated by an increase in JAK2 autophosphorylation. There was also an upper band that was phosphorylated after GH infusion in vitro and addition of ATP in vitro (Fig. 3D). Immunoblotting with aIRS-1 showed that the latter band corresponded to IRS-1, which was probably associated with JAK2 and was tyrosine phosphorylated by this kinase (data not shown).

GH stimulates tyrosyl phosphorylation of IRS-1, IRS-2, Shc, and JAK2 in heart, kidney, muscle, and adipose tissue of rats

To determine whether the same effects of GH could be observed in other tissues, fragments of heart, kidney, muscle, and adipose tissues were extracted 5 min after the injection of GH and immunoprecipitated with aIRS-1 (Fig. 4A), aIRS-2 (Fig. 4B), aShc (Fig. 4C), and aJAK2 (Fig. 4D). The behavior of these proteins in these tissues was similar to that seen in liver, i.e., an increase in the tyrosyl phosphorylation of IRS-1, IRS-2, Shc, and JAK2 after GH infusion.

Discussion

GH is known to mimic the actions of insulin in cells and tissues that have been deprived of GH (9). These insulin-like effects are transient and include acceleration of glucose transport (27), glycoegenesis (25), glycogenesis (27), antilipolysis (29), and accelerated conversion of pyruvate (30) and other lipogenic precursors to triglycerides (30, 31). The early responses to GH are similar to those of insulin and suggest that both hormones may activate some common signaling pathways. IRS-1 is a major cytoplasmic substrate of the insulin receptor (32), and the results presented here show that GH treatment leads to rapid changes in IRS-1 tyrosine phosphorylation in vivo, in agreement with studies using cultured adipocytes (10, 11) and 3T3-F442A fibroblasts (12). In contrast, in a recent study using liver and muscle from fed animals (19), the tyrosine phosphorylation of IRS-1 did not increase after GH stimulation. This discrepancy may reflect the fact that the high levels of insulin in nonfasted animals maintain IRS-1 sufficiently phosphorylated so that no effect of GH is seen. Despite the high basal phosphorylation of IRS-1 in fed animals, the administration of insulin can still
The results of the present study and those of a recent report on IRS-2 tyrosyl phosphorylation in 3T3-F442A fibroblasts (13) in response to GH suggest that both IRS family members have a role in GH signaling. Clearly, there is some overlap in function between IRS-1 and IRS-2, as both bind PI3K, Grb2, and SHP2 in response to GH. Although no downstream signaling molecules unique to either IRS-1 or IRS-2 have yet been identified, the potential for such molecules exists, because IRS-2 contains nine possible phosphorylation sites not shared with IRS-1, and IRS-1 contains five such sites not shared with IRS-2 (15). Distinct roles for IRS-1 and IRS-2 in GH signaling could arise from variations in the tissue expression of IRS-1 and IRS-2 (15).

In insulin-stimulated cells, the association of PI3K with tyrosyl-phosphorylated IRS-1 (33) and tyrosyl-phosphorylated IRS-2 (15) activates this enzyme. Thus, the ability of GH to stimulate the association of IRS-1 and IRS-2 with the 85-kDa regulatory subunit suggests that GH activates PI3K. A potential role for PI3K in activating the insulin-like metabolic effects of GH is suggested by the finding that the PI3K inhibitor wortmannin blocks the ability of GH to stimulate lipid synthesis (34). In contrast, a recent study has shown that PI3K activity stimulated by GH has no effect on glucose uptake or on the translocation of GLUT4 (35). On the other hand, PI3K may be involved in the regulation of protein kinase C. Phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, products of PI3K, have recently been shown to activate the Ca⁡²⁺-independent protein kinase C isoforms γ, ε, and η (36). The observations that GH induces the translocation of the ε isoform of protein kinase C from the cytoplasm to the membrane in 3T3-F442A fibroblasts (37) and that the PI3K inhibitor wortmannin inhibits GH-dependent diacylglycerol formation in rat adipocytes (38) raise the possibility that in some cell types, PI3K may be involved in the GH-dependent activation of protein kinase C.

The role of SHP2 in GH signaling has been examined by several approaches, but is still not well defined. SHP2 is probably activated during association with IRS-1 and IRS-2 and may dephosphorylate signaling intermediates located either in the IRS-1 signaling complex or at distant sites, thus down-regulating signaling (39). It seems unlikely that SHP2 bound to IRS-1 or IRS-2 is the primary phosphatase responsible for dephosphorylating JAK2 and/or GH-R. The over-expression of a catalytically inactive form of SHP2 blocks, rather than stimulates, the induction by PRL of a β-casein reporter gene; the latter is a JAK2- and PRL-dependent event (40). Thus, the role of SHP2 in GH signaling seems to include the same functions regulated by this protein when activated by insulin. SHP2 has been implicated in insulin, IGF-I, and epidermal growth factor-dependent stimulation of Ras, MAP kinase, DNA synthesis, and c-fos reporter gene expression (41–44). These responses are blocked by the overexpression of a catalytically inactive form of SHP2, suggesting that SHP2 is a positive regulator of these functions.

Our data indicate that in addition to its important role in coupling to IRS-1 signaling pathway, GH activates pathways involving Shc. This conclusion is in accordance with a report...
showing that in 3T3-F442A fibroblasts there is an increase in Shc tyrosine phosphorylation after GH treatment (18). Shc is thought to function as an adaptor molecule to recruit Grb2- mSos1 complexes to the activated receptor (45). The nucleotide exchange factor mSos1 then promotes the formation of p21 ras (GTP), thereby initiating a cascade of phosphorylation events that culminates with the phosphorylation of specific transcription factors in the nucleus (45, 46). The finding in this study that Grb2 coprecipitates with both the IRSs and Shc proteins is consistent with GH activation of MAP kinase. These results suggest that in addition to the mitogenic effects that are induced by IGF (insulin growth factor) stimulation with GH, GH can play a direct pivotal role in the regulation of cellular growth and differentiation.

Some of the phosphorylations induced by GH and described here in animal tissues are catalyzed by a GHR-associated kinase identified as JAK2 (8, 47, 48), whereas others are catalyzed by downstream kinases (7). Our experiments have shown that in liver, the time course of IRS-1 tyrosyl phosphorylation reflected the JAK2 tyrosyl phosphorylation induced by GH, and this correlation suggests that JAK2 activation may be necessary for IRS-1 tyrosyl phosphorylation. Moreover, communoprecipitation between JAK2 and IRS-1 and the use of an in vitro kinase assay demonstrated that a GH-activated kinase, presumably JAK2, was significantly increased after a portal infusion of GH. Consistent with the involvement of JAK2 in IRS-1 tyrosyl phosphorylation, a previous study demonstrated that the abilities of various mutant forms of the GHR to mediate GH-dependent tyrosyl phosphorylation of JAK2 correlated with the amount of IRS-1 tyrosyl phosphorylation detected (12).

The increased tyrosyl phosphorylation of IRS-1, IRS-2, JAK2, and Shc in liver, heart, kidney, muscle, and adipose tissue after GH stimulation agrees with the finding that the GHR is a protein expressed in the cell membrane of all of these tissues (4, 5). The ability of GH to activate these signaling pathways in different tissues allows this hormone to exert its diverse metabolic and growth effects.

In summary, our results show that IRSs and Shc proteins serve as signaling molecules for GH in fasted rat tissues. Furthermore, the activation by GH of the tyrosine kinase activity of JAK2 toward IRS-1 as well as the correlation between JAK2 tyrosyl phosphorylation and IRS-1 tyrosyl phosphorylation suggest that IRS-1 may interact primarily with JAK2, which mediates the tyrosyl phosphorylation of this substrate.

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G120K-PEG, A GH ANTAGONIST, DECREASES GH SIGNAL TRANSDUCTION IN THE LIVER OF MICE

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ABSTRACT

Growth hormone (GH) exerts diverse growth-promoting and metabolic effects mediated by the GH receptor (GHR). After receptor binding, GH induces GHR dimerization. JAK2, a member of the Janus family of tyrosine kinases, is activated after its association with a dimerized GHR, stimulating the tyrosyl phosphorylation of insulin receptor substrate-1 (IRS-1), IRS-2 and Shc proteins. GHR dimerization is a critical step in mediating biological activities of GH. G120K-PEG, a GH antagonist, is a genetically-engineered analog of GH produced by a mutation that blocks GH action by preventing the GHR dimerization. This study shows that in mice treated with G120K-PEG, GH-induced tyrosyl phosphorylation of JAK2, IRS-1, IRS-2 and SHC was reduced compared with the controls. These data suggest that G120K-PEG modulates GH signal transduction and might be a potential drug therapy in diseases caused by excess of GH.

INTRODUCTION

Growth hormone (GH) is a single polypeptide hormone that is produced and excreted by cells of the anterior pituitary gland and exerts diverse growth-promoting and metabolic effects (Isaksson et al., 1985). As GH circulates in the blood, it binds to GH receptors (GHR), a transmembrane protein expressed on the surface of liver, adipose, kidney, heart, intestine, lung and muscle cells (Mathews et al., 1989; Tiong and Herington, 1991). GHR, a member of the cytokine/hematopoietin receptor superfamily (Kelly et al., 1993), lacks intrinsic tyrosine kinase activity (Leung et al., 1987). The crystal structure of the ligand-bound complex (DeVos et al., 1992) shows that GH binds to two identical receptor (hGHBP) subunits to induce receptor dimerization which occurs sequentially, i.e. GH binds to the first GHR through Site 1 of GH and subsequently binds to the second GHR through Site 2 of GH establishing a GH(GHBP)_2 complex (Cunningham et al., 1991).

In cultured cells, JAK2, a member of the Janus family of tyrosine kinases, is activated after its association with a dimerized GH receptor in response to hormone binding (Argetsinger et al., 1993). As a consequence of the kinase activation, GH has been shown
to stimulate the tyrosyl phosphorylation of some molecules primarily described as insulin receptor substrates, such as insulin receptor substrate-1 (IRS-1) (Souza et al., 1994; Ridderstrale et al., 1995; Argetsinger et al., 1995), IRS-2 (Argetsinger et al., 1996) and Shc proteins (Vanderkuur et al., 1995). We have recently reported that GH is able to induce JAK2 tyrosine phosphorylation and kinase activation and, as a consequence, tyrosine phosphorylation of IRS-1, IRS-2 and Shc in liver, adipose tissue, heart, muscle and kidney of the intact rat in vivo (Thirone et al., 1999).

Thus, after receptor binding and GHR dimerization, multiple signaling events occur that may mediate the actions of GH, including the tyrosine phosphorylation of multiple cellular polypeptides, making the production of the active GH (GHBP)₂ complex a critical step in mediating biological activities of GH. It has clearly been demonstrated that the third α-helix of the GH molecule is critical for GHR dimerization (Cunningham et al., 1991; Chen et al., 1991a; Chen et al., 1991b; Fuh et al., 1992; Okada et al., 1992). Dimerization can be prevented by making mutations in the portion of the third α-helix corresponding to Site 2 that sterically hinder the GH:GHR binding event, thus creating a GH receptor antagonist (Chen et al., 1995b). G120K-PEG has also been conjugated with polyethylene glycol (PEG) polymers, which increase the hydrodynamic volume of the molecule and thereby slow its systemic clearance (Zalipsky and Lee, 1992).

Therefore, the purpose of this study was to investigate the effects of G120K-PEG on tyrosyl phosphorylation of proteins involved in GH signal transduction. We first assessed the effect of GH on JAK2, IRS-1, IRS-2 and SHC tyrosyl phosphorylation in the liver of this animal and subsequently tested the ability of G120K-PEG to inhibit GH-stimulated tyrosine phosphorylation of those proteins in liver of mice in vivo.

MATERIALS AND METHODS

Materials

The reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiothreitol (DTT), Triton X-100, Tween 20 and glycerol were from Sigma Chemical Co. (St. Louis, MO). Sodium amobarbital was from Lilly. Human biosynthetic GH (Norditropin) was
purchased from Novo Nordisk (Bagsvaerd, Denmark). G120K-PEG was kindly provided by Sensus Drug Development Corporation (Austin, TX). 125I-Protein A was from Amersham (Amersham, UK) and Protein A Sepharose 6 MB from Pharmacia (Uppsala, Sweden). Nitrocellulose (BA85, 0.2 um) was from Schleicher & Schuell. Male Swiss mice were from the UNICAMP Central Breeding Center (Campinas, SP). Monoclonal antiphosphotyrosine antibody (αPY/ clone 4G10) was from Upstate Biotechnology Incorporated (UBI, Lake Placid, NY). Anti-IRS-1 (αIRS-1/ C-20), Anti-IRS-2 (αIRS-2/ A-19), Anti-Shc (αShc/ C-20) and Anti-JAK2 (αJAK2/ HR-758) antibodies were from Santa Cruz Technology (Santa Cruz, CA).

Animals

Six week-old male Swiss mice were provided with standard rodent chow and water ad libitum. Food was withdrawn 12-14 h before the experiments.

Methods

Mice were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.), and were used 10-15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein exposed, and 0.5 ml of normal saline (0 time) or GH was injected at a dose of 1.0 mg/kg body weight. At 1, 5 and 15 min after GH injection the liver was removed, coarsely minced and immediately homogenized in extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/ml) at 4 °C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 sec. The extracts were centrifuged at 15,000 rpm and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA) for 45 min to remove insoluble material, and the supernatant of this tissue was used for immunoprecipitation with αJAK2, αIRS-1, αIRS-2 and αSHC and Protein A Sepharose 6 MB or Protein A/G plus (Santa Cruz Technology, Santa Cruz, CA). The second part of the experiment was performed with 6-week old mice divided in two groups: one in which the antagonist was administered s.c. in a dose of 1mg/Kg and the other received saline s.c. at the same time, the studies being performed in parallel using the
control and treated mice. After one hour, the animals were anesthetized and 0.5 ml of normal saline (0 time) or GH was injected at a dose from 0.01 to 1.0 mg/kg body weight (as indicated) into the portal vein. At 5 min after GH injection, liver extracts were removed and processed as described above.

Protein analysis by immunoblotting

The precipitated proteins were treated with Laemmli sample buffer (Laemmli, 1970) containing 100 mM DTT and heated in a boiling water bath for 4 min, after which they were subjected to SDS-PAGE (6% bis-acrylamide) in a Bio-Rad miniature slab gel apparatus (Mini-Protean).

Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin et al. (1979), except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high molecular mass proteins. Non-specific protein binding to the nitrocellulose was reduced by pre-incubating the filter overnight at 4°C in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose blot was incubated with antiphosphotyrosine antibody, diluted in blocking buffer (0.3% BSA instead of nonfat dry milk) overnight at 4°C and then washed for 60 min with blocking buffer without milk. The blots were subsequently incubated with 2 μCi of 125I-protein A (30 μCi/μg) in 10 ml of blocking buffer for 2 h at room temperature and then washed again for 30 min as described above. 125I-Protein A bound to the antiphosphotyrosine and anti-peptide antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at -80°C for 12-48 h. Band intensities were quantitated by optical densitometry (Hoefer Scientific Instruments, San Francisco; model GS 300) of the developed autoradiographs.

Statistical Analysis

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

GH stimulates tyrosyl phosphorylation of JAK2 in liver of mice

To ascertain whether JAK2 protein was tyrosyl phosphorylated by GH stimulation in liver of mice, we performed a Western blot assay employing αJAK2 and αPY. We infused GH into the portal vein of fasted mice, removed and homogenized the liver and then whole tissues lysates were immunoprecipitated with αJAK2. When the blots were probed with αPY, GH-dependent tyrosyl phosphorylation of a protein with an Mr of 130,000, appropriate for JAK2, was detectable within 1 min after exposure to GH and reached maximal level (15-fold above basal) 5 min after GH stimulation (Fig 1A). When the same membranes were probed with αJAK2, the level of this protein was the same during the time-course of the experiments (Fig 1B).

GH stimulates tyrosyl phosphorylation of IRS-1 in liver of mice

GH can induce tyrosine phosphorylation of IRS-1 in adipocytes (Souza et al., 1994; Ridderstrale et al., 1995), 3T3-F442A fibroblasts (Argetsinger et al., 1995) and in liver of rats (Thirone et al., 1999). To determine if IRS-1 is tyrosyl phosphorylated following stimulation by GH in mice, solubilized proteins from livers of animals that had received GH into the portal vein were immunoprecipitated with αIRS-1. These immunoprecipitates were analyzed for tyrosyl phosphorylation by immunoblotting with αPY. The presence of phosphorylated IRS-1 was detectable 1 min after the GH infusion and was maximal (10-fold above basal) 5 min after GH injection. The signal diminished by 15 min post-GH (Fig 1C). Figure 1D shows that there was no change in protein levels following the administration of the hormone.

GH stimulates tyrosyl phosphorylation of IRS-2 in liver of mice

IRS-2 has substantial structural similarity to IRS-1, including multiple potential tyrosyl phosphorylation sites that can be tyrosyl phosphorylated following stimulation with GH of 3T3-F442A fibroblasts (Argetsinger et al., 1996) and in liver of rats (Thirone et al.,
1999). To investigate whether IRS-2 is tyrosyl phosphorylated after stimulation by GH in mice, we infused GH into the portal vein of fasted mice, removed and homogenized the liver and immunoprecipitated it with αIRS-2 antibody and immunoblotted it with αPY. Increased protein tyrosyl phosphorylation with a Mr appropriate for IRS-2 (180,000 – 190,000) was detected within 1 min post-GH, with maximum stimulation (12-fold above basal) detected at 5 min (Fig. 1E). As with IRS-1, there was no change in the levels of IRS-2 protein in the liver after GH stimulation during the time-course of the experiments (Fig. 1F).

GH stimulates tyrosyl phosphorylation of SHC in liver of mice

GH has been demonstrated to promote tyrosyl phosphorylation of Shc proteins in 3T3-F442A fibroblasts (VanderKuur et al., 1996) and in liver of rats (Thirone et al., 1999). We examined whether GH could induce Shc tyrosine phosphorylation in mice liver. After infusion of GH into the portal vein, liver extracts were removed and homogenized and the solubilized proteins were immunoprecipitated with αShc. The presence of phosphorylated tyrosines was assessed by Western blotting with αPY. Increased tyrosyl phosphorylation of a protein migrating at Mr ~52,000 (appropriate for Shc) was observed within 1 min and was maximal (10-fold above basal) 15 min after the infusion of GH (Fig. 1G). When the same blot was reprobed with αShc antibody, no differences were observed in the level of this protein after acute GH stimulation (Fig. 1H).

Effect of G120K-PEG on GH-induced JAK2 phosphorylation levels in liver of mice

The effect of G120K-PEG on GH-induced JAK2 tyrosyl phosphorylation was investigated 1h after i.p. injection of saline or the antagonist (1mg/Kg B.W.). Then the animals were anesthetized, the abdominal cavity was opened and GH was injected into the portal vein. The tissue was extracted and homogenized as described, immunoprecipitated with αJAK2 and blotted with αPY.

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We investigated the tyrosyl phosphorylation of JAK2 stimulated by different doses of GH in liver of mice previously treated with saline or with 1mg/Kg B.W. of G120K-PEG. Figure 2 (lanes 1-4) shows that GH-induced tyrosyl phosphorylation of JAK2 occurs in a dose-dependent manner. The phosphorylated JAK2 was detectable after injection of as little as 0.01mg/Kg (lane 2) and half-maximal stimulation occurred with 0.1mg/Kg of the hormone (lane 3). The maximal stimulation occurred with 1mg/Kg B.W. (lane 4) and no further stimulation was observed with a dose of 10mg/Kg body weight (data not shown). Lanes 5 to 8 refer to mice previously treated with G120K-PEG (1mg/Kg B.W.). When GH was injected at a dose of 0.01mg/Kg body weight (GH:G120K-PEG=1:100) no tyrosyl phosphorylation of JAK2 was observed in the liver of mice treated with G120K-PEG (lane 6). When GH was infused in a dose of 0.1mg/Kg body weight (GH:G120K-PEG=1:10) the inhibitory effect of G120K-PEG was not complete and there was a 36% reduction in JAK2 tyrosyl phosphorylation compared to animals treated with the same dose of GH without the antagonist (lane 7 vs. lane 3). When the same dose of GH and of antagonist was used (1mg/Kg body weight) (GH:G120K-PEG=1:1) the reduction in JAK2 tyrosyl phosphorylation was 39% compared to animals treated only with 1mg/Kg body weight of GH (lane 8 vs. lane 4).

G120K-PEG treatment produced no significant changes in the JAK2 protein level in mice livers when the same blots were probed with αJAK2 (Fig. 2B).

**Effect of G120K-PEG on GH-induced IRS-1 phosphorylation levels in liver of mice**

After G120K-PEG (1mg/Kg B.W.) treatment, changes were observed in stimulated phosphorylation of IRS-1 in liver. Before GH infusion there was no immunoreactivity in the basal state in controls and in mice treated with G120K-PEG. After stimulation with GH (1mg/Kg B.W.) at a ratio of GH:G120K-PEG of 1:1, the intensity of this band increased in both groups of animals (Fig. 3A). However, comparison of the bands stimulated by GH revealed that the extent of phosphorylation of IRS-1 was reduced to 50 ± 7% (n=7; p=0.003) in G120K-PEG-treated rats, compared to controls (100 ± 11%; n=7) (Fig. 3B). Using the specific anti-peptide antibody against IRS-1 (Fig. 3C), the level of this
protein was found to be unchanged in the liver of mice treated with G120K-PEG. We also investigated the tyrosyl phosphorylation of IRS-1 stimulated by GH in liver of mice previously treated with G120K-PEG in a ratio of 1:100. The results showed that there is no tyrosyl phosphorylation of IRS-1 when the ratio GH:G120K-PEG is 1:100 (data not shown).

**Effect of G120K-PEG on GH-induced IRS-2 phosphorylation levels in liver of mice**

To better define the effect of G120K-PEG on IRS-2 phosphorylation, we performed a Western blot analysis of the tyrosyl-phosphorylated proteins in anti-IRS-2 immunoprecipitates before and after stimulation with GH in both groups at a ratio of GH:G120K-PEG of 1:1 (Fig. 3D). Accordingly with the behavior of IRS-1, the level of GH stimulated phosphorylation of IRS-2 was reduced to $41 \pm 7.3\%$ ($n=7$; $p=0.03$) in the presence of GH antagonist (Fig. 3E) compared to controls ($100 \pm 14\%$; $n=7$). There was no change in the IRS-2 protein levels in the liver of mice treated with G120K-PEG, as determined by immunoblotting with anti-IRS-2 antibody (Fig. 3F). Using a ratio of GH:G120K-PEG of 1:100 no tyrosyl phosphorylation of IRS-2 was observed (data not shown).

**Effect of G120K-PEG on GH-induced SHC phosphorylation levels in liver of mice**

To investigate the extent of Shc phosphorylation, we performed a Western blot analysis of tyrosyl-phosphorylated proteins in anti-Shc immunoprecipitates before and after stimulation with GH (1mg/Kg B.W.) at a ratio of GH:G120K-PEG of 1:1 in both groups of animals (Fig. 5A). Shc phosphorylation was decreased in G120K-PEG-treated mice to $59 \pm 8\%$ ($n=10$; $p=0.006$) as compared to the control group ($100 \pm 10\%$; $n=10$) (Fig. 5B), although Shc protein level was not significantly different from that in the controls (Fig. 5C). Using a ratio of GH:G120K-PEG of 1:100 no tyrosyl phosphorylation of IRS-2 was observed (data not shown).
DISCUSSION

GH displays a variety of biological activities and has short-term effects that mimic the actions of insulin (Davidson, 1987). The GH insulin-like activities can result in transient hypoglycemia, increased glucose and aminoacid transport into tissue, and increased glucose and aminoacid metabolism (Goodman, 1968; Honeyman and Goodman, 1980; Press, 1988; Smal et al., 1987). These acute GH effects, similar to those of insulin, suggest that this hormone and insulin may activate some common signaling pathways. GH binds to GHR and activates the GHR-associated tyrosine kinase JAK2, whereupon both GHR and JAK2 become tyrosyl phosphorylated (Argtsinger et al., 1993). It has already been shown that in cultured cells (Souza et al., 1994; Ridderstrale et al., 1995; Argtsinger et al., 1995; Argtsinger et al., 1996) and in rats (Thirone et al., 1999), JAK2 kinase activation leads to tyrosyl phosphorylation of multiple intracellular signaling molecules, first described as insulin receptor substrates, including IRS-1, IRS-2 and SHC. We have studied the GH signal transduction in mice and the results presented here show that GH treatment leads to rapid changes in JAK2, IRS-1, IRS-2 and SHC tyrosine phosphorylation, in agreement with those studies related above.

Dimerization of receptors induced by ligand binding is a common means of triggering signal transduction in mammalian cells (Ulrich and Schlessinger, 1990). In the cytokine receptor superfamily, including GHR, one ligand molecule promotes dimerization of heterologous receptor subunits or dimerization of two identical subunits (Wells, 1994; Kishimoto et al., 1994), which is followed by activation of the tyrosine kinase receptor. GHR dimerization occurs sequentially, i.e. GH binds to the first GHR through site 1 of GH and subsequently binds to the second GHR through site 2 of GH (Cunningham et al., 1991). GHR dimerization is thought to activate JAK2 kinase, which than phosphorylates itself, GHR and downstream signaling components (Argtsinger and Carter-Su, 1996). G120K-PEG is a GH variant that has a mutation (a lysine for arginine substitution at the 120 position) made in Site 2, resulting in binding at Site 2 that is significantly disrupted, and dimerization of the GHRs is thus prevented. Our results, demonstrating that G120K-PEG decreased GH induced tyrosyl phosphorylation of JAK2, corroborate the studies that demonstrate the importance of receptor dimerization in signal transduction (Silva et al., 1993).
In our experiments, the inhibitory effect of G120K-PEG was maximal with a 
GH:G120K-PEG ratio of 1:100, as no JAK2 tyrosyl phosphorylation was observed with 
this dose of GH. When the dose of GH was increased and with a GH:G120K-PEG ratio of 
1:10 some tyrosyl phosphorylation of JAK2 could be observed. Additionally, GH-induced 
JAK2, IRS-1, IRS-2 and SHC tyrosyl phosphorylation was inhibited ~50% at equimolar 
centrations of the antagonist of GH. These results, demonstrating that GH-induced 
tyrosine phosphorylation of downstream signaling components can be reduced by G120K-
PEG reinforces the idea that GH signal transduction is linked to dimer formation.

The first biologic action attributed to GH was promotion of skeletal and organ 
growth (Isaksson et al., 1987; Green et al., 1985), as observed in the clinical syndromes of 
acromegaly. Acromegaly is a hormonal disorder that results when a tumor in the pituitary 
gland produces excess growth hormone and serious health consequences of excessive GH 
include diabetes mellitus, hypertension, increased risk of cardiovascular disease and cancer 
(Melman, 1990). Currently, treatment options include surgical removal of the tumor, 
radiation therapy of the pituitary gland and drug therapy. Our results showed that G120K-
PEG is able to impair the effect of GH at the cellular level, making this drug particularly 
useful in cases in which the tumor does not express somatostatin receptors and is 
unresponsive to somatostatin inhibitors. Another GH receptor antagonist has recently been 
reported to be effective in the treatment of acromegaly (Trainer et al., 2000).

In poorly controlled diabetes, retinopathy and nephropathy are two major 
complications (Marks and Krall, 1971; Klein et al., 1989; Friedman, 1995). A role for GH 
has been suggested in the development of various long-term diabetic end organ damage, 
including diabetic kidney disease (Lundback et al., 1970; Merimee et al., 1973; Press et al., 
1984; Orskov, 1985; Yang et al., 1993; Flyvbjerg, 2000) and diabetic retinopathy (Deckert 
et al., 1967; Merimee et al., 1970; Smith et al., 1997). The implicated effect of GH in 
diabetic end organ damage may be mediated by GH receptor (GHR) or postreceptor events 
in GH signal transduction (Flyvbjerg et al., 1989; Flyvbjerg, 2000). Recently published 
studies have described renoprotective effects of GH antagonists in long-term diabetic 
transgenic mice that express GH antagonists (Chen et al., 1995a; Chen et al., 1996; 
Esposito et al., 1996) and in STZ-diabetic mice that received G120K-PEG (Flyvbjerg et al.,
1999). Connecting these previous studies with our results we can suggest that the reduced GH signal transduction induced by GH antagonist can contribute to the renoprotective effect of the drug in these animals. This is a new concept in the treatment of diabetic kidney disease.

In summary, the ability of G120K-PEG to modulate the signal transduction of GH in mice in vivo by impairing JAK2, IRS-1, IRS-2 and SHC tyrosyl phosphorylation stimulated by this hormone suggests that this GH antagonist might be a potential drug therapy in diseases associated with undesirable effects of GH.

REFERENCES


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**FIGURE LEGENDS**

**Figure 1**: Time course of GH-stimulated tyrosine phosphorylation and protein levels of JAK2, IRS-1, IRS-2 and SHC in the liver of normal fasted mice. Liver extracts from mice injected with saline (-, 0 min) or GH (+, 1, 5, 15 min) were prepared as described in Material and Methods. (A): Tissue extracts were immunoprecipitated with αJAK2 (2 μg/ml) and immunoblotted with αPY (1 μg/ml). (B): Protein levels of JAK2 in liver. The same blots were incubated with αJAK2 (1 μg/ml). (C): Tissue extracts were immunoprecipitated with αIRS-1 (2 μg/ml) and immunoblotted with αPY (1 μg/ml). The same blot was incubated with αIRS-1(1 μg/ml) (D). Liver extracts were also immunoprecipitated with αIRS-2 (2 μg/ml) and immunoblotted with αPY (1 μg/ml) (E). The same membrane was incubated with αIRS-2 (1 μg/ml) (F). Tissue extracts were immunoprecipitated with αShc (2 μg/ml) and immunoblotted with αPY (1 μg/ml) (G). The same blot was also incubated with αShc (1 μg/ml) (H). These results are representative of seven independent experiments.
Figure 2: Different doses of GH-stimulated JAK2 tyrosine phosphorylation in intact livers from controls and G120K-PEG-treated mice. Mice treated with saline were injected with different doses of GH (lanes 1-4). (A): Mice treated with G120K-PEG were injected with different doses of GH (lanes 5-8). Tissue extracts were immunoprecipitated with αJAK2 (2 μg/ml) and immunoblotted with αPY (1 μg/ml). (B): Protein levels of JAK2. The same blots were incubated with αJAK2 (1 μg/ml). These results are representative of five independent experiments.

* P<0.05 vs lane 2; † P<0.05 vs lane 3; ‡ P<0.05 vs lane 4.

Figure 3: GH-stimulated IRS-1 and IRS-2 tyrosine phosphorylation in intact livers from controls (C) and G120K-PEG-treated mice (A). Liver extracts from mice injected with saline (-) or GH (+) were prepared as described in Material and Methods. Tissue extracts were immunoprecipitated with αIRS-1 (2 μg/ml) and immunoblotted with αPY (1 μg/ml) (A). The values for IRS-1 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 7 experiments (B). (C): Protein levels of IRS-1. The same blots were incubated with αIRS-1 (1 μg/ml). Tissue extracts were immunoprecipitated with αIRS-2 (2 μg/ml) and immunoblotted with αPY (1 μg/ml) (D). The values for IRS-2 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 7 experiments (E). (F): Protein levels of IRS-2. The same blots were incubated with αIRS-2 (1 μg/ml). * P<0.05 vs C.

Figure 4: GH-stimulated SHC tyrosine phosphorylation in intact livers from controls (C) and G120K-PEG-treated mice (A). Liver extracts from mice injected with saline (-) or GH (+) were prepared as described in Material and Methods. Tissue extracts were immunoprecipitated with αSHC (2 μg/ml) and immunoblotted with αPY (1 μg/ml) (A). The values for SHC phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 10 experiments (B). (C): Protein levels of SHC. The same blots were incubated with αSHC (1 μg/ml). * P<0.05 vs C.
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<td><strong>B. IP: α JAK2</strong>&lt;br&gt;<strong>IB: α JAK2</strong></td>
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| **C. IP: α IRS-1**<br>**IB: α PY** | **D. IP: α IRS-1**<br>**IB: α IRS-1** |
| Time (min) GH  | Time (min) GH |
| 0 1 5 15       | 0 1 5 15     |
| + + + +        | + + + +      |

| **E. IP: α IRS-2**<br>**IB: α PY** | **F. IP: α IRS-2**<br>**IB: α IRS-2** |
| Time (min) GH  | Time (min) GH |
| 0 1 5 15       | 0 1 5 15     |
| + + + +        | + + + +      |

| **G. IP: α SHC**<br>**IB: α PY** | **H. IP: α SHC**<br>**IB: α SHC** |
| Time (min) GH  | Time (min) GH |
| 0 1 5 15       | 0 1 5 15     |
| + + + +        | + + + +      |

FIGURE 1
FIGURE 2
FIGURE 3
FIGURE 4

IP: αSHC

A. IB: αPY

C  A
GH - + - +

B.

Arbitrary units

120
100
80
60
40
20
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Phosphorylation

C. IB: αSHC

C  A
GH - + - +

Protein

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66
GH SIGNAL TRANSDUCTION IN KIDNEY OF UNINEPHRECTOMIZED RATS

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Running Title: GH signal transduction in kidney of uninephrectomized rats.
ABSTRACT

The kidney is a target organ for a number of polypeptide growth factors. Growth factors are thought to mediate adaptations in renal function that occur in chronic reduction of functional renal mass and to promote regeneration following acute renal injury. In experimental animals, the reduction of functional renal mass leads to a compensatory renal growth (CRG), as well as to an adaptive increase in glomerular and tubular functions. Recent investigations have suggested a role for growth hormone (GH) and insulin-like growth factor-I (IGF-I) in the pathogenesis of experimental renal growth and scarring. GH signal transduction involves GHR dimerization. The GHR lacks intrinsic tyrosine kinase activity but JAK2, a member of the Janus family of tyrosine kinases, is activated after its association with a dimerized GHR. As a consequence of the kinase activation, GH has been shown to stimulate the tyrosyl phosphorylation of insulin receptor substrate-1 (IRS-1), IRS-2 and Shc proteins. The results of this study demonstrated that GH-induced tyrosyl phosphorylation of JAK2, IRS-1, IRS-2 and SHC in kidney of UNx rats were similar to that of control animals. These data suggest that no alteration in GH signal transduction is necessary for the compensatory hypertrophy response to unilateral nephrectomy.

INTRODUCTION

The kidney is a target organ for a number of polypeptide growth factors. Certain of these peptides interact with sensitive renal cells as classical endocrine hormones. Others are produced within the kidney and exert actions on the cell of origin or on adjacent cells in an autocrine or paracrine fashion. A number of studies support roles for one or more of these agents as causative of the increase in renal size and changes in renal function that occur in conditions such as hypersomatotropism, compensatory hypertrophy and diabetes mellitus. Growth factors are thought to mediate adaptations in renal function that occur in chronic reduction of functional renal mass and to promote regeneration following acute renal injury. While the adaptations induced by growth factors are probably initially salutary, the effects of continuing renal growth factor expression may be maladaptive (1).
In experimental animals, the reduction of functional renal mass leads to a compensatory renal growth (CRG), as well as to an adaptive increase in glomerular and tubular functions. Recent data suggest a causal link between CRG and the development of renal scarring (2). If such an association is confirmed, the characterization of the mediators involved in the early renal growth response may allow their manipulation and the prevention of subsequent scarring. Recent investigations have suggested a role for growth hormone (GH) and insulin-like growth factor-I (IGF-I) in the pathogenesis of experimental renal growth and scarring (3, 4).

Considerable information has been obtained concerning GH signaling. The initial event in GH signal transduction is the binding of GH to its receptor. GHR, a transmembrane protein expressed on the surface of liver, adipose, kidney, heart, intestine, lung and muscle cells (5, 6), is a member of the cytokine/hematopoietin receptor superfamily (7). The GHR itself is not a tyrosine kinase (8), but after receptor binding, JAK2, a member of the Janus family of tyrosine kinases, is activated after its association with a dimerized GH receptor in response to hormone binding (9). As a consequence of the kinase activation, GH has been shown to stimulate the tyrosyl phosphorylation of some molecules primarily described as insulin receptor substrates, as insulin receptor substrate-1 (IRS-1) (10-12), IRS-2 (13) and Shc proteins (14). We have recently reported that GH is able to induce JAK2 tyrosine phosphorylation and kinase activation and, as a consequence, tyrosine phosphorylation of IRS-1, IRS-2 and Shc in liver, adipose tissue, heart, muscle and kidney of the intact rat in vivo (15).

Unilateral nephrectomy is followed by CRG, with rapid kidney growth that is manifest within 24-48h and involves both hypertrophy and hyperplasia (16). Considering that recent investigations have suggested that CRG might be GH dependent and with the acknowledge of the cellular mechanisms by which GH elicits these effects, the aim of the present study was to examine the effects of uninephrectomy (Unx) on GH-induced tyrosine phosphorylation of JAK2, IRS-1, IRS-2 and SHC in kidney of rats.
MATERIALS AND METHODS

Materials

The reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiothreitol (DTT), Triton X-100, Tween 20 and glycerol were from Sigma Chemical Co. (St. Louis, MO). Sodium amobarbital was from Lilly. Human biosynthetic GH (Norditropin) was purchased from Novo Nordisk ( Bagsvaerd, Denmark). ^125^I-Protein A was from Amersham (Amersham, UK) and Protein A Sepharose 6 MB from Pharmacia (Uppsala, Sweden). Nitrocellulose (BA85, 0.2 um) was from Schleicher & Schuell. Male Wistar rats were from the UNICAMP Central Breeding Center (Campinas, SP). Monoclonal antiphosphotyrosine antibody (αPY/ clone 4G10) was from Upstate Biotechnology Incorporated (UBI, Lake Placid, NY). Anti-JAK2 (αJAK2/ HR-758), Anti-IRS-1 (αIRS-1/ C-20), Anti-IRS-2 (αIRS-2/ A-19) and Anti-Shc (αShc/ C-20) antibodies were from Santa Cruz Technology (Santa Cruz, CA).

Animals

Six week-old male Wistar rats were divided into two groups: the control (C) and uninephrectomized rats (Unx). All groups received standard rodent chow and water ad libitum. Under sodium amobarbital anaesthesia one group underwent left uninephrectomy (Unx) and one group was shamoperated by flank incision and gentle manipulation of left kidney (C). This day of the surgery was considered day 0.

Methods

Experiments were performed in three different days: days 1, 4 and 7. At each day the animals were again divided into two groups and the studies were performed in parallel using the control (C) and uninephrectomized rats (Unx). Rats were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.), and were used 10-15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the cava vein exposed, and 0.5 ml of normal saline (negative) or GH (positive) was injected at a dose of 1.8 mg/kg body weight. At 5 min after GH injection, a
fragment from the right kidney tissue was removed, minced coarsely and homogenized immediately in extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/ml) at 4 °C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 sec. The extracts were centrifuged at 15,000 rpm and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA) for 45 min to remove insoluble material, and the supernatant of these tissues was used for immunoprecipitation with αJAK2, αIRS-1, αIRS-2 and αShc and Protein A Sepharose 6 MB or Protein A/G plus (Santa Cruz Technology, Santa Cruz, CA).

Protein analysis by immunoblotting

The precipitated proteins were treated with Laemmli sample buffer (17) containing 100 mM DTT and heated in a boiling water bath for 4 min after which they were subjected to SDS-PAGE (6% bis-acrylamide) in a Bio-Rad miniature slab gel apparatus (Mini-Protean).

Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin et al. (18) except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high molecular mass proteins. Non-specific protein binding to the nitrocellulose was reduced by pre-incubating the filter overnight at 4°C in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose blot was incubated with antiphosphotyrosine antibody, diluted in blocking buffer (0.3% BSA instead of nonfat dry milk) overnight at 4°C and then washed for 60 min with blocking buffer without milk. The blots were subsequently incubated with 2 μCi of 125I-protein A (30 μCi/μg) in 10 ml of blocking buffer for 2 h at room temperature and then washed again for 30 min as described above. 125I-Protein A bound to the antiphosphotyrosine and anti-peptide antibodies was detected by autoradiography using prefleashed Kodak XAR film with Cronex Lightning Plus intensifying screens at -80°C for 12-48 h. Band intensities were quantitated by optical densitometry (Hoefer Scientific Instruments, San Francisco; model GS 300) of the developed autoradiographs.
Statistical Analysis

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

RESULTS

Regulation of JAK2, IRS-1, IRS-2 and Shc proteins in kidney of rats 1 day after uninephrectomy

The first experiments were performed one day after the initial surgery using one group of animals that was submitted to uninephrectomy (UnX1) and one group that was submitted to laparotomy (C1). To investigate JAK2 phosphorylation following GH stimulation, we infused GH into the cava vein and then removed the right kidney and homogenized the tissue and immunoprecipitated the proteins with anti-JAK2 antibody. The JAK2 immunoprecipitates were analyzed for tyrosyl phosphorylation by immunoblotting with a monoclonal anti-phosphotyrosine antibody (Fig.1A). There was no statistically significant difference in the GH stimulated phosphorylation of JAK2 in uninephrectomy rats when compared to the controls (UnX1=101 ± 14 vs. C1=100 ± 9%; P=0.917; n=6) (Fig.1B).

To determine the level of IRS-1 phosphorylation, solubilized proteins from rat kidney from both groups were immunoprecipitated with αIRS-1 and immunoblotted with antiphosphotyrosine antibody before and after stimulation with GH (Fig.1C). In accordance with the behavior of JAK2, comparison of the bands stimulated by GH revealed that tyrosyl phosphorylation of IRS-1 was not statistically different in uninephrectomy rats when compared to the controls (UnX1=132 ± 20 vs. C1=100 ± 10%; P=0.232; n=6) (Fig.1D).

To estimate the rate of GH-induced IRS-2 phosphorylation, kidney samples from the two groups of rats were immunoprecipitated with anti-IRS-2 antibody and immunoblotted with antiphosphotyrosine antibody (Fig.1E). GH-induced IRS-2 tyrosine phosphorylation in uninephrectomized rats was not different compared with that of control animals (UnX1= 92 ± 10 vs. C1=100 ± 16%; P=0.227; n=6) (Fig.1F).
To better define the extent of Shc phosphorylation, we performed a Western blot analysis of tyrosyl-phosphorylated proteins in anti-Shc immunoprecipitates before and after stimulation with GH in both groups of animals (Fig. 1G). After stimulation with GH, comparison of the bands stimulated by GH revealed that Shc phosphorylation showed no difference between the uninephrectomized compared to control animals \((Unx1=99 \pm 11 \text{ vs. C1}=100 \pm 8\%\,; \, P=0.950; \, n=6)\) (Fig. 1H).

**Regulation JAK2, IRS-1, IRS-2 and Shc proteins in kidney of rats 4 days after uninephrectomy**

The same studies were performed four days after the surgeries using uninephrectomized \((Unx4)\) and control \((C4)\) animals. To investigate the effects of uninephrectomy on GH-induced JAK2 tyrosyl phosphorylation in kidneys of rats, right kidney samples from the two groups of animals were immunoprecipitated with anti-JAK2 antibody and immunoblotted with antiphosphotyrosine antibody (Fig. 2A). The results showed that GH-stimulated JAK2 tyrosyl phosphorylation of rats submitted to uninephrectomy 4 days before was similar to that of the control group \((Unx4=114 \pm 10 \text{ vs. C4}=100 \pm 9\%\,; \, P=0.323; \, n=6)\) (Fig. 2B).

To determine the level of IRS-1 phosphorylation, solubilized proteins from rat right kidney were immunoprecipitated with αIRS-1 and immunoblotted with with antiphosphotyrosine antibody (Fig.2C). After exposure to GH there was no statistically significant differences in the GH stimulated phosphorylation of IRS-1 in uninephrectomized rats when compared to the controls \((Unx4=122 \pm 12 \text{ vs. C4}=100 \pm 25\%\,; \, P=0.8; \, n=6)\) (Fig. 2D).

Using a specific anti-peptide antibody against IRS-2 (Fig. 2E), the level of tyrosyl phosphorylation of this protein after GH stimulation was found to be unchanged in the kidney of uninephrectomized rats when compared to the controls \((Unx4=94 \pm 11 \text{ vs. C4}=100 \pm 11\%\,; \, P=0.728; \, n=6)\) (Fig. 2F).
To investigate the effects of uninephrectomy on GH-induced Shc tyrosyl phosphorylation in kidney of rats samples from the two group of animals were immunoprecipitated with anti-Shc antibody and immunoblotted with antiphosphotyrosine antibody (Fig.2G). The results showed that GH-stimulated Shc tyrosyl phosphorylation in kidney of rats submitted to uninephrectomy 4 days before was similar to that of the control group (Unx4= 108 ± 15 vs. C4=100 ± 20%; P=0.78; n=6) (Fig. 2H).

Regulation of JAK2, IRS-1, IRS-2 and Shc proteins in kidney of rats 7 days after uninephrectomy

The last studies were with animals that underwent uninephrectomy seven days before (Unx7) and a control group was used in parallel (C7). We performed a Western blot analysis of the tyrosyl-phosphorylated proteins in anti-JAK2 immunoprecipitates before and after stimulation with GH in the two groups of animals (Fig. 3A). GH-stimulated JAK2 tyrosyl phosphorylation was not different in uninephrectomized rats when compared with control animals (Unx7= 122 ± 20 vs. C7=100 ± 5%; P=0.355; n=6) (Fig. 3B).

Similarly, no significant change occurred in the level of IRS-1 tyrosyl phosphorylation in the kidney of uninephrectomized rats when compared to the controls (Fig.3C). In samples from kidney previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antiphosphotyrosine antibody, there was no change in GH-stimulated IRS-1 tyrosine phosphorylation in uninephrectomized rats when compared with control animals (Unx7= 101 ± 18 vs. C7=100 ± 15%; P=0.994; n=6) (Fig. 3D).

Uninephrectomy had no effect on the IRS-2 tyrosyl phosphorylation level in the contralateral kidney as determined by immunoprecipitated of cell lysates with anti-IRS-2 antibody (Fig.3E). In samples from kidney previously immunoprecipitated with anti-IRS-2 antibody and immunoblotted with antiphosphotyrosine antibody, there was no change in GH-stimulated IRS-2 tyrosine phosphorylation in uninephrectomized rats when compared with control animals (Unx= 127 ± 17 vs. C7=100 ± 17%; P=0.325; n=6) (Fig. 3F).
In order to examine if uninephrectomy exerts any effect in GH-induced tyrosyl phosphorylation of Shc, kidney samples from the two group of rats were immunoprecipitated with anti-SHC antibody and immunoblotted with antiphosphotyrosine antibody (Fig. 3G). Accordingly with the behavior of IRS-1 and IRS-2, there was no significant change in GH-stimulated Shc tyrosyl phosphorylation in kidney of uninephrectomized rats compared with control animals (Unx7= 99 ± 11 vs. C7=100 ± 8%; P=0.950; n=6) (Fig. 3H).

DISCUSSION

The compensatory growth of the contralateral kidney, which follows loss of a kidney in humans and unilateral nephrectomy of experimental animals (19), is demonstrable within 1-2 days. The growth involves primarily the glomerulus and proximal tubule (20). The glomeruli are morphologically and functionally normal after nephrectomy in rats unless the remaining renal mass is severely reduced, in which case progressive glomerulosclerosis ensues (21). The hormonal regulation of compensatory hypertrophy is not fully understood, however total kidney insulin-like growth factor-I (IGF-I) mRNA levels are increased following unilateral nephrectomy (3,19). This suggests a role for this hormone in hypertrophy of the adult kidney as well as in normal development (22). The growth factors operative in the disorderly growth characteristic of some glomerular disease processes have not been elucidated. However, there are abnormalities in the circulating levels of growth hormone (GH) in some diseases associated with increases in glomerular extracellular matrix and cell number (23-25) and among other neuropeptides and neurohormones, GH has been shown to modulate the development of glomerular injury in various renal diseases (26).

GH signal transduction involves GHR dimerization. The GHR lacks intrinsic tyrosine kinase activity but JAK2, a member of the Janus family of tyrosine kinases, is activated after its association with a dimerized GHR (8,9). As a consequence of the kinase activation, GH has been shown to stimulate the tyrosyl phosphorylation of insulin receptor substrate-1 (IRS-1), IRS-2 and Shc proteins (10-15). Our results demonstrated that GH-
induced tyrosyl phosphorylation of JAK2, IRS-1, IRS-2 and SHC were not altered in kidney of rats after one, four or seven days after they were submitted to unilateral nephrectomy. Thus, our observations shows that these first steps of GH signal transduction do not seem to be involved in the renal hypertrophy observed after unilateral nephrectomy. A role for GH in compensatory renal growth has initial been postulated because hypophysectomy inhibits the compensatory response (20). However, our results are in accordance with some data of literature the observed that compensatory enlargement and increases in renal IGF-I content do occur in hypophysectomized rats (3) and in GH-deficient rats (27, 28), consistent with a GH-independent process by which kidney growth is mediated.

Recent studies has shown that the early, accelerated remnant kidney growth following uninephrectomy occurs through alternate mechanisms in juvenile and adult male rats, which may govern the type of renal growth hat occurs after UNx. Early CRG in the adult male rat is GH dependent, but independent of changes in the renal IGF-I system. In contrast, CRG is GH independent in the juvenile male rat, but is associated with significant increases in the renal IGF-I system, and hyperplastic kidney growth (29). Our data are in accordance with this recent study as our experiments were performed with juvenile rats and we observed that GH signal transduction was not altered in kidney of UNx rats.

In summary, the results of this study demonstrated that GH-induced tyrosyl phosphorylation of JAK2, IRS-1, IRS-2 and SHC in kidney of UNx rats were similar to that of control animals. These data suggest that no alteration in GH signal transduction is necessary for the compensatory hypertrophy response to unilateral nephrectomy.
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FIGURE LEGENDS:

**Figure 1:** GH-stimulated JAK2, IRS-1, IRS-2 and SHC tyrosine phosphorylation in kidney from control (C1) and uninephrectomized rats (Unx1) one day postsurgery. Kidney extracts from rats injected with saline (-) or GH (+) were prepared as described in Material and Methods. (A): Tissue extracts were immunoprecipitated with αJAK2 (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for JAK2 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (B). (C): Tissue extracts were immunoprecipitated with αIRS-1 (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for IRS-1 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (D). (E): Tissue extracts were immunoprecipitated with αIRS-2 (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for IRS-2 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (F). (G): Tissue extracts were immunoprecipitated with αSHC (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for SHC phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (H).

**Figure 2:** GH-stimulated JAK2, IRS-1, IRS-2 and SHC tyrosine phosphorylation in kidney from control (C4) and uninephrectomized rats (Unx4) four days postsurgery. Kidney extracts from rats injected with saline (-) or GH (+) were prepared as described in Material and Methods. (A): Tissue extracts were immunoprecipitated with αJAK2 (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for JAK2 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (B). (C): Tissue extracts were immunoprecipitated with αIRS-1 (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for IRS-1 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (D). (E): Tissue extracts were immunoprecipitated with αIRS-2 (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for IRS-2 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (F). (G): Tissue extracts were immunoprecipitated with αSHC (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for SHC phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (H).
αPY (1μg/ml). The values for SHC phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (H).

**Figure 3:** (A): GH-stimulated JAK2, IRS-1, IRS-2 and SHC tyrosine phosphorylation in kidney from control (C7) and uninephrectomized rats (Unx7) seven days postsurgery. Kidney extracts from rats injected with saline (-) or GH (+) were prepared as described in Material and Methods. (A): Tissue extracts were immunoprecipitated with αJAK2 (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for JAK2 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (B). (C): Tissue extracts were immunoprecipitated with αIRS-1 (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for IRS-1 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (D). (E): Tissue extracts were immunoprecipitated with αIRS-2 (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for IRS-2 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (F). (G): Tissue extracts were immunoprecipitated with αSHC (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for SHC phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 6 experiments (H).
FIGURE 1

A. IP: α JAK2  
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B. 


C. IP: α IRS-1  
   IB: α PY  

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D. 


E. IP: α IRS-2  
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FIGURE 2
FIGURE 3
MODULATION OF GH SIGNAL TRANSDUCTION IN KIDNEYS OF STREPTOZOTOCIN DIABETIC ANIMALS: EFFECT OF A GROWTH HORMONE RECEPTOR ANTAGONIST

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Running Title: GH signaling in kidneys of diabetic animals.
ABSTRACT

Although controversial, a role for GH and IGF-I has been suggested in the development of diabetic kidney disease. The implicated effect of GH in diabetic end organ damage may be mediated by GH receptor (GHR) or postreceptor events in GH signal transduction. After receptor binding, GH induces GHR dimerization. The GHR lacks intrinsic tyrosine kinase activity but JAK2, a member of the Janus family of tyrosine kinases, is activated after its association with a dimerized GHR. As a consequence of kinase activation, GH has been shown to stimulate the tyrosyl phosphorylation of insulin receptor substrate-1 (IRS-1), IRS-2 and Shc proteins, which can associate and activate proteins containing SH2 domains, leading to activation of MAPK and Akt. The aim of the present study was to investigate the effects of diabetes induced by STZ on early steps in GH signal transduction in kidney of rats. Our results demonstrated that GH-induced phosphorylation of JAK2, IRS-1, Shc, MAPK and Akt and association of IRS-1/PI3K, IRS-1/Grb2 and Shc/Grb2 were increased in kidney STZ-diabetic rats. GHR dimerization is a critical step in mediating biological activities of GH and G120K-PEG, a GH antagonist, can prevent GHR dimerization. Administration of G120K-PEG in diabetic mice showed inhibitory effects on diabetic renal enlargement and reversed the alterations in GH signal transduction observed in diabetic animals. The present study demonstrates a role for GH in the pathogenesis of early diabetic renal changes and suggests that specific GHR blockade may present a new concept in the treatment of diabetic kidney disease.

INTRODUCTION

In poorly controlled diabetes, retinopathy and nephropathy are two major complications (1-3). Although controversial, a role for GH and insulin-like growth factor I (IGF-I) has been suggested in the development of various long-term diabetic end organ damage, including diabetic kidney disease (4-8). Renal and glomerular hypertrophy and increased renal function are observed in the early phase of human and experimental diabetes (9-12). In early experimental diabetes, an initial transient increase in kidney IGF-1 is observed, followed by renal and glomerular hypertrophy (13, 14), indicating a possible renotropic role for IGF-1 in diabetic renal changes. The effect of a long-acting somatostatin...
analog, octreotide, which is a potent inhibitor of GH and IGF-1, has been investigated in experimental diabetes and its administration during the first 7 days after diabetes induction eliminated the early diabetic kidney IGF-1 accumulation and the renal and glomerular hypertrophy (13). On the other hand, although high GH levels have been frequently observed in insulin-dependent diabetes mellitus patients (15-18), decreased IGF-1 and retarded growth have been reported in diabetic rats and humans (19-22). Therefore, the importance of IGF-1 in the pathogenesis of microvascular disease has been questioned and the implicated effect of GH in diabetic end organ damage may be mediated by GH receptor (GHR) or postreceptor events in GH signal transduction, but not by IGF-1.

Our understanding of GH signaling has rapidly expanded in the past several years. Considerable information has been obtained concerning the initial event in GH signal transduction, the binding of GH to its receptor. GHR, a transmembrane protein expressed on the surface of liver, adipose, kidney, heart, intestine, lung and muscle cells (23, 24), is a member of the cytokine/hematopoietin receptor superfamily (25). The GHR itself is not a tyrosine kinase (26), but after receptor binding, JAK2, a member of the Janus family of tyrosine kinases, is activated after its association with a dimerized GH receptor in response to hormone binding (27). As a consequence of the kinase activation, GH has been shown to stimulate the tyrosyl phosphorylation of some molecules primarily described as insulin receptor substrates, as insulin receptor substrate-1 (IRS-1) (28-30), IRS-2 (31) and Shc proteins (32). We have recently reported that GH is able to induce JAK2 tyrosine phosphorylation and kinase activation and, as a consequence, tyrosine phosphorylation of IRS-1, IRS-2 and SHC in liver, adipose tissue, heart, muscle and kidney of the intact rat in vivo (33). Tyrosyl phosphorylation of IRS-1 and IRS-2 in response to GH provides binding sites for specific proteins containing SH2 domains, including the 85kDa regulatory subunit of phosphatidylinositol (PI) 3-kinase (PI3K) (28-30, 33). Downstream PI 3-kinase, the PH domain-containing serine-threonine kinase Akt is activated and its phosphorylation appears to be the primary mechanism by which enzymatic activity is regulated (35). Similarly, GH promotes the binding of growth factor receptor bound 2 (Grb2) to IRS-1 (33, 34) and Shc proteins (32, 33). It was also demonstrated that GH stimulates the mitogen-activated protein (MAP) kinase (37) and that one of the pathways that leads GHR to MAP kinase involves Grb2, son of Sevenless (SOS), Ras, Raf and MAP-ERK kinase (MEK) (37, 38).
Considering that GH may play a role in diabetic nephropathy and with the acknowledgement of the cellular mechanisms by which GH elicits these effects, plus the recently information that these signal pathways may be stimulated in various tissues in vivo, including kidney, the first aim of the present study was to examine the effects of diabetes induced by streptozotocin on GH-induced phosphorylation of JAK2, IRS-1, Shc, MAPK and Akt and on the association of IRS-1/PI3 kinase, IRS-1/Grb2 and Shc/Grb2 in kidney of rats.

GHR dimerization is a critical step in mediating biological activities of GH. G120K-PEG, a GH antagonist, is a genetically engineered analog of GH produced by a mutation in the third α-helix that blocks GH action preventing GHR dimerization. Thus, the second aim of this study was to investigate the effect of G120K-PEG on renal enlargement and on GH signal transduction in kidneys of diabetic mice.

MATERIALS AND METHODS

Materials

The reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonfylfluoride (PMSF), aprotinin, dithiothreitol (DTT), Triton X-100, Tween 20, glycerol and streptozotocin (STZ) were from Sigma Chemical Co. (St. Louis, MO). Sodium amobarbital was from Lilly. Human biosynthetic GH (Norditropin) was purchased from Novo Nordisk (Bagsvaerd, Denmark). [125]I-Protein A was from Amersham (Amersham, UK) and Protein A Sepharose 6 MB from Pharmacia (Uppsala, Sweden). Nitrocellulose (BA85, 0.2 um) was from Schleicher & Schuell. G120K-PEG was kindly provided by Sensus Drug Development Corporation (Austin, TX). Male Wistar rats and male Swiss mice were from the UNICAMP Central Breeding Center (Campinas, SP). Monoclonal antiphosphotyrosine antibody (αPY/ clone 4G10) was from Upstate Biotechnology Incorporated (UBI, Lake Placid, NY). Anti-JAK2 (αJAK2/ HR-758), Anti-IRS-1 (αIRS-1/ C-20), Anti-Shc (αShc/ C-20), Anti-PI3K (αPI3K/ p85 Z-8) and Anti-Grb2 (αGrb2/ C-23) antibodies were from Santa Cruz Technology (Santa Cruz,
CA). Anti-phospho-MAPK (αMAPK/ p44-42/ Thr183 and Tyr 185) and Anti-phospho-Akt (serine 473) antibodies were from New England Biolabs (Beverly, MA).

**Animals**

For the first part of the experiments, six week-old male Wistar rats were divided into two groups and the studies were performed in parallel using the control and treated rats (STZ group). All groups received standard rodent chow and water *ad libitum*.

The second part of the experiments was performed with six week-old male Swiss mice divided in three groups: one with control animals, one with animals treated with STZ and the last one with animals treated with STZ and G120K-PEG.

**STZ treatment**

One group of overnight fasted rats was rendered diabetic by a single intravenous injection of STZ (Sigma; 60 mg/Kg in citric buffer, pH 4.5). Age-matched normal rats received an equivalent volume of citric buffer, pH 4.5, and served as a control group.

In order to induce diabetes in two groups of overnight fasted mice, a single intraperitoneal injection of STZ (Sigma; 100 mg/Kg in citric buffer, pH 4.5) was administered. Age-matched normal mice received an equivalent volume of citric buffer, pH 4.5, and served as a control group.

Rats and mice were used for the experiments 4 days after receiving STZ injection. The body weight of the animals was recorded and diabetes was confirmed by blood glucose >250 mg/dl. Plasma glucose levels were determined by the glucose oxidase method using blood samples obtained from the animal tail before the experiments were performed.
G120K-PEG treatment

One diabetic group of mice was treated with subcutaneous injections of a pegylated GHR antagonist (G120K-PEG) (Sensus, Austin, TX). Treatment was started 24h after STZ injection with a dose of 1mg/Kg body wt and repeated 48h later to maintain high diurnal levels of the GHR antagonist. Untreated diabetic and nondiabetic control mice were injected with an equivalent volume of saline.

Methods

Rats or mice were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.), and were used 10-15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein exposed, and 0.5 ml of normal saline (0 time) or GH was injected at a dose of 1.8 mg/kg body weight. At 5 min after GH injection, a fragment from the right kidney tissue was removed, coarsely minced and immediately homogenized in extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/ml) at 4°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 sec. The extracts were centrifuged at 15,000 rpm and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA) for 45 min to remove insoluble material, and the supernatant of these tissues was used for immunoprecipitation with αIRS-1, αShc and αJAK-2 and Protein A Sepharose 6 MB or Protein A/G plus (Santa Cruz Technology, Santa Cruz, CA).

As for the group of mice, after the extraction of a fragment from the right kidney, the left kidney was rapidly removed and carefully cleaned and weighed.
Protein analysis by immunoblotting

The precipitated proteins were treated with Laemmli sample buffer (39) containing 100 mM DTT and heated in a boiling water bath for 4 minutes, after which they were subjected to SDS-PAGE (6% bis-acrylamide) in a Bio-Rad miniature slab gel apparatus (Mini-Protean).

To determine the phosphorylation of MAPK and Akt, whole tissue extracts from all animals were mixed with Laemmli buffer and similar sized aliquots (200μg protein) were subjected to SDS-PAGE.

Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean), as described by Towbin et al. (40), except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high molecular mass proteins. Non-specific protein binding to the nitrocellulose was reduced by pre-incubating the filter overnight at 4°C in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose blot was incubated with antiphosphotyrosine antibody, anti-PI3K antibody, anti-Grb2 antibody, anti-phospho-MAPK antibody or anti-phospho-Akt antibody diluted in blocking buffer (0.3% BSA instead of nonfat dry milk) overnight at 4°C and then washed for 60 min with blocking buffer without milk. The blots were subsequently incubated with 2 μCi of 125I-protein A (30 μCi/μg) in 10 ml of blocking buffer for 2 h at room temperature and then washed again for 30 min as described above. 125I-Protein A bound to the antiphosphotyrosine and anti-peptide antibodies was detected by autoradiography using prefocused Kodak XAR film with Cronex Lightning Plus intensifying screens at -80°C for 12-48 h. Band intensities were quantitated by optical densitometry (Hoefer Scientific Instruments, San Francisco; model GS 300) of the developed autoradiographs.

Statistical Analysis

All data are expressed as means ± SE. Comparisons between different groups were performed using analysis of variance followed by Student’s t if appropriate. The level of significance employed was p<0.05.
RESULTS

Rats characteristics

At the moment of the experiments, 4 days after the STZ injection, body weight of STZ-treated diabetic rats had significantly decreased when compared with the control group (D=140 ± 13 vs. C=180 ± 12 g; P<0.05). Data concerning plasma glucose concentrations are summarized in Table 1. There was a markedly higher fasting plasma glucose concentration in STZ-treated diabetic rats than in controls (D=323 ± 18 vs. C=112 ± 4 mg/dl; P<0.001).

Effect of STZ-induced diabetes on JAK2 phosphorylation levels in the kidney of rats

To determine the level of JAK2 phosphorylation, solubilized proteins from rat kidney were immunoprecipitated with αJAK2 and immunoblotted with antiphosphotyrosine antibody. After exposure to GH there was an increase in the phosphorylation of a protein with a Mr of 130,000 appropriate for JAK2. There was a significant increase in the GH stimulated phosphorylation of JAK2 in STZ-induced diabetic rats when compared to the controls (D=193 ± 12 vs. C=100 ± 25%; P<0.01) (Fig.1).

Effect of STZ-induced diabetes on IRS-1 phosphorylation and on IRS-1/PI3K and IRS-1/Grb2 association levels in the kidney of rats

We did not observe differences in basal IRS-1 tyrosine phosphorylation levels in STZ-induced diabetic rats when compared to the controls. There was a significant increase in the GH stimulated phosphorylation of IRS-1 in STZ-induced diabetic rats when compared to the control animals (D=189 ± 13 vs. C= 100 ± 21%; P<0.005) (Fig. 2A).

In kidney samples previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted against the 85 kDa subunit of PI 3-kinase (Fig. 2B), after stimulation with GH, a band with the expected molecular mass (85kDa) of the PI 3-kinase regulatory subunit was observed in anti-IRS-1 antibody immunoprecipitates of kidney from the two groups of rats. This finding is consistent with a stable association between IRS-1 and PI 3-.
kinase. Comparison of the bands stimulated by GH revealed that the amount of PI 3-kinase associated with IRS-1 was increased in STZ-induced diabetic rats when compared to the controls (D=150 ± 10 vs. C=100 ± 17%; P<0.05) (Fig.2B).

Tyrosyl phosphorylation of IRS-1 in response to GH provides binding sites for Grb2, a protein linked to mitogenic pathways (33, 34). Similarly, there was an increase in the amount of Grb2 which co-precipitated with IRS-1 in STZ-induced diabetic rats when compared to the controls (D=145 ± 9 vs. C=100 ± 15%; P<0.05) (Fig.2C).

Effect of STZ-induced diabetes on SHC and on SHC/Grb2 association levels in the kidney of rats

To better define the extent of Shc phosphorylation, we performed a Western blot analysis of tyrosyl-phosphorylated proteins in anti-Shc immunoprecipitates before and after stimulation with GH in both groups of animals. We have recently demonstrated that the 52kDa Shc isoform has a higher level of tyrosine phosphorylation than the 46kDa species when stimulated by GH (33), probably as a consequence of the higher amounts of the 52 kDa Shc isoform in rat tissues compared with other isoforms. Basal Shc phosphorylation showed no difference between the two groups. After stimulation with GH, increased tyrosyl phosphorylation of a protein migrating at Mr ~52,000 (appropriate for Shc) was observed in both groups of animals, but comparison of the bands stimulated by GH revealed that the extent of phosphorylation of Shc was increased in STZ-induced diabetic rats compared to controls (D=189 ± 29 vs. C=100 ± 16%; P<0.05) (Fig. 3A).

In intact rats in vivo (33), GH promotes the association of Shc with Grb2. To investigate the effect of diabetes induced by STZ treatment on Shc/Grb2 association in kidney of rats, Shc and associated proteins were immunoprecipitated with anti-Shc and Western blotted with anti-Grb2. A protein recognized by αGrb2 in Western blots and migrating with the appropriate size for Grb2 (Mr 23,000) was precipitated by αShc. We observed that after GH stimulation the amount of Grb2 associated with Shc was increased in STZ-diabetic rats compared to controls (D=143 ± 10 vs. C=100 ± 11%; P<0.05) (Fig. 3B).
Effect of STZ-induced diabetes on MAPK and Akt phosphorylation levels in the kidney of rats

To estimate the rate of GH-induced MAPK phosphorylation in the kidney of STZ-diabetic rats, we performed experiments with whole tissue extracts from all animals with similar sized aliquots (200μg protein) that were subjected to SDS-PAGE and electrotransfer of proteins from the gel to nitrocellulose was performed. Immunoblotting the nitrocellulose membranes with anti-phospho-MAPK antibody we observed that GH-stimulated MAPK phosphorylation was increased in the diabetic rats compared with control animals (D=155 ± 12 vs. C=100 ± 13%; P<0.05) (Fig. 4A).

We also established the level of Akt serine phosphorylation in the kidney of STZ-diabetic rats by immunoblotting the nitrocellulose membranes with samples obtained from the whole tissue extracts separated by SDS-PAGE with anti-phospho-Akt antibody (Fig. 4B). Comparison of the bands stimulated by GH revealed that the extent of serine phosphorylation of Akt was increased in STZ-induced diabetic rats compared to controls (D=167 ± 20 vs. C=100 ± 11%; P<0.05).

Mice characteristics

G120K-PEG is a genetically engineered analog of GH produced by a mutation in the third α-helix that blocks GH action preventing GHR dimerization and has specificity for mice. For this reason we performed the subsequent experiments using mice.

The plasma glucose determinations from the experimental animals are illustrated in Table 1. Blood glucose levels were markedly elevated in the diabetic group compared to controls (D=423 ± 11 vs. C=135 ± 4 mg/dl; P<0.001). G120K-PEG administration in diabetic animals did not affect blood glucose when compared with the diabetic animals that did not receive the antagonist (D+Tx=396 ± 13 vs. D=423 ± 11 mg/dl; P=0.14).
The relation kidney weight/body weight is illustrated in Figure 5. Kidney weight in the untreated diabetic mice was higher compared with that in nondiabetic control animals ($D=8.7 \pm 0.3$ vs. $C=7 \pm 0.3$ mg/g body wt; $P<0.005$). Diabetic animals subjected to G120KPEG-treatment had a great inhibition of the diabetes-associated renal enlargement as kidney weight was similar to that of controls ($D+Tx=7.2 \pm 0.1$ vs. $C=7 \pm 0.3$ mg/g body wt; $P=0.44$).

Effect of G120K-PEG on GH-induced JAK2 tyrosine phosphorylation level in the kidneys of diabetic mice

To investigate the effects of G120K-PEG on GH-induced JAK2 tyrosyl phosphorylation in kidneys of diabetic mice, kidney samples from the three groups of mice were immunoprecipitated with anti-JAK2 antibody and immunoblotted with antiphosphotyrosine antibody. The results showed that GH-stimulated JAK2 tyrosyl phosphorylation was increased in the diabetic mice that did not receive G120K-PEG compared to control animals ($D=188 \pm 26$ vs. $C=100 \pm 8$; $P<0.05$). When diabetic mice were treated with G120K-PEG, GH-induced JAK2 tyrosyl phosphorylation was similar to that of control mice ($D+Tx=108 \pm 8$ vs. $C=100 \pm 8$; $P=0.5$) (Fig.6).

Effect of G120K-PEG on GH-induced IRS-1 tyrosine phosphorylation level and on IRS-1/PI3K and IRS-1/Grb2 associations in the kidneys of diabetic mice

We also tested the effect of G120K-PEG on IRS-1 phosphorylation in kidney of diabetic mice performing a Western blot analysis of the tyrosyl-phosphorylated proteins in anti-IRS-1 immunoprecipitates, before and after stimulation with GH in the three groups of animals. In accordance with the behavior of JAK2, GH-stimulated IRS-1 tyrosyl phosphorylation was increased in the diabetic mice compared to control animals ($D=198 \pm 15$ vs. $C=100 \pm 6$; $P<0.001$) and returned to values similar to those of the control group, when diabetic mice were also treated with G120K-PEG ($D+Tx=112 \pm 10$ vs. $C=100 \pm 6$; $P=0.33$) (Fig. 7A).
The blots that had been previously immunoprecipitated with antibody against IRS-1 were subsequently incubated with antibodies against the 85 kDa subunit of PI 3-kinase (Fig. 7B), and the comparison of the bands stimulated by GH revealed that the amount of PI 3-kinase associated with IRS-1 was increased in STZ-induced diabetic mice when compared to the controls \( (D=155 \pm 10\% \text{ vs. } C=100 \pm 11\%; \ P<0.01) \) and was indistinguishable from controls in G120K-PEG treated animals \( (D+Tx=95 \pm 6 \text{ vs. } C=100 \pm 11\%; \ P=0.7) \).

To determine the extent of IRS-1/Grb2 association in kidney extracts of the three groups of animals, we performed experiments by stripping and reprobing the same membrane above with anti-Grb2 antibody. The level of IRS-1/Grb2 association was increased in the diabetic mice compared to control animals \( (D=148 \pm 14 \text{ vs. } C=100 \pm 8\%; \ P<0.05) \) and did not differ from controls in G120K-PEG treated mice \( (D+Tx=99 \pm 13 \text{ vs. } C=100 \pm 8\%; \ P=0.94) \) (Fig. 7C).

**Effect of G120K-PEG on GH-induced SHC tyrosine phosphorylation level and on SHC/Grb2 association in the kidneys of diabetic mice**

In order to examine the effect of G120K-PEG on GH-induced SHC tyrosyl phosphorylation in kidneys of diabetic animals, kidney samples from the three groups of mice were immunoprecipitated with anti-SHC antibody and immunoblotted with antiphosphotyrosine antibody. GH-induced SHC tyrosine phosphorylation in diabetic mice was increased compared with that of control animals \( (D=192 \pm 31 \text{ vs. } C=100 \pm 13\%; \ P<0.05) \). This increase in SHC tyrosine phosphorylation was almost eliminated in the G120K-PEG-treated diabetic mice \( (D+Tx=111 \pm 10 \text{ vs. } C=100 \pm 13\%; \ P=0.52) \) (Fig. 8A).

Since after GH stimulation Shc can associate with Grb2, blots with samples that had been previously immunoprecipitated with anti-Shc were reprobed with anti-Grb2. The GH-induced association between Shc/Grb2 increased in the diabetic mice compared with control animals \( (D=150 \pm 10 \text{ vs. } C=100 \pm 10\%; \ P<0.01) \) and decreased to the control level when animals were treated with G120K-PEG \( (D+Tx=92 \pm 12 \text{ vs. } C=100 \pm 10\%; \ P=0.62) \) (Fig. 8B).
Effect of G120K-PEG on GH-induced MAPK and Akt phosphorylation levels in the kidneys of diabetic mice

We also tested the effect of G120K-PEG on GH-induced MAPK phosphorylation in the kidneys of STZ-diabetic rats by immunoblotting the nitrocellulose membranes with anti-phospho-MAPK antibody. The results showed that MAPK phosphorylation was increased in the kidney of diabetic mice compared to control animals (D=154 ± 15 vs. C=100 ± 11%; P<0.05) and G120K-PEG reversed the effect of diabetes and decreased the phosphorylation of MAPK to control levels (D+Tx=103 ± 13 vs. C=100 ± 11%; P=0.86) (Fig. 9A).

Similar results were observed for Akt phosphorylation, as GH-induced Akt serine phosphorylation in the kidney of untreated diabetic mice was increased compared with that of control animals (D=174 ± 22 vs. C=100 ± 13%; P<0.05). This increase in GH-induced Akt serine phosphorylation was almost eliminated in the G120K-PEG-treated diabetic mice (D+Tx=109 ± 15 vs. C=100 ± 13%; P=0.66) (Fig. 9B).

DISCUSSION

The onset of diabetes mellitus in humans and in experimental diabetes is accompanied by a rapid increase in renal mass and enhanced glomerular filtration rate and renal plasma flow (41). Kidneys from diabetic rats are morphologically characterized by early glomerular hypertrophy followed by an increase in proximal tubular cell size (42, 43). Glomerular hypertrophy is thought to be one of the key early changes in the development of diabetic nephropathy (44, 45). Several hormonal systems have been implicated as factors in early diabetic nephropathy. Involvement of GH and IGF-1 is strongly suggested from studies of the impact of GH excess (46, 47) and deficiency (48, 49) on kidney growth and function in various animal models, both diabetic (47-49) and nondiabetic (46). In these studies, a direct relationship has been noted between activity of GH and IGF-1 and renal hypertrophy (46-49) and between GH and glomerulosclerosis (46, 47).
GH signal transduction involves GHR dimerization and JAK2 is activated after its association with a dimerized GHR (26, 27). As a consequence of the kinase activation of JAK2, GH has been shown to stimulate the tyrosyl phosphorylation of insulin receptor substrate-1 (IRS-1), IRS-2 and SHC proteins (28-33). In the present study it was demonstrated that GH-induced tyrosyl phosphorylation of JAK2, IRS-1 and SHC was increased in kidneys of STZ-diabetic rats and mice, suggesting that these increases in the first steps of GH signal transduction may play a role in diabetic nephropathy. Our report is in accordance with other studies that support a role for GH in kidney diabetic disease given that GH-deficient rats with diabetes are relatively protected from typical renal effects of diabetes seen in GH-sufficient rats (48, 49), while transgenic mice expressing excess GH develop glomerular hypertrophy, albuminuria and glomerulosclerosis, a sequence of events similar to the evolution of diabetic nephropathy (44, 46). Similarly, transgenic mice expressing excess IGF-1 binding protein have elevated GH levels and developed mesangial hypertrophy and glomerulosclerosis, despite a decrease in plasma IGF-1 levels (50). These studies are consistent with a GH-dependent process by which diabetic nephropathy is mediated, and our results provide data in GH signal transduction that reinforces earlier information.

Recent studies have identified that increased diacylglycerol (DAG) levels initiated by hyperglycemia and the activation of protein kinase C (PKC) are associated with many vascular abnormalities in retinal, renal and cardiovascular tissues (51-54). Growth hormone has been shown to elicit rapid transient increases in DAG in multiple cell types (55-57). In rat adipocytes, GH-induced DAG production is blocked by a tyrosine kinase inhibitor, genistein (57), which also inhibits JAK2 (58). This is consistent with GH-dependent PKC activation lying downstream of JAK2 in these cells. Our results, showing that in diabetic animals kidneys there is an increase in GH-induced tyrosine phosphorylation levels of JAK2 may suggest another pathway that may lead to DAG-PKC activation in these animals.

In this report we also demonstrated that there is an increase in GH-induced IRS-1 tyrosine phosphorylation and IRS-1/PI 3-kinase association in kidneys of diabetic rats. PI 3-kinase is the best studied signaling molecule activated by IRS-1. It plays an important
role in the regulation of a broad array of biological responses including membrane ruffling, mitogenesis and differentiation (59). Different approaches have demonstrated that Akt/PKB, a serine-threonine kinase with a pleckstrin homology domain, is functionally located downstream of PI 3-kinase (60, 61, 35). Akt/PKB has been implicated in the mediation of some of the insulin and GH action, including glycogen synthesis (62-64). We observed an increase in GH-induced phosphorylation levels of Akt in diabetic animals and since Akt/PKB phosphorylation is closely related to Akt/PKB activity, we can suggest that in diabetic rats kidneys Akt/PKB activity induced by GH is probably increased.

In microvessels, increased activity of nitric oxide (NO), a potent vasodilator, may enhance glomerular filtration (65). Urinary excretion of NO$_2$ and NO$_3$, stable metabolites of NO, have been reported to be increased in diabetes of short duration (65-67), possibly due to enhanced expression of inducible NO Synthase (iNOS) gene and increased production of NO in mesangial cells (68). Recent evidences suggest that activation of Akt is an important step to activate NO synthase and induce NO production (69, 70). The present study, demonstrating that GH-induced Akt phosphorylation is increased in kidney of diabetic animals, suggests one possible mechanism that can contribute to increase NO production in these animals.

In addition to PI 3-kinase, tyrosine phosphorylated motifs in the IRS-protein bind to the SH2-domains in several small adapter proteins, including Grb2 (33, 34). Flanking its SH2 domain, Grb2 contains SH3-domains that associate constitutively with mSos, a guanine nucleotide exchange protein that stimulates GDP/GTP exchange on p21ras (37, 38). The recruitment by growth receptors of Grb2/mSos to membranes containing p21ras is one of the mechanisms employed to activate the MAP kinase cascade. During GH stimulation Grb2 engages IRS-1 and She, which will result in MAP kinase activation (36). Activation of this enzyme is believed to lead to selective changes in gene transcription and protein synthesis and is therefore likely to be crucial for cellular responses to GH (36, 38). Early diabetic nephropathy is characterized by excessive growth and fractional expansion of the glomerular mesangium (11). MAPK activation has been implicated in the development of proliferative glomerular disease (71-75), resulting in increased gene expression and enhanced synthesis of extracellular matrix proteins, including type I and IV
collagens, fibronectin, and laminin (76). Our results showed that GH-induced IRS-1/Grb2, Shc/Grb2 associations and MAPK phosphorylation were increased in kidneys of STZ-diabetic animals. Since a signal driven by SHC/Grb2 and IRS-1/Grb2 leads to Ras and MAP kinase activation and positively modulates multiple cellular events linked to development, growth and mitogenesis, these results suggest that increased activity of the IRS-1 and Shc branches of the GH signaling pathway can be an addition to the mitogenic effect of GH playing a role in the diabetic nephropathy.

By recognizing the potential role that GH may play in various pathophysiological conditions, including diabetic nephropathy, a series of highly specific antagonists of GH action has been recently developed for potential therapeutic use (77). Thus, previous studies have described renoprotective effects of GH antagonists in long-term diabetic transgenic mice that express GH antagonists (47, 78, 79). Our results demonstrates normalization of diabetic renal enlargement in diabetic mice treated with G120K-PEG and an inhibitory effect on GH-induced tyrosyl phosphorylation of the proteins involved in GH signal transduction, showing that the increase in JAK2, IRS-1, SHC, MAPK and Akt phosphorylation and that the increase in IRS-1/PI 3-kinase, IRS-1/Grb2 and SHC/Grb2 associations in diabetic animals can be prevented by this GHR antagonist. Accordingly, two recent studies showed protection against diabetes-associated kidney damage: in transgenic mice, expressing a disruption in the GH receptor/binding protein gene (80) and after treating nonobese diabetic mice with a polyethylene-glycol GHR antagonist (81). Therefore, the results argue for an important role of GH in the development of diabetes induced end-organ damage, implying that modulation of GH effects may have beneficial therapeutic implications in diabetic nephropathy.

In summary, the results of this study demonstrated that GH-induced phosphorylation of JAK2, IRS-1, SHC, MAPK and Akt, and IRS-1/PI 3kinase, IRS-1/Grb2 and SHC/Grb2 associations were increased in kidneys of STZ-diabetic rats. These data suggest that diabetes is characterized by an increase in the GH signaling that might contribute to the development of diabetic kidney disease. Also, administration of G120K-PEG in diabetic mice has inhibitory effects on diabetic renal enlargement and in the increased activity of the GH signaling pathway observed in diabetic animals. The present
study demonstrates alterations in the early steps of GH signal transduction in kidneys of diabetic rats and mice and suggests that specific GHR blockade may prevent these alterations and present a new concept in the treatment of diabetic kidney disease.

ACKNOWLEDGMENTS

This work was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and Conselho Nacional de Pesquisa (PRONEX). The authors wish to thank Luiz Janeri for his technical assistance.

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FIGURE LEGENDS:

**Figure 1:** GH-stimulated JAK2 tyrosine phosphorylation in kidney of control (C) and STZ-diabetic (D) rats. Kidney extracts from rats injected with saline (-) or GH (+) were prepared as described in Material and Methods. Tissue extracts were immunoprecipitated with αJAK2 (2μg/ml) and immunoblotted with αPY (1μg/ml). The values for JAK2 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 7 experiments. *P<0.05 vs. C.

**Figure 2:** GH-stimulated IRS-1 tyrosine phosphorylation and the association of this substrate with SH2 domain-containing proteins in kidney of control (C) and STZ-diabetic (D) rats. Kidney extracts from rats injected with saline (-) or GH (+) were prepared as described in Material and Methods. Tissue extracts were immunoprecipitated with αIRS-1 (2μg/ml) and immunoblotted with αPY (1μg/ml) (A). The same blot was incubated with αPI3K(1μg/ml) (B) and αGrb2 (1μg/ml) (C). The values for IRS-1 phosphorylation or association levels are represented as the mean ± SEM of scanning densitometry of 7 experiments. *P<0.005 vs. C.

**Figure 3:** GH-stimulated Shc tyrosine phosphorylation and the association of this substrate with Grb2 in kidney of control (C) and STZ-diabetic (D) rats. Kidney extracts from rats injected with saline (-) or GH (+) were prepared as described in Material and Methods. Tissue extracts were immunoprecipitated with αShc (2μg/ml) and immunoblotted with αPY (1μg/ml) (A). The same blot was reprobed with αGrb2 (B). The values for Shc phosphorylation or association levels are represented as the mean ± SEM of scanning densitometry of 7 experiments. *P<0.05 vs. C.
Figure 4: GH-stimulated MAPK and Akt phosphorylation in kidney of control (C) and STZ-diabetic (D) rats. Kidney extracts from rats injected with saline (-) or GH (+) were prepared and equal quantities of lysate protein were run on SDS-PAGE as described in Material and Methods. Following transfer to nitrocellulose, blots were probed with αMAPKp (1 μg/ml) (A) or αAkt (B). The values for MAPK or Akt phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 7 experiments. * P<0.05 vs. C.

Figure 5: Kidney weight:body weight ratio of control (C), STZ-diabetic (D) and G120K-PEG treated STZ-diabetic (D+Tx) mice on the day of the experiments. The values are represented as the mean ± SEM of 8 animals in each group. * P<0.05 vs C; † P<0.05 vs (D).

Figure 6: GH-stimulated JAK2 tyrosine phosphorylation in kidney of control (C), STZ-diabetic (D) and G120K-PEG treated STZ-diabetic (D+Tx) mice. Kidney extracts from mice injected with GH (+) were prepared as described in Material and Methods. Tissue extracts were immunoprecipitated with αJAK2 (2 μg/ml) and immunoblotted with αPY (1 μg/ml). The values for JAK2 phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 5 experiments. * P<0.05 vs C; † P<0.05 vs (D).

Figure 7: GH-stimulated IRS-1 tyrosine phosphorylation and the association of this substrate with SH2 domain-containing proteins in kidney of control (C), STZ-diabetic (D) and G120K-PEG treated STZ-diabetic (D+Tx) mice. Kidney extracts from rats injected with GH (+) were prepared as described in Material and Methods. Tissue extracts were immunoprecipitated with αIRS-1 (2 μg/ml) and immunoblotted with αPY (1 μg/ml) (A). The same blot was incubated with αPI3K(1 μg/ml) (B) and αGrb2 (1 μg/ml) (C). The values for IRS-1 phosphorylation or association levels are represented as the mean ± SEM of scanning densitometry of 5 experiments. * P<0.05 vs C; † P<0.05 vs (D).

Figure 8: GH-stimulated Shc tyrosine phosphorylation and the association of this substrate with Grb2 in kidney of control (C), STZ-diabetic (D) and G120K-PEG treated STZ-diabetic (D+Tx) mice. Kidney extracts from rats injected with GH (+) were prepared as described in Material and Methods. Tissue extracts were immunoprecipitated...
with αShc (2μg/ml) and immunoblotted with αPY (1μg/ml) (A). The same blot was incubated with and αGrb2 (1μg/ml) (B). The values for Shc phosphorylation or association levels are represented as the mean ± SEM of scanning densitometry of 5 experiments. * P<0.05 vs C; † P<0.05 vs (D).

**Figure 9:** GH-stimulated MAPK and Akt phosphorylation in kidney of control (C), STZ-diabetic (D) and G120K-PEG treated STZ-diabetic (D+Tx) mice. Kidney extracts from rats injected with GH (+) were prepared and equal quantities of lysate protein were run on SDS-PAGE as described in Material and Methods. Following transfer to nitrocellulose, blots were probed with αMAPKp (1μg/ml) (A) or αAkt (B). The values for MAPK or Akt phosphorylation levels are represented as the mean ± SEM of scanning densitometry of 5 experiments. * P<0.05 vs C; † P<0.05 vs (D).

**Table 1:** Blood glucose levels of control (C) and STZ-diabetic(D) rats and control (C), STZ-diabetic (D) and G120K-PEG treated STZ-diabetic (D+Tx) mice on the day of the experiments. The values are represented as the mean ± SEM of 17 control rats and 20 STZ diabetic rats. For mice, the values are represented as the mean ± SEM of 10 animals in each group. * P<0.05 vs C.

<table>
<thead>
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<th>TABLE 1: Characteristics of the experimental animals.</th>
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FIGURE 1
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FIGURE 5
**FIGURE 6**

**IP α JAK2**

**Blot α PY**

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Arbitrary units

- C
- D
- D+Tx

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Trabalhos publicados/a publicar

118
FIGURE 7
**FIGURE 8**

**IP α She**

**Blot α PY**

- **A)**
  - C  D  D+Tx
  - GH
  - Arbitrary units

**B)**

**Blot α Grb2**

- **C**  D  D+Tx
  - GH
  - Arbitrary units

Trabalhos publicados/a publicar

120
FIGURE 9

A) Blot α MAPK

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Arbitrary units

B) Blot α Akt

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Arbitrary units
GROWTH HORMONE SIGNAL TRANSDUCTION IN LIVER AND ADIPOSE TISSUE OF OLD RATS

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Running Title: Aging, IRS-1, IRS-2, JAK2 and Shc phosphorylation.
ABSTRACT

Biological aging is a catabolic state characterized by a decline in tissue protein synthesis and tissue function. These structural changes are associated with a reduction in high amplitude growth hormone (GH) secretion and a concomitant decline in plasma concentrations of insulin-like growth factor I (IGF-1), being the latter event recognized as a major contributing factor to the decline in functional status with age. GH stimulates the tyrosine phosphorylation of various cellular polypeptides, including the GH receptor itself, IRS-1, IRS-2 and Shc proteins. Some of these phosphorylations are catalyzed by a GH receptor-associated kinase identified as JAK2, a member of the Janus family of tyrosine kinases. GH-induced phosphorylation of GHR and JAK2 were assessed in vitro in liver of old mice and it was observed that there was a decline in JAK2 and GHR phosphorylation in livers of 31-month-old animals. This study investigated the effects of aging in JAK2, IRS-1, IRS-2 and Shc in liver and adipose tissue of rats. GH-induced tyrosyl phosphorylation of JAK2, IRS-1 and IRS-2 decreased in adipose tissue of 18-month-old compared with 6-week-old rats, while no change was observed in Shc tyrosyl phosphorylation after GH stimulation. Aging had no effect in GH-stimulated tyrosyl phosphorylation of JAK2, IRS-1, IRS-2 and Shc proteins in the liver. These data suggest that the early steps of GH signal transduction is impaired in adipose tissue of old rats and this may contribute to the decline in tissue function present in aging.

INTRODUCTION

Biological aging is associated with decreased lean body mass, expansion of fat mass and decreased strength. This catabolic state is characterized by a decline in tissue protein synthesis and tissue function (1-3). It has been well established that these structural changes are associated with a reduction in high amplitude growth hormone (GH) secretion and a concomitant decline in plasma concentrations of insulin-like growth factor I (IGF-1) (4-10). Previous studies indicate that a decline in IGF-1 is a major contributing factor to the decline in functional status with age (11, 12). Considering that liver is the major source of IGF-1 (13) and that studies in aged Long-Evans rats demonstrated a striking rise in the number of hepatic GH receptors (GHR) compared to that in young animals and that
nevertheless, plasma IGF-1 levels remain 40-50% lower in these aged animals (14), the question concerning potential impairment in post-receptor GH signal transduction must be raised.

GHR, a transmembrane protein expressed on the surface of liver, adipose, kidney, heart, intestine, lung and muscle cells (15), is a member of the cytokine/hematopoietin receptor superfamily (16). The GHR lacks intrinsic tyrosine kinase activity (17), but after receptor binding, JAK2, a member of the Janus family of tyrosine kinases, is activated after its association with a dimerized GH receptor in response to hormone binding (18). As a consequence of the kinase activation, GH has been shown to stimulate the tyrosyl phosphorylation of some molecules primarily described as insulin receptor substrates or insulin receptor substrate-1 (IRS-1) (19-21), IRS-2 (22) and Shc proteins (23). We have recently reported that GH is able to induce Jak2 tyrosine phosphorylation, kinase activation and, as a consequence, tyrosine phosphorylation of IRS-1, IRS-2 and Shc in the liver, adipose tissue, heart, muscle and kidney of the intact rat in vivo (24).

It has been hypothesized that reduced signal transduction through the GHR is a contributing factor in the increased resistance of tissue to growth hormone with age (25). It was demonstrated that despite an increase in the GHR density and GHR binding in livers of old mice, IGF-1 decreased dramatically with age (25, 26). GH-induced phosphorylation of GHR and JAK2 were also assessed in vitro in the liver of those old mice and it was observed that there was a decline in JAK2 and GHR phosphorylation in the liver of 31-month-old animals (25, 26). The GHR signal transduction pathway involving IRSs and Shc proteins, which are known to be phosphorylated by GHR through JAK2 kinase activity (19-24), has not yet been elucidated in the aging process. It was described that insulin-stimulated IRS-1 and IRS-2 tyrosyl phosphorylation decreased with aging (27), but the status of GH-induced tyrosyl phosphorylation of these substrates has not been investigated.

Therefore, the purpose of this study was to investigate whether other signaling molecules involved in GH signal transduction should also contribute to the loss of protein synthesis and tissue function that is associated with normal aging. We assessed the GH effect on JAK2, IRS-1, IRS-2 and Shc tyrosyl phosphorylation in liver and adipose tissue of old rats in vivo.
MATERIALS AND METHODS

Materials

The reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA). Tris, phenylmethysulfonylfluoride (PMSF), aprotinin, dithiothreitol (DTT), Triton X-100, Tween 20 and glycerol were from Sigma Chemical Co. (St. Louis, MO). Sodium amobarbital was from Lilly. Human biosynthetic GH (Norditropin) was purchased from Novo Nordisk (Bagsvaerd, Denmark). $^{125}$I-Protein A was from Amersham (Amersham, UK) and Protein A Sepharose 6 MB from Pharmacia (Uppsala, Sweden). Nitrocellulose (BA85, 0.2 um) was from Schleicher & Schuell. Male Wistar rats were from the UNICAMP Central Breeding Center. Monoclonal antiphosphotyrosine antibody ($\alpha$PY) was from Upstate Biotechnology Incorporated (UBI, Lake Placid, NY). Anti-IRS-1 ($\alpha$IRS-1), Anti-IRS-2 ($\alpha$IRS-2), Anti-Shc ($\alpha$Shc) and Anti-JAK2 ($\alpha$JAK2) antibodies were from Santa Cruz Technology (Santa Cruz, CA).

Animals

Six week- and 18-month-old male Wistar rats were provided with standard rodent chow and water ad libitum. Food was withdrawn 12-14 h before the experiments.

Methods

Rats were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.), and were used 10-15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein exposed, and 0.5 ml of normal saline (0 time) or GH was injected at a dose of 1.8 mg/kg body weight. We previously performed a time course experiment and observed that maximal tyrosyl phosphorylation occurred within 5 min of the GH stimulation (data not shown). Therefore, at 5 min after GH injection, liver and adipose tissues were removed, minced coarsely and
immediately homogenized in extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/ml) at 4°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 sec. The extracts were centrifuged at 15,000 rpm and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA) for 45 min to remove insoluble material, and the supernatant of these tissues was used for immunoprecipitation with αIRS-1, αIRS-2, αShc and αJAK-2 and Protein A Sepharose 6 MB or Protein A/G plus (Santa Cruz Technology, Santa Cruz, CA).

Protein analysis by immunoblotting

The precipitated proteins were treated with Laemmli sample buffer (28) containing 100 mM DTT and heated in a boiling water bath for 4 min, after which they were subjected to SDS-PAGE (6% bis-acrylamide) in a Bio-Rad miniature slab gel apparatus (Mini-Protean).

Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by Towbin et al. (29), except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high molecular mass proteins. Non-specific protein binding to the nitrocellulose was reduced by pre-incubating the filter overnight at 4°C in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose blot was incubated with antiphosphotyrosine antibody diluted in blocking buffer (0.3% BSA instead of nonfat dry milk) overnight at 4°C and then washed for 60 min with blocking buffer without milk. The blots were subsequently incubated with 2 μCi of 125I-protein A (30 μCi/μg) in 10 ml of blocking buffer for 2 h at room temperature and then washed again for 30 min as described above. 125I-Protein A bound to the antiphosphotyrosine and anti-peptide antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at -80°C for 12-48 h. Band intensities were quantitated by optical densitometry (Hoefer Scientific Instruments, San Francisco; model GS 300) of the developed autoradiographs.
Statistical Analysis

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

RESULTS

Regulation of JAK-2, IRS-1, IRS-2 and Shc proteins in adipose tissue of old rats

To investigate JAK2 phosphorylation following GH stimulation in old rats, we infused GH into the portal vein of 6-week- and 18-month-old rats and then removed and homogenized the adipose tissue and immunoprecipitated the proteins with anti-JAK2 antibody. The JAK2 immunoprecipitates were analyzed for tyrosyl phosphorylation by immunoblotting with a monoclonal anti-phosphotyrosine antibody. We performed previously a timer course experiment and observed that maximal phosphorylation of a protein with a Mr of 130,000 appropriate for JAK2 occurred 5 min after GH stimulation. Comparison of the bands from GH-stimulated tissues revealed that the extent of JAK2 phosphorylation was reduced to 50 ± 11% (n= 5; p<0.05) in old rats compared with controls (100 ± 16%; n=5 ) (Fig 1A).

GH can induce tyrosine phosphorylation of IRS-1 in cultured cells (19-21). To investigate whether IRS-1 was tyrosyl phosphorylated following stimulation by GH, we infused GH into the portal vein of rats and then removed and homogenized the adipose tissue and immunoprecipitated the proteins with αIRS-1. These immunoprecipitates were analyzed for tyrosyl phosphorylation by immunoblotting with αPY. The presence of phosphorylated IRS-1 was maximal 5 min after the GH infusion (data not shown). To compare young and old rats, extracts of adipose tissue from both groups were immunoprecipitated with αIRS-1 and immunoblotted with αPY after 5 min of GH infusion into portal vein. The level of GH-stimulated tyrosyl phosphorylation of IRS-1 decreased to 25 ± 7% (n=5; p<0.05) in 18-month-old rats compared to the 6-week-old animal (100 ± 24%; n=7) (Fig 1B).
IRS-2 tyrosyl phosphorylation following GH stimulation was also observed in cultured cells (22). The time course experiment showed that maximal phosphorylation of IRS-2 was coincident with that of IRS-1 (data not shown). To estimate the IRS-2 tyrosyl phosphorylation induced by GH, after 5 min of hormone infusion into portal vein, extracts from adipose tissue were obtained and immunoprecipitated with anti-IRS-2 antibody and immunoblotted with antiphosphotyrosine antibody. The GH-stimulated phosphorylation of IRS-2 was reduced to \( 24 \pm 8\% \) (\( n=5 \)) in old rats compared with the young group (\( 100 \pm 24\% \); \( n=6 \); \( p<0.05 \)) (Fig 1C).

Since the involvement of Shc in tyrosine kinase signaling pathways appears to require its phosphorylation, and considering that GH has already been demonstrated to promote the tyrosyl phosphorylation of Shc proteins in 3T3-F442A fibroblasts (23), we examined whether GH could induce Shc tyrosine phosphorylation in rat liver. Adipose tissue extracts were removed and homogenized after the portal vein infusion of GH. The solubilized proteins were immunoprecipitated with \( \alpha \)Shc and the presence of phosphorylated tyrosines was assessed by Western blotting with \( \alpha \)PY. Increased tyrosyl phosphorylation of a protein migrating at Mr ~52,000 (appropriate for Shc) was observed within 1 min and was 3-fold above basal 5 min after the infusion of GH (data not shown). In contrast to what was observed with the other proteins, tyrosyl phosphorylation of Shc stimulated by GH was not different in old rats (\( 102 \pm 26\% \); \( n=4 \)) when compared with young animals (\( 100 \pm 34\% \); \( n=4 \); \( p=0.959 \)) (Fig 1D).

Regulation of JAK-2, IRS-1, IRS-2 and Shc proteins in liver of old rats

The results of time course experiments with liver were quite similar to that obtained with adipose tissue extracts (data not shown). Therefore, extractions of liver were performed after 5 min of GH infusion into portal vein.

In liver samples previously immunoprecipitated with anti-JAK2 antibody and immunoblotted with antiphosphotyrosine antibody, there was a slight, but no statistically significant, decrease in GH-stimulated JAK2 tyrosyl phosphorylation of 18-month-old rats (\( 80 \pm 10\% \); \( n=6 \)) when compared with 6-week-old rats (\( 102 \pm 11\% \); \( n=5 \); \( p=0.224 \)). (Fig 2A).
To better define the level of IRS-1 phosphorylation, we performed a Western blot analysis of the tyrosyl-phosphorylated proteins in anti-IRS-1 immunoprecipitates before and after stimulation with GH in both group of animals. Fig 2B shows that IRS-1 tyrosyl phosphorylation after GH stimulation was unaltered in old animals (122 ± 21%; n=6) compared with control group (100 ± 19%; n=6; p=0.446). (Fig 2B).

In agreement with the behavior of IRS-1, the GH-induced tyrosine phosphorylation of IRS-2 was not change in liver of old rats (108 ± 28%; n=6) compared with the young animals (100 ± 26%; n=6; p=0.84) (Fig 2C).

GH-stimulated Shc tyrosine phosphorylation in liver was determined by immunoblotting of cell lysates with anti-antiphosphotyrosine antibody after samples from liver were previously immunoprecipitated with anti-Shc antibody. Similar to that observed in adipose tissue, aging had no effect in insulin-stimulated Shc tyrosine phosphorylation and the values were 101 ± 11% for 18-month-old rats (n=6) and 100 ± 9% for the controls (n=6; p=0.918) (Fig 2D).

DISCUSSION

Aging is associated with a series of progressive alterations in body composition characterized by a contraction in lean body mass and an expansion of adipose tissue (1,2). Previous studies indicated that a decline in plasma IGF-1 contributes to the decrease of protein synthesis and tissue function that is associated with normal aging (4, 6,10). The decrease in IGF-1 with age appears to result from a deficiency in high-amplitude growth hormone secretion (5-9). On the other hand, studies in aged Long-Evans rats demonstrated a striking rise in the number of hepatic GH receptors (GHR) in old animals despite decreased plasma IGF-1 levels in these group (14).

Binding of GH to its specific receptor determines dimerization of two GHR and stimulation of JAK2 kinase activity with subsequent phosphorylation of itself and the GHR, as an early response (17, 18, 30). Secondary events include GH-stimulated tyrosine
phosphorylation of a number of intracellular signaling molecules, including IRS-1, IRS-2 and Shc (19-24). Recently, it was demonstrated that first steps of GH-signal transduction were impaired in liver of 30-months-old mice, characterized by a decreased tyrosyl phosphorylation of GHR and JAK2 (25, 26). In agreement with these findings, our study showed a decrease in GH-stimulated JAK2 tyrosyl phosphorylation in adipose tissue of 18-month-old rats. In liver of the same animals, GH-induced JAK2 tyrosyl phosphorylation showed a tendency to decrease, without statistic significance. Considering that there is an increase in protein level of hepatic GHR in aged rats (14) and that liver contains the highest GHR expression (18), it is possible that these facts contribute to the stable JAK2 tyrosyl phosphorylation observed in liver of 18-months-old rats.

Adipose tissue has long been recognized as a target for GH action. Data derived from both experimental animals and clinical studies provide compelling evidence that GH influences the formation and the function of fat cells and that it has an imprecisely defined role in the overall regulation of fat metabolism (31). The 50-year-old report that rats treated with growth-hormone pituitary extracts resulted in a decrease in carcass fat (32) has been repeatedly confirmed with purified GH (33). On the other hand, GH-deficient well nourished children present a characteristic mildly obese state that is reversed by replacement therapy (34). GH-deficient children posses fewer but larger adipocytes and with a higher fat content than normal children. After long-term treatment with GH, such findings reverse (35), with an increase in adipocyte cell number, probably due to the hastening effects of GH in the conversion of fibroblasts to adipocytes (36). In middle and late adulthood, the lean body mass shrinks and the mass of adipose tissue expands (1, 2). It has been proposed that reduced availability of GH in late adulthood may contribute to such changes (2).

GH action upon adipocytes seems to be exerted directly without IGF-1 participation, as GH-mediated lipolysis is not altered when incubating tissue with anti-IGF-1 antibodies (31). Ours results showed that aging in rats is associated with a decreased GH-stimulated JAK2, IRS-1 and IRS-2 tyrosyl phosphorylation in adipose tissue, characterizing a state of impairment in GH signal transduction. The signaling pathway of GH downstream IRS-1 and IRS-2 phosphorylation is not yet understood and seems to differ somewhat from
that activated by insulin. Tyrosyl phosphorylation of IRS-1 and IRS-2 in response to insulin provides binding sites for specific proteins containing SH2 domains, including phosphatidylinositol (PI) 3-kinase (37, 38), Grb2 (39) and tyrosine phosphatase SHPTP2 (40). It is well known that GH can stimulate binding of IRS-1 to PI 3-kinase, but a recent study has shown that PI 3-kinase activity stimulated by GH, in contrast from what was observed for PI 3-kinase activity stimulated by insulin, has no effect on glucose uptake or on the translocation of GLUT4 (41). Therefore, it seems reasonable to speculate that decreased GH-induced tyrosyl phosphorylation of JAK2, IRS-1 and IRS-2 in adipose tissue might contribute to the decrease in anti-lipolytic action of GH observed in aging, but further studies will be necessary to clarify the specific mechanisms and proteins involved in the decreased GHR signal transduction with age.

In addition to its important role in coupling to IRSs signaling pathway, GH activates pathways involving Shc (23, 24). Shc is thought to function as an adaptor molecule to recruit Grb2-mSos1 complexes to the activated receptor (42). Recruitment of Grb2 in response to GH initiates one pathway that leads to the activation of MAP kinases that are believed to be important in the regulation of growth and differentiation (43). We observed that GH-induced tyrosyl phosphorylation of Shc proteins did not change in liver and adipose tissue of 18-month-old rats. These results correlates with a study that investigated the GH-stimulated MAPkinase activation in 17-month- and 30-month-old mice and observed that activation of this protein was only decreased in liver of 30-month-old mice (24).

In summary, the results of this study demonstrated that GH-induced tyrosyl phosphorylation of JAK2, IRS-1 and IRS-2 were decreased in adipose tissue of 18-month-old rats, while no change was observed in tyrosyl phosphorylation of Shc in adipose tissue of old animals after GH stimulation. Otherwise, no change was observed in GH-stimulated JAK2, IRS-1, IRS-2 and Shc tyrosyl phosphorylation in liver of the same animals. These data suggest that aging is characterized by impairment in the GH signaling and there is a substrate- and tissue-specific regulation in the signal transduction pathway for GH that might contribute to the decline in tissue functions that is associated with normal aging.
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FIGURE LEGENDS

Figure 1 - GH-stimulated tyrosine phosphorylation of JAK2, IRS-1, IRS-2 and Shc in intact adipose tissue of 6 weeks-old (C) and 18-months-old (O) rats. Saline (0.9%, lanes 1 and 3 or GH (1.8 mg/kg body weight, lanes 2 and 4) was administered into the portal vein as a bolus injection and 5 min later adipose tissue was excised. Aliquots containing the same amount of protein were immunoprecipitated with anti-JAK2 antibody (A), anti-IRS-1 antibody (B), anti-IRS-2 antibody (C) and anti-Shc antibody (D). All the membranes were immunoblotted with anti-phosphotyrosine antibody(A). Scanning densitometry of autoradiograms was performed on at least 5 experiments. The black bars represent the control group and the empty bars represent the old group. The data are expressed as the mean ± SEM. * At least p<0.05.
Figure 2 - GH-stimulated tyrosine phosphorylation of JAK2, IRS-1, IRS-2 and Shc in intact liver of 6 weeks-old (C) and 18-months-old (O) rats. Saline (0.9%, lanes 1 and 3) or GH (1.8 mg/kg body weight, lanes 2 and 4) was administered into the portal vein as a bolus injection and 5 min later liver was excised. Aliquots containing the same amount of protein were immunoprecipitated with anti-JAK2 antibody (A), anti-IRS-1 antibody (B), anti-IRS-2 antibody (C) and anti-Shc antibody (D). All the membranes were immunoblotted with anti-phosphotyrosine antibody (A). Scanning densitometry of autoradiograms was performed on at least 5 experiments. The black bars represent the control group and the empty bars represent the old group. The data are expressed as the mean ± SEM. * At least p<0.05.
FIGURE 1
FIGURE 2
4. DISCUSSÃO
O hormônio do crescimento (GH) é necessário para o crescimento linear normal, (CASANUEVA, 1992) e também interfere em muitos aspectos do metabolismo tendo sido descrito como um hormônio que exerce efeitos anabólicos, lipolíticos e diabetogênicos (DAVIDSON, 1987). A fim de exercer seus efeitos biológicos, o GH deve ser reconhecido e especificamente ligado ao seu receptor de membrana (GHR) das células-alvo que não contêm um sitio tirosina quinase intrínseco (LEUNG et al., 1987). Entretanto, a ligação do GH a seu receptor determina rápido aumento de fosforilação em tirosina de múltiplas proteínas celulares (CAMPBELL, CHRISTIAN, CARTER-SU, 1993). Em cultura de células, está bem demonstrado que em resposta à ligação do GH a seu receptor dimerizado ocorre fosforilação e ativação da proteína JAK-2 (ARGETSINGER et al., 1993; SILVA et al., 1994) e estímulo à fosforilação em tirosina dos substratos 1 e 2 do receptor de insulina (IRS-1 e IRS-2) (SOUZA et al., 1994; RIDDERSTRALE, DEGERMAN, TORNQVIST, 1995; ARGETSINGER et al., 1995; ARGETSINGER et al., 1996) e SHC (VANDERKURR et al., 1995).

Embora estudos em cultura de células tenham fornecido importantes informações dos mecanismos intracelulares de sinalização do GH, os achados neste sistema experimental podem não refletir as reações de sinalização que ocorrem em resposta ao GH em tecidos animais intactos. Assim, a caracterização da transmissão do sinal do GH em animais in vivo e sua modulação em situações fisiopatológicas adquirem importância para melhor compreensão da transmissão do sinal deste hormônio.

Tendo conhecimento dos mecanismos celulares de sinalização do GH podemos considerar que a dimerização do GHR é um passo crítico na mediação dos efeitos biológicos deste hormônio. Tendo acesso a um antagonista, G120K-PEG que é capaz de bloquear a dimerização do GHR, seria interessante verificar os efeitos do G120K-PEG na fosforilação em tirosina de proteínas envolvidas na transmissão do sinal do GH colaborando para avaliação deste composto como possível droga na terapia de doenças causadas por excesso de GH.

Os conhecimentos atuais apontam o GH como um hormônio capaz de exercer importantes ações em tecido renal (HAMMERMAN et al., 1993). Vários estudos indicam que o GH possa exercer algum papel no aumento renal e alterações nas funções renais que
ocorrem em condições como hipersomatotropismo, hipertrofia compensatória e diabetes mellitus. Nesse sentido, torna-se importante investigação da transmissão do sinal do GH em tecido renal em modelos fisiopatológicos de crescimento deste tecido, como no crescimento renal compensatório a uninefrectomia e em rins de animais diabéticos.

Estudos prévios indicam que o estado catabólico, característico de animais e homens senis, está associado a uma redução na alta amplitude de secreção de GH, com um concomitante declínio na concentração plasmática de IGF-1 (FLORINI et al., 1981; SONNTAG et al., 1980; RUDMAN et al., 1981; BREESE, INGRAM, SONNTAG, 1991; KAHLER et al., 1986), atribuindo a estas alterações principal fator na queda da síntese protéica com o aumento da idade (RUDMAN et al., 1990; SONNTAG, HYLKA, MEITES, 1984; JOHANSON & BIZZARD, 1981). Embora um declínio na secreção de GH seja um importante aspecto no declínio da IGF-1, seria interessante investigar se alguma alteração na transmissão do sinal do GH seria importante neste estado catabólico da senilidade.

Desta maneira, desenvolvemos nossos estudos a fim de caracterizar a transmissão do sinal do GH em diversos tecidos de animais intactos in vivo, bem como a modulação desta sinalização pela droga bloqueadora do GH, G120K-PEG. Posteriormente, analisamos o comportamento da sinalização deste hormônio em diferentes situações fisiopatológicas. Trabalhamos então com modelos animais de crescimento renal, avaliando a transmissão do sinal do GH em rins contralaterais de ratos submetidos a uninefrectomia e em rins de ratos diabéticos. Outro modelo animal estudo foi o rato senil, onde descrevemos a sinalização do GH em tecidos adiposo e hepático de ratos de 18 meses.

4.1. TRANSMISSÃO DO SINAL DO HORMÔNIO DO CRESCIMENTO (GH) EM TECIDOS ANIMAIS.

Sabe-se que o GH mimetiza as ações da insulina (DAVIDSON, 1987), sendo que estes efeitos são transitórios e incluem aceleração do transporte de glicose (GOODMAN, 1968), glicogênese (HONEYMAN & GOODMAN, 1980), glicólise (GOODMAN, 1968), antilipólise (PRESS, 1988) e aceleração da conversão de piruvato e
outros precursores lipogênicos a triglicerídeos (GOODMAN, 1981; SMAL et al., 1987). Estas respostas iniciais do GH semelhantes às da insulina sugerem que ambos os hormônios possam ativar algumas vias comuns de sinalização.

IRS-1 é o principal substrato do receptor de insulina (WHITE & KAHN, 1994) e os resultados apresentados aqui mostram que o tratamento com GH leva a uma rápida mudança na fosforilação em tirosina do IRS-1 in vivo, em concordância com os estudos usando cultura de adipócitos (SOUZA et al., 1994; RIDDERSTRALE et al., 1995) e fibroblastos 3T3-F442A (ARGETSINGER et al., 1995). Em contraste, um estudo anterior mostrou que em figado e músculo de ratos alimentados não houve aumento na fosforilação em tirosina do IRS-1 após estímulo com GH (CHOW et al., 1996). Esta discrepância pode refletir o fato de que o alto nível de insulina nestes animais alimentados manteve o IRS-1 suficientemente fosforilado fazendo com que nenhum efeito do GH tenha sido visto. A despeito da alta fosforilação basal do IRS-1 em animais alimentados, a administração de insulina ainda pode aumentar a fosforilação em tirosina deste substrato. Esta observação está de acordo com os achados de que em cultura de células a fosforilação do IRS-1 foi estimulada mais intensamente pela insulina do que por concentrações elevadas de GH (SOUZA et al., 1994; ARGETSINGER et al., 1995). Maior estimulação do IRS-1 pela insulina do que pelo GH pode também refletir diferenças nas afinidades relativas do IRS-1 com o receptor quinase de insulina e com a quinase ativada pelo GH. Então, em condições fisiológicas, o GH parece estimular a fosforilação em tirosina do IRS-1 quando o nível plasmático de insulina está baixo.

Os resultados do presente estudo e aqueles da publicação que evidenciou fosforilação em tirosina do IRS-2 em fibroblastos 3T3-F442A (ARGETSINGER et al., 1996) em resposta ao GH sugerem que ambos os membros da família IRS têm papel na sinalização do GH. Claramente, existe alguma sobreposição das funções do IRS-1 e IRS-2, já que ambos se ligam a PI 3-quinase, Grb2 e SHP2 em resposta ao GH. Devem existir papéis distintos para o IRS-1 e IRS-2 na sinalização do GH que advenham das variações na expressão tecidual destas proteínas.

Discussão

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Em células estimuladas pela insulina, as associações da PI 3-quinase com IRS-1 (BACKER et al., 1992) e IRS-2 (SUN et al., 1995) fosforilados em tirosina ativam esta enzima. Assim, a capacidade do GH em estimular a associação do IRS-1 e IRS-2 com a subunidade regulatória de 85 kDa da PI 3-quinase sugere que o GH ativa a PI 3-quinase. Um potencial papel para a PI 3-quinase na ativação dos efeitos metabólicos “insulina-like” do GH é sugerido pelos achados que o inibidor da PI 3-quinase, “wortmannin”, bloqueia a capacidade do GH em estimular a síntese lipídica (RIDDERSTRALE & TORNQVIST, 1994). Em contraste, outro estudo mostrou que a atividade da PI 3-quinase estimulada pelo GH não tem nenhum efeito na captação de glicose ou na translocação do GLUT4 (SAKAUE et al., 1997). Por outro lado, a PI 3-quinase pode estar envolvida na regulação da proteína quinase C. Demonstrou-se que os produtos da PI 3-quinase, fosfatidilinositol 3,4-bífósfato e fosfatidilinositol 3,4,5-trífósfato ativam as isoformas γ, ε e η da proteína quinase C independente de Ca²⁺ (TOKER et al., 1994). As observações de que o GH induz a translocação da isoforma ε da proteína quinase C do citoplasma para a membrana em fibroblastos 3T3-F442A (OKADA & KOPCHICK, 1995) e que o inibidor da PI 3-quinase, wortmannin, inibe a formação de diacilglicerol dependente de GH em adipócitos de ratos (ROUPAS & HERRINGTON, 1995) aumenta a possibilidade de que em algumas células a PI 3-quinase possa estar envolvida na ativação da proteína quinase C dependente de GH.

O papel da SHP2 na sinalização do GH tem sido avaliado de várias formas, mas permanece não definido. SHP2 é provavelmente ativada durante associação com IRS-1 e IRS-2 e pode desfosforilar sinalizadores intermediários localizados tanto no complexo sinalizador do IRS-1 quanto em sítios distantes, desta forma regulando o sinal (MYERS & WHITE, 1996). O papel da SHP2 na sinalização do GH parece incluir as mesmas funções reguladas por esta proteína quando ativada pela insulina.

Nossos dados indicam que em adição ao seu importante efeito em ativar as vias de sinalização do IRS-1 e IRS-2, GH ativa as vias de sinalização envolvendo o SHC. Esta conclusão está de acordo com os relatos mostrando que em fibroblastos 3T3-F442A há um aumento na fosforilação em tirosina do SHC após tratamento com GH (VANDERKUUR et al., 1995). Acredita-se que o SHC funcione como uma molécula adaptadora que recruta o complexo Grb2-mSos1 ao receptor ativado (ARONHEIM et al., 1994). O fator nucleotídeo
de troca mSos1 então promove a formação de p21 Ras (GTP), iniciando, desta forma, a cascata de eventos de fosforilação de fatores transcricionais específicos no núcleo (ARONHEIM et al., 1994; HILL & TREISMAN, 1995). Os achados neste estudo de que Grb2 co-precipita com ambas as proteínas IRSs e SHC são consistentes com a ativação da MAP quinase pelo GH. Estes dados sugerem que em adição aos efeitos mitogênicos que são induzidos pela IGF após estímulo com GH, GH pode ter papel central direto na regulação do crescimento e diferenciação celulares.

Algumas das fosforilações induzidas pelo GH e descritas aqui em tecidos animais são catalisadas pela quinase associada ao GHR identificada como JAK2 (ARGETSINGER et al., 1993; SILVA et al., 1994; WANG et al., 1993), enquanto outras são catalisadas por quinases a jusante (CAMPBELL, CHRISTIAN, CARTER-SU, 1993). Nossos experimentos mostraram que em figado, os tempos de fosforilação em tirosina do IRS-1 refletiram aqueles da fosforilação em tirosina do JAK2 induzida pelo GH, e esta correlação sugere que a ativação do JAK2 possa ser necessária para fosforilação em tirosina do IRS-1. Mais ainda, co-imunoprecipitação entre JAK2 e IRS-1 e os resultados do ensaio quinase in vitro demonstraram que uma quinase ativada pelo GH, presumivelmente JAK2, esteve significativamente aumentada após infusão de GH na veia porta. Consistente com o envolvimento do JAK2 na fosforilação em tirosina do IRS-1, demonstrou-se em estudo anterior que a capacidade de vários mutantes do GHR em mediar a fosforilação em tirosina do JAK2 dependente de GH correlacionou-se com a quantidade detectada de IRS-1 fosforilado em tirosina (ARGETSINGER et al., 1995).

O aumento da fosforilação em tirosina de IRS-1, IRS-2, JAK2 e SHC em figado, coração, rim, músculo e tecido adiposo após estímulo com GH está de acordo com os achados de que o GHR é uma proteína expressa na membrana das células de todos estes tecidos (MATHEWS, ENBERG, NORSTEDT, 1989; TIONG & HERINGTON, 1991). A capacidade do GH de ativar estas vias de sinalização em diferentes tecidos permite a este hormônio exercer seus diversos efeitos metabólicos e de crescimento.
4.2. G120K-PEG, UMA ANTAGONISTA DO GH.

Na superfamília de receptores de citocinas, incluindo o GHR, uma molécula ligante promove dimerização de subunidades heterólogas de receptores ou dimerização de duas subunidades idênticas (WELLS, 1994; KISHIMOTO, TAGA, AKIRA, 1994). A dimerização do GHR ocorre sequencialmente, isto é, o GH se liga ao primeiro GHR através do sítio 1 do GH e subsequentelemente se liga ao segundo GHR através do sítio 2 do GH (CUNNINGHAM et al., 1991). Acredita-se que a dimerização do GH ativa a quinase JAK2, a qual fosforila a mesma, o GHR e componentes sinalizadores a jusante (ARGENTSINGER & CARTER-SU, 1996).

G120K-PEG é uma variante do GH que tem uma mutação (uma lisina ou arginina na posição 120) realizada no sítio 2, resultando em interrupção significante na ligação a este sítio e, assim, a dimerização dos GHRs é evitada. Nossos resultados demonstraram que o G120K-PEG diminuiu a fosforilação em tirosina do JAK2 induzida pelo GH, estando de acordo com os estudos que demonstraram a importância da dimerização do receptor na transmissão do sinal (SILVA, WEBER, THORNER, 1993).

Em nossos experimentos, o efeito inibitório do G120K-PEG foi máximo com uma proporção de 1:100 de GH:G120K-PEG, já que nenhuma fosforilação em tirosina do JAK2 foi observada com está dose de GH. Quando se aumentou a dose de GH atingindo-se uma proporção de 1:10 de GH:G120K-PEG, alguma fosforilação do JAK2 pode ser observada. Adicionalmente, as fosforilações em tirosina induzidas por GH de JAK2, IRS-1, IRS-2 e SHC mostraram-se inibidas em aproximadamente 50% a concentrações equimolares do antagonista do GH. Estes resultados demonstrando que as fosforilações em tirosina de componentes sinalizadores a jusante podem ser reduzidas por G120K-PEG, reforçando a ideia de que a sinalização do GH está ligada a formação de dímeros do GHR.

A primeira ação biológica atribuída ao GH foi promoção de crescimento esquelético e orgânico (ISAKSSON et al., 1987; GREEN, MORIKAWA, NIXON, 1985), como observado em síndromes clínicas como acromegalia. Acromegalia é uma alteração hormonal resultante de um tumor hipofisário produtor de excesso de GH, e que leva a sérias consequências incluindo diabetes mellitus, hipertensão, aumento de risco de doenças cardiovasculares e câncer (MELMED, 1990).
As opções de tratamento disponíveis incluem ressecção cirúrgica do tumor, radiação e terapêutica com drogas. Nossos resultados mostraram que o G120K-PEG é capaz de atenuar os efeitos do GH em nível celular, tornando esta droga particularmente útil em casos em que o tumor não expressa receptores para somatostatina e não é responsiva a inibidores da somatostatina. Outro antagonista do GHR mostrou-se ser efetivo no tratamento da acromegalia (TRAINER et al., 2000).


4.3. REGULAÇÃO DA TRANSMISSÃO DO SINAL DO GH EM TECIDO RENAL.

4.3.1. Nefrectomia.

O crescimento compensatório do rim contralateral que se segue a nefrectomia unilateral em humanos ou animais experimentais (FAGIN & MELMED, 1987) é demonstrável ao fim de 1 a 2 dias. O crescimento envolve primariamente os glomérulos e
túbulos proximais (FINE, 1986). Os glomérulos são morfologicamente e funcionalmente normais após nefrectomia em ratos a não ser que o tecido renal remanescente esteja muito reduzido, quando neste caso glomeruloesclerose se estabelece (HOSTETTER et al., 1981). A regulação hormonal da hipertrofia compensatória não é totalmente compreendida, entretanto os níveis totais de mRNA de IGF-1 estão aumentados após nefrectomia unilateral (STILES, SOSENKO, D’ERCOLE, 1985; FINE, 1986). Isto sugere um papel para este hormônio na hipertrofia do rim adulto bem como no crescimento normal (SCHOENLE et al., 1982). Os fatores de crescimento que atuam no crescimento desordenado característico de algumas doenças glomerulares não estão bem elucidados. Entretanto, existem anormalidades nos níveis circulantes de GH em algumas doenças associadas com aumentos na matriz extracelular glomerular e número de células (IKKOS, LJUNGGREN, LUFT, 1956; CHRISTIANSEN et al., 1981; CHRISTIANSEN, 1984) e dentre outros neuropeptídeos e neurohormônios, tem se mostrado que o GH modula o desenvolvimento do dano glomerular em várias doenças renais (BAUD et al., 1999).

Nossos resultados demonstraram que as fosforilações em tirosina das proteínas JAK2, IRS-1, IRS-2 e SHC induzidas por GH não se alteraram em rins de ratos um, quatro ou sete dias após nefrectomia unilateral. Assim, nossas observações mostram que estas etapas iniciais da transmissão do sinal do GH não parecem estar envolvidas na hipertrofia renal observada após nefrectomia unilateral. Um papel para o GH no crescimento renal compensatório foi inicialmente postulado porque hipofisectomia inibe a resposta compensatória (FINE, 1986). Entretanto, nossos resultados estão de acordo com dados da literatura que observaram que o crescimento compensatório e o aumento no conteúdo de IGF-1 ocorrem em ratos hipofisectomizados (STILES, SOSENKO, D’ERCOLE, 1985) e em ratos deficientes de GH (EL NAHAS, LE CARPENTIER, BASSET, 1990; BASINGER & GITTES, 1975), consistente com o crescimento renal ser mediado por um processo independente de GH.

Estudos recentes mostraram que o crescimento precoce acelerado do rim remanescente após Unx ocorre através de mecanismos diversos em ratos machos jovens e adultos, o que pode direcionar o tipo de crescimento renal que ocorre após Unx. O CRC inicial em rato adultos machos é dependente de GH, mas independente de mudanças no sistema de IGF-1. Em contraste, o CRC é independente de GH em ratos machos jovens,
mas é associado com significante aumento na IGF-1 renal e crescimento hiperplásico do rim (MULRONEY & PESSE, 2000). Nossos dados estão de acordo com este estudo já que nossos experimentos foram realizados com ratos jovens e observamos que a transmissão do sinal do GH não se mostrou alterada em rins de ratos submetidos a Unx.

4.3.2. Diabetes Mellitus / G120K-PEG.

O estabelecimento de diabetes mellitus em humanos e animais experimentais é acompanhado por rápido crescimento do tecido renal, aumento na taxa de filtração glomerular e aumento no fluxo plasmático renal (HAMMERMAN, O’SHEA, MILLER, 1993). Rins de ratos diabéticos são morfologicamente caracterizados por aumento no tamanho das células do túbulo proximal (SEYER-HANSEN, 1980; SCHWEIGER & FINE, 1990). Acredita-se que a hiperтроfia glomerular seja uma das mudanças-chave iniciais no desenvolvimento da nefropatia diabética (CHEN et al., 1995a; FOGO & ICHIKAWA, 1991). Vários sistemas hormonais têm sido implicados como fatores na nefropatia diabética inicial. Tem se fortemente sugerido o envolvimento de GH e IGF-1 através de estudos do impacto de excesso ou déficit de GH (DOI et al., 1990; CHEN et al., 1995a; FLYVBJERG et al., 1992; MUCHANETA-KUBARA et al., 1994) no crescimento e na função renais em vários modelos animais, tanto de diabéticos (CHEN et al., 1995a; FLYVBJERG et al., 1992; MUCHANETA-KUBARA et al., 1994) e não diabéticos (DOI et al., 1990). Nestes estudos, tem se notado uma relação entre hipertrofia renal e atividade de IGF-1 e GH, e entre este último e glomeruloesclerose (DOI et al., 1990; CHEN et al., 1995a; FLYVBJERG et al., 1992; MUCHANETA-KUBARA et al., 1994).

No presente estudo demonstramos que as fosforilações em tirosina do JAK2, IRS-1 e SHC estimuladas por GH estão aumentadas em rins de ratos e camundongos diabéticos, sugerindo que estas alterações nos passos iniciais de transmissão do GH possam ter um papel na nefropatia diabética. Nossos resultados estão de acordo com outros estudos que dão suporte para um papel do GH na doença renal diabética, visto que ratos deficientes em GH com diabetes são relativamente protegidos dos efeitos renais típicos de diabetes presentes nos animais sem esta deficiência hormonal (FLYVBJERG et al., 1992;
MUCHANETA-KUBARA et al., 1994), enquanto animais transgênicos com expressão de excesso de GH desenvolvem hipertrofia glomerular, albuminúria e glomeruloesclerose, uma sequência de eventos semelhantes à evolução da nefropatia diabética (DOI et al., 1990; CHEN et al., 1995a). De forma similar, camundongos transgênicos expressando excesso de proteína ligante de IGF-1 tiveram elevados níveis de GH e desenvolveram hipertrofia mesangial e glomeruloesclerose, a despeito de diminuição dos níveis plasmáticos de IGF-1 (DOUBLIER et al., 1997). Estes estudos são consistentes com um processo dependente de GH através do qual a nefropatia diabética é mediada e nossos resultados fornecem dados sobre a sinalização do GH que reforçam estas informações anteriores.

Estudos recentes identificaram que níveis elevados de diacilglicerol (DAG) iniciados por hiperglicemia e a ativação da proteína quinase C (PKC) são associados com muitas anormalidades vasculares em retina, rins e tecidos cardiovasculares (SHIBA et al., 1993; INOGUCHI et al., 1992; CRAVEN, DAVIDSON, DERUBERTIS, 1990; ISHII et al., 1996). Tem-se mostrado que o GH determina rápido aumento transitório no DAG em múltiplos tipos celulares (DOGLIO et al., 1989; JOHNSON et al., 1990; ROUPAS & HERRINGTON, 1995). Em adipócitos de ratos, a produção de DAG induzida por GH é bloqueada por genisteína, um bloqueador de tirosina quinase, o que também inibe JAK2 (ARGETSINGER & CARTER-SU, 1996). Isto está de acordo com a ativação de PKC por GH situando-se a jusante do JAK2 nestas células. Nossos resultados, mostrando que ocorre aumento no nível de fosforilação em tirosina do JAK2 em rins de animais diabéticos pode sugerir outra via que leva a ativação de DAG-PKC nestes animais.

Também demonstramos que há aumentos na fosforilação do IRS-1 e na associação IRS-1/PI 3-quinase induzidas por GH em rins de ratos diabéticos. A PI 3-quinase é a molécula sinalizadora ativada pelo IRS-1 mais bem estudada, tendo importante papel na regulação de uma vasta quantidade de respostas biológicas incluindo franzimento da membrana, mitogênese e diferenciação (OGAWA, TAKASHI, KASUGA, 1998). Diferentes métodos têm demonstrado que Akt/ PKB, uma serina-treonina quinase com um domínio homólogo a plequstrina, está funcionalmente localizada a jusante da PI 3-quinase (KHON, KOVACINA, ROTH, 1995; FRANKE et al., 1995; BURGERING & COFFER,
1995). Akt/PKB tem sido implicada na mediação de algumas das ações de insulina e GH, incluindo síntese de glicogênio (KHWAJA et al., 1997; VAN WEEREN et al., 1998; COSTOYA et al., 1999). Observamos aumentos nos níveis de fosforilação de Akt em animais diabéticos e desde que a fosforilação de Akt/PKB está diretamente relacionada com sua ativação, podemos sugerir que em rins de ratos diabéticos a atividade de Akt/PKB induzida por GH esteja provavelmente aumentada.

Em microvasos, a atividade aumentada de óxido nítrico (NO), um potente vasodilatador, pode aumentar a taxa de filtração glomerular (BANK & AYNEDJIAN, 1993). Têm sido relatados aumentos na excreção urinária de NO₂ e NO₃, metabólitos estáveis do NO, em diabetes de curta duração (BANK & AYNEDJIAN, 1993; TOLINS et al., 1993; KOMERS, ALLEN, COOPER, 1994), possivelmente devido ao aumento na expressão do gene induzível da NO sintetase (iNOS) e aumento na produção de NO nas células mesangiais (SHARMA et al., 1995). Evidências recentes sugerem que a ativação do Akt é um passo importante na ativação da NO sintetase e indução da produção de NO (FULTON et al., 1999; LUO et al., 2000). O presente estudo, demonstrando que, em rins de animais diabéticos, a fosforilação de Akt induzida por GH encontra-se aumentada, sugere um possível mecanismo que possa contribuir para aumento na produção de NO nestes animais.

Em adição à PI 3-quinase, o IRS-1 fosforilado em tirosina liga-se a domínios SH2 de várias pequenas proteínas adaptadoras, incluindo Grb2 (THIRONE, CARVALHO, SAAD, 1999; MYERS et al., 1994). Grb2 contém também domínios SH3 que se associam constitucionalmente com mSos, um fator permutador de guanina que estimula permutação de GDP/GTP no p21Ras (ARONHEIM et al., 1994; HILL & TREISMAN, 1995). O recrutamento por receptores de crescimento do Grb2/Sos para membranas contendo p21Ras é um dos mecanismos necessários a ativação da cascata da MAP quinase. Durante estimulação com GH, Grb2 liga-se com IRS-1 e SHC, o que resulta em ativação de MAP quinase (VANDERKURR et al., 1997). Acredita-se que ativação desta enzima leve a mudanças seletivas em genes transcricionais e síntese protéica, e assim parece ser crucial para resposta celular do GH (VANDERKURR et al., 1997; HILL & TREISMAN, 1995). Nefropatia diabética inicial é caracterizada por crescimento excessivo e expansão do
mesângio glomerular (OSTERBY & GUNDERSEN, 1975). Ativação da MAP quinase tem sido implicada no desenvolvimento da doença proliferativa glomerular (BONVENTRE & FORCE, 1998; BOKEMEYER et al., 1997; BOKEMEYER et al., 1998; HANEDA et al., 1997; YAMAGUCHI et al., 1995), resultando em aumento da expressão gênica e aumento na síntese das proteínas da matriz extracelular, incluindo colágenos tipo I e IV, fibronectina e laminina (ROBERTS, MCCUNE, SPORN, 1995). Nossos resultados mostraram que as associações de IRS-1/Grb2 e Shc/Grb2 e fosforilação de MAP quinase induzidas por GH estão aumentadas em rins de animais diabéticos por STZ. Desde que o sinal direcionado por IRS-1/Grb2 e Shc/Grb2 levam a ativação de Ras e MAP quinase e positivamente modula múltiplos eventos celulares ligados a desenvolvimento, crescimento e mitogênese, estes resultados sugerem que aumento na atividade das vias de IRS-1 e SHC na sinalização do GH pode ser fator adicional nos efeitos mitogênicos do GH, tendo papel na nefropatia diabética.

Sendo reconhecido o potencial papel do GH em várias condições fisiopatológicas, incluindo nefropatia diabética, vários antagonistas específicos para ação do GH foram recentemente desenvolvidos para potencial uso terapêutico (TRAINER et al., 2000). Assim, estudos prévios descreveram efeitos reno-protetores de antagonistas de GH em camundongos diabéticos transgênicos que expressam antagonistas de GH (CHEN et al., 1995a; FLYVBJERG et al., 1992; ESPOSITO et al., 1996). Nossos resultados demonstram normalização do aumento renal nos camundongos tratados com G120K-PEG e efeito inibitório na fosforilação em tirosina das proteínas envolvidas na transmissão do sinal do GH, mostrando que o aumento nas fosforilações de JAK2, IRS-1, SHC, MAPK and Akt e os aumentos nas associações IRS-1/PI 3-quinase, IRS-1/Grb2 e SHC/Grb2 em animais diabéticos pode ser prevenida por este antagonista do GHR. Em concordância, dois estudos recentes mostraram proteção contra danos renais associados a diabetes: em camundongos nocautes para GHR (BELLUSH et al., 2000) e após tratamento de camundongos obesos diabéticos com um antagonista do GHR (SEGEV et al., 1999). Assim, nossos resultados sugerem um importante papel para a transmissão do sinal do GH no desenvolvimento das lesões orgânicas finais de diabetes, inferindo que modulação dos efeitos do GH possa ter implicações terapêuticas benéficas na nefropatia diabética.
4.4. REGULAÇÃO DA TRANSMISSÃO DO SINAL DO GH EM TECIDOS HEPÁTICO E ADIPOSO: EFEITO DO ENVELHECIMENTO.


Recentemente demonstrou-se que as etapas iniciais da transmissão do sinal do GH estão deterioradas em figado de camundongos de 30 meses, caracterizadas por decréscimo na fosforilação em tirosina do GHR e JAK2 (XU et al., 1995; XU & SONNTAG, 1996). De acordo com estes achados, nosso estudo mostrou diminuição na fosforilação em tirosina do JAK2 estimulada por GH em tecido adiposo de ratos de 18 meses. No figado destes mesmos animais, a fosforilação em tirosina do JAK2 estimulada por GH mostrou uma tendência a decrescer, sem significância estatística. Considerando que há aumento no nível protéico de GHR em ratos senis (TAKAHASHI & MEITES, 1987) e que o figado contém a mais alta expressão tecidual de GHR (ARGETSINGER et al., 1993), é possível que estes fatos contribuam para a ausência de alterações na fosforilação em tirosina do JAK2 observada no figado de ratos de 18 meses. Um paralelo pode ser feito quando analisamos a transmissão do sinal da insulina em animais diabéticos e em jejum, quando um aumento na expressão tecidual do receptor de insulina contribui para que não ocorram alterações na transmissão do sinal deste hormônio a jusante a sua ligação a seu receptor (SAAD et al., 1992; FOLLI et al., 1993).
Há muito tem se reconhecido que o tecido adiposo é alvo de ação do GH. Dados de estudos experimentais com animais e estudos clínicos fornecem evidências que o GH influencia a formação e função de células gordurosas e regulação do metabolismo de gorduras (GOODMAN et al., 1990). O estudo realizado há mais de 50 anos em que o tratamento de ratos com extratos de GH hipofisário resultou em diminuição da carcaça de gordura (LEE & SCHAFFER, 1934) tem sido repetidamente confirmado com GH purificado (ASTWOOD, 1955). Por outro lado, criança bem nutrida deficiente de GH apresenta uma característica obesidade moderada que é revertida com terapia de reposição (TANNER, HUGHES, WHITEHOUSE, 1977). Crianças deficientes em GH possuem menos, mas maiores adipócitos e têm um conteúdo de gordura maior que crianças normais. Após tratamento por longo prazo com GH tais achados se revertem (BROOK, 1973), com aumento no número de adipócitos, provavelmente devido aos rápidos efeitos do GH na conversão de fibroblastos em adipócitos (GREEN, MORIKAWA, NIXON, 1985). Na fase adulta jovem e tardia, a massa corpórea magra diminui e a massa de tecido adiposo expande (NOVAK, 1972; RUDMAN, 1985). Tem sido proposto que reduzida disponibilidade de GH na fase adulta possa contribuir para estas mudanças (RUDMAN, 1985).

A ação do GH em torno dos adipócitos parece ser exercida diretamente, sem participação de IGF-1, já que a lipólise mediada por GH não está alterada quando tecidos são incubados com anticorpos anti-IGF-1 (GOODMAN et al., 1990). Nossos resultados mostraram que senilidade em ratos está associada com diminuições das fosforilações em tirosina de JAK2, IRS-1 e IRS-2 estimuladas por GH em tecido adiposo, caracterizando um estado de diminuição na transmissão do sinal do GH. Assim podemos especular que diminuição nestas etapas iniciais de transmissão do GH em tecido adiposo pode contribuir para diminuição nas ações antilipônicas do GH observadas com evoluir da idade.

Em adição a seu importante papel na ligação às vias de sinalização de IRSs, GH ativa as vias envolvendo SHC (VANDERKUUR et al., 1995; THIRONE, CARVALHO, SAAD, 1999). Acredita-se que o SHC funcione como uma molécula adaptadora para recrutar o complexo Grb2-Sos ao receptor ativado (ARONHEIM et al., 1994). Recrutamento de Grb2 em resposta a GH inicia uma via que leva a ativação de MAP quinase, que se acredita ser importante para regulação de crescimento e diferenciação.
(CAMPBELL et al., 1992). Observamos que a fosforilações em tirosina do SHC estimuladas por GH não se modificaram em fígado e em tecido adiposo de ratos de 18 meses. Estes resultados correlacionam-se com um estudo em que a investigação da ativação da MAP quinase estimulada por GH em camundongos de 17 e 30 meses em que a ativação desta proteína mostrou-se somente diminuída em fígado de camundongos de 30 meses (XU et al., 1995; XU & SONNTAG, 1996).

Assim, nossos dados sugerem que envelhecimento é caracterizado por alterações na sinalização do GH e que existe regulação substrato específica e tecido específica nas vias de sinalização do GH que podem contribuir para o declínio das funções teciduais associadas com envelhecimento normal.
5. CONCLUSÕES
Nossos estudos em animais *in vivo* mostraram que as proteínas IRSs e SHC servem como moléculas sinalizadoras para o GH em tecidos de ratos. A ativação do GH pela atividade tirosina quinase do JAK2 em direção ao IRS-1 bem como a correlação temporal entre as fosforilações em tirosina do JAK2 e IRS-1, sugerem que o IRS-1 possa interagir primariamente com JAK2, o qual seria mediador da fosforilação em tirosina deste substrato.

G120K-PEG pode modular a transmissão do sinal do GH em camundongos *in vivo* através da sua capacidade de diminuir as fosforilações em tirosina do JAK2, IRS-1, IRS-2 e SHC estimuladas por este hormônio, sugerindo que este antagonista do GH possa ser uma droga potencial na terapêutica de doenças associadas com os efeitos indesejáveis do GH.

Em relação aos modelos de crescimento renal, observamos que as fosforilações em tirosina induzidas por GH das proteínas JAK2, IRS-1, IRS-2 e SHC no rim contralateral de ratos submetidos a uninefrectomia foram semelhantes àquelas dos animais controles. Estes dados sugerem que nenhuma alteração na transmissão do sinal do GH é necessária para a hipertrofia renal compensatória à nefrectomia unilateral. Por outro lado, as fosforilações das proteínas JAK2, IRS-1, SHC, MAPK e Akt e as associações IRS-1/PI 3 quinase, IRS-1/Grb2 e SHC/Grb2 induzidas por GH estão aumentadas em rins de ratos diabéticos por STZ. Estes dados sugerem que diabetes é caracterizado por aumento na sinalização do GH que pode contribuir para o desenvolvimento da doença renal diabética. Também, administração de G120K-PEG em camundongos diabéticos teve efeitos inibitórios no crescimento renal e no aumento da via de sinalização do GH observada em animais diabéticos. Demonstramos assim alterações nas etapas iniciais da transmissão do sinal do GH em rins de ratos e camundongos diabéticos e sugerimos que um bloqueador específico do GHR pode prevenir estas alterações e representar um novo conceito no tratamento da doença renal diabética.

Considerando o modelo de senilidade, as fosforilações estimuladas por GH das proteínas JAK2, IRS-1 e IRS-2 estão diminuídas em tecido adiposo de ratos de 18 meses, enquanto nenhuma alteração foi observada na fosforilação em tirosina da proteína SHC em tecido adiposo de ratos senis após estímulo com GH. Por outro lado, nenhuma alteração foi
observada nas fosforilações em tirosina de JAK2, IRS-1, IRS-2 e SHC estimuladas por GH no figado destes mesmos animais. Assim, nossos dados sugerem que envelhecimento é caracterizado por alterações na sinalização do GH que podem contribuir para o declínio das funções teciduais associadas com envelhecimento normal.
6. SUMMARY
Growth hormone (GH) stimulates the tyrosine phosphorylation of various cellular polypeptides, including the GH receptor itself, in an early part of the intracellular response. This study demonstrated that GH increased the tyrosine phosphorylation of IRS-1, IRS-2, JAK2 and Shc proteins in the liver, heart, kidney, muscle and adipose tissue of rats and that GH stimulated the association of IRS-1/2 with PI 3-kinase, Grb2 and SHP2 and of Shc with Grb2. The correlation between JAK2 tyrosyl phosphorylation and IRS-1 tyrosyl phosphorylation in response to GH, together with the results of the in vitro tyrosine kinase assay, is consistent with that JAK2 might mediate the phosphorylation of IRS-1 induced by GH.

G120K-PEG, a GH antagonist, is a genetically-engineered analog of GH produced by a mutation that blocks GH action by preventing the GHR dimerization. This study showed that in mice treated with G120K-PEG, the GH-induced tyrosyl phosphorylation of JAK2, IRS-1, IRS-2 and SHC was reduced compared with the controls. These data suggest that G120K-PEG modulates the signal transduction of GH and might be a potential drug therapy in diseases caused by excess of GH.

Recent investigations have suggested a role for growth hormone (GH) and insulin-like growth factor-I (IGF-I) in the pathogenesis of experimental renal growth and scarring. The results of this study demonstrated that GH-induced tyrosyl phosphorylation of JAK2, IRS-1, IRS-2 and SHC in kidney of Unx rats were similar to that of control animals. These data suggest that no alteration in GH signal transduction is necessary for the compensatory hypertrophy response to unilateral nephrectomy.

Although controversial, a role for GH and IGF-I has been suggested in the development of diabetic kidney disease. The implicated effect of GH in diabetic end organ damage may be mediated by GH receptor (GHR) or postreceptor events in GH signal transduction. Our results demonstrated that GH-induced phosphorylation of JAK2, IRS-1, Shc, MAPK and Akt and association of IRS-1/PI3K, IRS-1/Grb2 and Shc/Grb2 were increased in kidney STZ-diabetic rats and the administration of G120K-PEG in diabetic mice showed inhibitory effects on diabetic renal enlargement and reversed the alterations in GH signal transduction observed in diabetic animals. The present study demonstrates a role for GH in the pathogenesis of early diabetic renal changes and suggests that specific GHR blockade may present a new concept in the treatment of diabetic kidney disease.
Biological aging is a catabolic state characterized by a decline in tissue protein synthesis and tissue function, associated with a reduction in high amplitude growth hormone (GH) secretion and a concomitant decline in plasma concentrations of insulin-like growth factor I (IGF-1). This study demonstrated that GH-induced tyrosyl phosphorylation of JAK2, IRS-1 and IRS-2 decreased in adipose tissue of 18-month-old compared with 6-week-old rats, while no change was observed in Shc tyrosyl phosphorylation after GH stimulation. Aging had no effect in GH-stimulated tyrosyl phosphorylation of JAK2, IRS-1, IRS-2 and Shc proteins in the liver. These data suggest that the early steps of GH signal transduction is impaired in adipose tissue of old rats and this may contribute to the decline in tissue function present in aging.
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