TIAGO GOMES ARAÚJO

“CARACTERIZAÇÃO DO PAPEL DO HGF COMO ELO ENTRE O AUMENTO DA MASSA DA ILHOTA/HIPERINSULINEMIA E A RESISTÊNCIA À INSULINA”

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UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE CIÊNCIAS MÉDICAS

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Orientador(a): Prof. Dr. Mario Jose Abdalla Saad

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SUMÁRIO

LISTA DE ABREVIATURAS

IRS-1 – substrato do receptor de insulina – 1

IRS-2 - substrato do receptor de insulina – 2

IR – receptor de insulina

IGF-I – fator do crescimento do tipo insulina I

PI3K – fosfatidilinositol-3-quinase

Domínio PH – *pleckstrin homology*

HGF – fator de crescimento do hepatócito

HAI-1 – inibidor do ativador de HGF

INKs – cinases inibitórias

CIPs – proteínas inibitórias de ciclinas

KIPs - proteínas inibitórias de cinases

GLUT-4 – *Glucose Transporter 4*

GLUT-2 – *Glucose Transporter 2*

PDX-1 – pancreatic and duodenal homeobox-1

NF-κB – Fator de Transcrição Nuclear κB

Resumo

A resistência à insulina está presente na obesidade e na diabetes tipo 2, e está associada à hiperplasia das ilhotas pancreáticas e hiperinsulinemia como resposta compensatória, entretanto, as forças motrizes por trás desse mecanismo compensatório não são totalmente compreendidos. Dados anteriores sugeriram o envolvimento de um fator circulante desconhecido que na resistência à insulina atua como um fator de crescimento das células β. Neste contexto, procurando por candidatos a serem este fator circulante, percebemos que o fator de crescimento de hepatócitos (HGF) é um forte candidato a ser este elo entre a resistência à insulina e o aumento da massa de ilhotas / hiperinsulinemia. Nossa abordagem teve como objetivo mostrar uma possível relação de causa-efeito entre o aumento dos níveis circulantes de HGF e a hiperplasia da ilhota / hiperinsulinemia compensatória, assim mostrando a força da associação. Ainda, se esta associação, apresenta ou não uma resposta dose-dependente, temporalidade, consistência, plausibilidade e reversibilidade. Nesse sentido, os nossos dados mostraram: a) uma correlação forte e consistente entre o HGF e o mecanismo de compensação em três modelos animais de resistência à insulina; b) o HGF aumenta a massa de célula β de uma forma dose-dependente; c) o bloqueio do HGF interrompe os mecanismos de compensação; d) o aumento nos níveis de HGF precede a resposta compensatória associada com a resistência à insulina, indicando que estes eventos ocorrem em um modo sequencial. Além disso, o bloqueio do receptor de HGF (Met) piorou a já prejudicada sinalização da insulina no fígado de ratos obesos induzidos por dieta. Em geral, os nossos dados indicam que o HGF é um fator de crescimento que desempenha um papel fundamental no aumento da massa de ilhotas e hiperinsulinemia em ratos obesos induzidos por dieta, e sugerem um efeito protetor da interação HGF-Met na sinalização de insulina no fígado.

Abstract

Insulin resistance is present in obesity and in type 2 diabetes, and is associated with islet cell hyperplasia and hyperinsulinemia but the driving forces behind this compensatory mechanism are incompletely understood. Previous data have suggested the involvement of an unknown circulating insulin resistance-related β-cell growth-factor. In this context, looking for candidates to be a circulating factor, we realized that hepatocyte growth factor (HGF) is a strong candidate as a link between insulin resistance and increased mass of islets/hyperinsulinemia. Our approach aimed to show a possible cause-effect relationship between increase in circulating HGF levels and compensatory islet hyperplasia/hyperinsulinemia by showing the strength of the association, whether or not is a dose-dependent response, the temporality, consistency, plausibility and reversibility of the association. In this regard, our data showed: a) a strong and consistent correlation between HGF and the compensatory mechanism in three animal models of insulin resistance; b) HGF increases β-cell mass in a dose-dependent manner; c) blocking HGF shuts down the compensatory mechanisms; d) an increase in HGF levels seems to precede the compensatory response associated with insulin resistance, indicating that these events occur in a sequential mode. Additionally, blockages of HGF receptor (Met) worsen the impaired insulin-induced insulin signaling in liver of diet-induced obesity rats. Overall, our data indicate that HGF is a growth factor playing a key role in islet mass increase and hyperinsulinemia in diet-induced obesity rats, and suggest a protective effect of the HGF–Met axis on insulin signaling in the liver.

Introdução

Diversas doenças que apresentam alta prevalência na sociedade ocidental incluindo obesidade, diabetes mellitus tipo 2, várias endocrinopatias, hipertensão e dislipidemias estão associadas com a resistência periférica à insulina (Reaven 1988; Martin, Warram et al. 1992; Goldberg 2000). Em pacientes com resistência insulínica pode ocorrer um aumento da massa de células β pancreáticas, como resposta compensatória. Essa resposta causará um aumento da secreção de insulina e conseqüentemente a hiperinsulinemia, muitas vezes observada na diabetes tipo 2 e outras situações de resistência insulínica (Kahn 1994; Bruning, Winnay et al. 1997; Prentki and Nolan 2006). Modelos de diabetes em roedores, como os camundongos *ob/ob* e *db/db* e o rato *fatty* Zucker, e ainda humanos hiperinsulinêmicos com obesidade e/ou diabetes tipo 2 exibem de moderada a acentuada hiperplasia das ilhotas (Gapp, Leiter et al. 1983; Tomita, Doull et al. 1992; Tokuyama, Sturis et al. 1995; Pinar, Pinar et al. 2000). Alguns modelos de camundongos que apresentam resistência insulínica, esses criados pela inativação do gene para o substrato do receptor de insulina – 1 (IRS – 1) ou nocaute para heterozigotos duplos de receptor de insulina (IR) e IRS – 1 exibem acentuada hiperplasia da ilhota (Araki, Lipes et al. 1994; Bruning, Winnay et al. 1997; Kulkarni, Winnay et al. 1999). Os fatores que contribuem para hiperplasia de células β em estado de resistência insulínica são pouco estudados. É conhecido que a glicose estimula a proliferação de célula β, porém em nocautes para heterozigotos duplos e IRS -1, e camundongos *ob/ob* e *db/db* é observado que a hiperplasia/hipertrofia da ilhota se manifesta antes de se detectar a hiperglicemia (Edvell and Lindstrom 1999). Portanto, a resposta hiperplásica na maioria dos casos não apresenta uma relação com o nível de hiperglicemia, sugerindo que fatores independentes de glicose

podem contribuir para a proliferação das células beta (Figura 1) (Flier, Kulkarni et al. 2001).

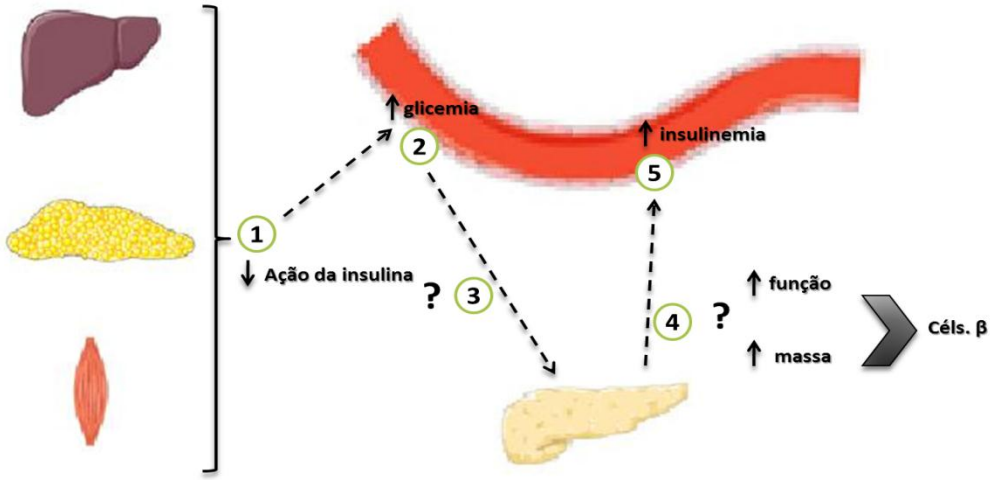

Figura 1. Esquema demonstra que para proteger o indivíduo do desenvolvimento da diabetes ocorre uma resposta compensatória realizada pelo pâncreas (aumento massa de célula β e da capacidade secretória). Entretanto, esta resposta precede o desenvolvimento da hiperglicemia, levando a crer na existência de um fator circulante que cumpriria este papel de elo entre a resistência à insulina e a resposta compensatória do pâncreas.

Com base em outras situações de resistência hormonal e em alças endócrinas de retroalimentação, é importante examinar a hipótese que a resistência insulínica induz o crescimento de células β pancreáticas por meio de um fator "mitogênico" circulante que é independente de glicose (Flier, Kulkarni et al. 2001). Dentre os possíveis candidatos a fator circulante que na resistência à insulina induz crescimento das células β pancreáticas merecem menção o hormônio de crescimento, o IGF-I (fator do crescimento do tipo insulina I), a prolactina, o lactogênio placentário e a leptina. O hormônio de crescimento e o IGF-I tornam-se fracos candidatos porque estão normais ou reduzidos em camundongos

nocautes para IRS-1 e *ob/ob*. O lactogênio placentário e a prolactina são candidatos inadequados, visto que as situações de resistência à insulina ocorrem indistintamente em camundongos fêmeas e machos. A leptina que também é sugerida como promotora do crescimento de células β, apresenta-se deficiente em camundongos *ob/ob*, e significantemente reduzida em camundongos heterozigotos duplos de receptor de insulina (IR) e IRS – 1 (Flier, Kulkarni et al. 2001). Assim, estes dados citados acima excluem esses fatores como principais candidatos.

A procura de um fator circulante que na resistência insulínica fosse capaz de induzir aumento da massa de células β deve ter como etapa essencial a determinação do(s) tecido(s) fonte(s) desse(s) fator(es).

A comunicação metabólica entre órgãos e tecidos é essencial para manutenção da glicose circulante e homeostasia energética (Imai, Katagiri et al. 2008). O comprometimento da sinalização de insulina no fígado (Michael, Kulkarni et al. 2000; Okada, Liew et al. 2007), mas não no músculo (Bruning, Michael et al. 1998) ou tecido adiposo (Bluher, Michael et al. 2002), induz hiperplasia das ilhotas e hiperinsulinemia. Ainda, em estudo no qual se utilizaram camundongos nocaute especifico para o receptor IR no fígado (LIRKO), foi observado que este animal apresenta hiperinsulinemia sem desenvolver diabetes, isto, causado pelo acentuado aumento na massa de células beta (Okada, Liew et al. 2007). Neste contexto, sugere-se que o fígado exerce um importante papel na regulação do crescimento das ilhotas (Imai, Katagiri et al. 2008). Em nosso laboratório, Prada e cols. (2005) procurando investigar a instalação da resistência à insulina em tecidos de ratos tratados com dieta cafeteria, demonstraram que as alterações da sinalização de insulina no músculo e hipotálamo são mais precoces e não são suficientes

para induzir hiperinsulinemia compensatória. Entretanto, quando a resistência insulínica se instala no fígado paralelamente ocorre a hiperinsulinemia compensatória.

No fígado, a resistência insulínica é manifestada pela diminuição da capacidade da insulina ativar sua via de sinalização (Saad, Araki et al. 1992; Biddinger, Hernandez-Ono et al. 2008). Em nível molecular, a insulina inicia sua atividade biológica ao ligar-se a seu receptor localizado na membrana plasmática das células, que pertence à subfamília de receptores tirosina quinase, possuindo duas subunidades α e duas β (Patti and Kahn 1998). Esse receptor possui a capacidade de se auto-fosforilar em resíduos de tirosina (Pessin and Saltiel 2000). A partir dessa ativação do receptor, proteínas da família dos substratos do receptor de insulina (IRS) são fosforiladas em resíduos de tirosina (Sesti, Federici et al. 2001), ativando uma cascata de sinalização, como a via da PI3K (fosfatidilinositol-3-quinase) e a via da MAPK (Saltiel and Kahn 2001).

A PI3K é uma enzima constituída por uma subunidade catalítica, a p110, e por uma subunidade regulatória, a p85, que possuem 2 domínios SH2, e interagem com as proteínas IRS. Estas são fosforiladas formando o PIP3, que recruta as proteínas serina quinases que possuem o domínio PH: PDK-1, Akt e PKC (Vanhaesebroeck and Alessi 2000). Akt é uma serina/treonina quinase constituída de dois domínios, sendo um domínio PH (*pleckstrin homology*) que se liga a fosfatidilinositol (3, 4,5) P3 fosfato (PIP3) e um domínio catalítico contendo um resíduo de treonina (Tre^{308}), o qual sua fosforilação é necessária para ativação de Akt. Próximo ao domínio catalítico existe uma terminação c-terminal contendo um segundo sítio regulado por fosforilação (Ser^{473}). Esses dois sítios de fosforilação são necessários para a completa atividade da Akt (Elghazi, Balcazar et al. 2006). A ativação da via PI3K/Akt resulta na fosforilação de muitos substratos que controlam várias cascatas de sinais biológicos, tais como: o transporte de glicose mediado por insulina no tecido adiposo

e muscular, a síntese de proteína e de glicogênio, a lipogênese (Girard, Perdereau et al. 1994; Elghazi, Balcazar et al. 2006), proliferação, crescimento celular, diferenciação e sobrevivência (Woodgett 2005).

A via da MAPK também é ativada por insulina, por ambas as associações de SHC com o receptor de insulina e de Grb2 com o receptor e com moléculas de IRS, levando à ativação de ERK. Essa proteína ativada pode translocar-se para o núcleo e iniciar um programa transcricional que leva à proliferação e/ou diferenciação celulares (Boulton, Nye et al. 1991).

Recentemente, Imai e cols. (2008) investigando mecanismos responsáveis pela hiperinsulinemia compensatória em animais com resistência à insulina demonstraram que a ativação da proteína MAPK/ERK quinase, exclusivamente no fígado, era capaz de induzir controle neuronal que possibilitava a indução da proliferação de células β através da sinalização neuronal mediada por sinais metabólicos, mas não isoladamente. Esta evidência indica que mecanismos neuronais são responsáveis, em parte, por essa hiperinsulinemia compensatória, mas há ainda fator(es) circulante(s) que contribuem para essa regulação fisiopatológica (Flier, Kulkarni et al. 2001). Assim, com o objetivo de identificar o possível fator circulante envolvido na reposta compensatória das ilhotas à resistência insulínica, é importante estudar proteínas que apresentam supraregulação ou ativação, principalmente no fígado, em situações de resistência à insulina.

Nesse sentido o fator de crescimento do hepatócito (HGF) apresenta-se como um possível candidato, por apresentar pelo menos três características, que o tornam elo fisiopatológico entre resistência à insulina e hiperplasia de ilhotas/hiperinsulinemia:

a) O HGF é predominantemente produzido pelo fígado (Nakamura, Sakai et al. 2011);
b) É regulado pela via da MAPK/ERK (Motoki, Sugiura et al. 2008);

c) Tanto *in vitro* quanto *in vivo* o HGF estimula a secreção de insulina e o aumento da massa da ilhota (Otonkoski, Beattie et al. 1994; Otonkoski, Cirulli et al. 1996; Garcia-Ocana, Takane et al. 2000; Dai, Huh et al. 2005; Mellado-Gil, Rosa et al. 2011);
d) Os níveis deste fator estão elevados na situação de resistência insulínica mais comum, que é a obesidade (Hiratsuka, Adachi et al. 2005; Vistoropsky, Trofimov et al. 2008).

Essas três características serão descritos mais detalhadamente abaixo.

Biologia do HGF e de seu receptor (c-Met)

O HGF é uma proteína derivada do mesênquima e originalmente identificada como um fator circulante envolvido na regeneração do fígado depois de uma lesão hepática ou hepatectomia (Nakamura, Nishizawa et al. 1989; Garcia-Ocana, Takane et al. 2000). Atualmente é reconhecido que HGF também exibe atividades mitogênicas, morfogênicas e motogênicas em uma ampla variedade de órgãos, incluindo o fígado, rins, cérebro e ilhotas pancreáticas (Fiaschi-Taesch, Berman et al. 2008).

O lócus do gene do HGF localiza-se no cromossomo 7q21.1, onde um gene composto de 18 exons e 17 íntrons abrange aproximadamente 70 Kb (Seki, Hagiya et al. 1991). A tradução de um único transcrito de 6 Kb resulta em um pré-pró-polipeptídeo de 728 aminoácidos (Stella and Comoglio 1999). No fígado, o HGF é sintetizado e secretado na forma de um precursor inativo de cadeia simples (proHGF, 92 kDa). O proHGF é ativado por uma única clivagem proteolítica entre Arg^{494} e Val^{495}, assim convertendo para forma heterodimeríca ativa (HGF maduro ou o HGF propriamente dito), constituída de uma cadeia pesada (cadeia α, 62 kDa) e uma cadeia leve (cadeia β, 32-36 kDa) ligadas por meio de uma ponte dissulfeto (Naka, Ishii et al. 1992; Miyazawa, Shimomura et al. 1994).

Quatro proteases têm sido descritas capazes de ativar HGF *in vitro*, incluindo o fator de coagulação XIIa, a uroquinase, o ativador de plasminogênio e o ativador de HGF. Entre essas proteases citadas, o ativador de HGF (34 kDa) cliva proHGF em HGF maduro mais eficientemente (Shimomura, Miyazawa et al. 1995). O ativador de HGF é sintetizado principalmente no fígado e em menor intensidade, no tecido gastrointestinal (Itoh, Hamasuna et al. 2000; Yanagida, Kaibori et al. 2006). Essa protease está presente no plasma como um precursor inativo (ativador proHGF, 96 kDa). O ativador proHGF é convertido para uma forma ativa (ativador de HGF, 34 kDa) por trombina e pelo próprio ativador de HGF (Shimomura, Kondo et al. 1993). Esses dados sugerem que o ativador de HGF apresenta um importante papel na regulação da atividade de HGF. Além disso, recentemente foi isolado um inibidor do ativador de HGF (HAI – 1), o qual atuaria como inibidor fisiológico do ativador do HGF na superfície celular (Hayashi, Nishioka et al. 2007). Entretanto, seu papel precisa ser melhor elucidado.

A forma madura de HGF é necessária para suas atividades biológicas, como a mitogênese, que é mediada através da ligação do HGF com o seu receptor (c-Met), sendo este um receptor com atividade tirosina quinase transmembrana codificado pelo protooncogene c-met ($p190^{MET}$) (Roccisana, Reddy et al. 2005). O receptor c-Met é um polipeptídeo transmembrana dimérico, constituído de uma cadeia α exposta na superfície celular e a cadeia β integrada à membrana plasmática. A cadeia α (50kDa) é altamente glicosilada e apresenta uma ligação dissulfídica com a cadeia β (145 kDa), essa que contém o domínio quinase, o sítio de autofosforilação de tirosina e o sítio de ligação multifuncional que apresenta uma cadeia específica de aminoácidos localizados na parte carboxi-terminal da proteína (Hartmann, Naldini et al. 1992).

A ligação do HGF ocorre na presença de ATP e íons Mg^{2+}, dessa forma induzindo a dimerização do receptor e a transfosforilação do domínio catalítico. Isso resulta numa forte ativação da atividade enzimática do receptor c-Met, principalmente por uma mudança conformacional do receptor que permite a ligação e fosforilação de diferentes transdutores intracelulares ao sítio multifuncional. Esses transdutores são proteínas, tais como: a subunidade de PI3K (p85), Ras Gap, PLC-γ, tirosinas quinases relacionadas com Src, Grb-2, Gab-1 e IRS-1 (Figura 2) (Stella and Comoglio 1999; Rosario and Birchmeier 2003; Roccisana, Reddy et al. 2005).

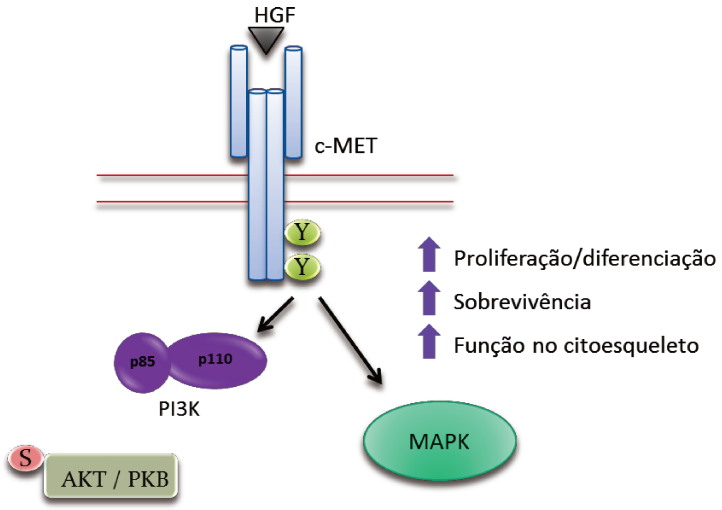

Figura 2. O HGF liga-se ao seu receptor especifico c-Met que autofosforila-se em resíduos de tirosina, desta forma levando a ativação de vias metabólicas chaves, tais como: PI3K/Akt e MAPK.

O HGF amplifica o sinal da insulina

Estudos demonstram, que assim como para o receptor de insulina (IR), o c-Met é um heterodímero consistindo de uma subunidade α e uma β, mantidas juntas por ligações dissulfeto. Nesse sentido, o c-Met, é o receptor mais semelhante ao IR em termos da estrutura geral do receptor e da sequência do domínio quinase em comparação com

qualquer outro membro da subclasse de receptores com atividade tirosina quinase (De Meyts 2004).

Recentemente, Fafalios e colaboradores (2011) demonstraram que a ativação de c-Met no fígado induz a formação de um complexo c-Met-IR, o qual aumenta o recrutamento de substratos do receptor de insulina, o IRS-1 e principalmente o IRS-2, e assim amplifica o sinal. Ainda, foi demonstrado nesse estudo que a ativação desse complexo melhora o metabolismo hepático de glicose, através do aumento da captação de glicose e diminuição da produção hepática de glicose e de sua liberação (Figura 3) (Fafalios, Ma et al. 2011).

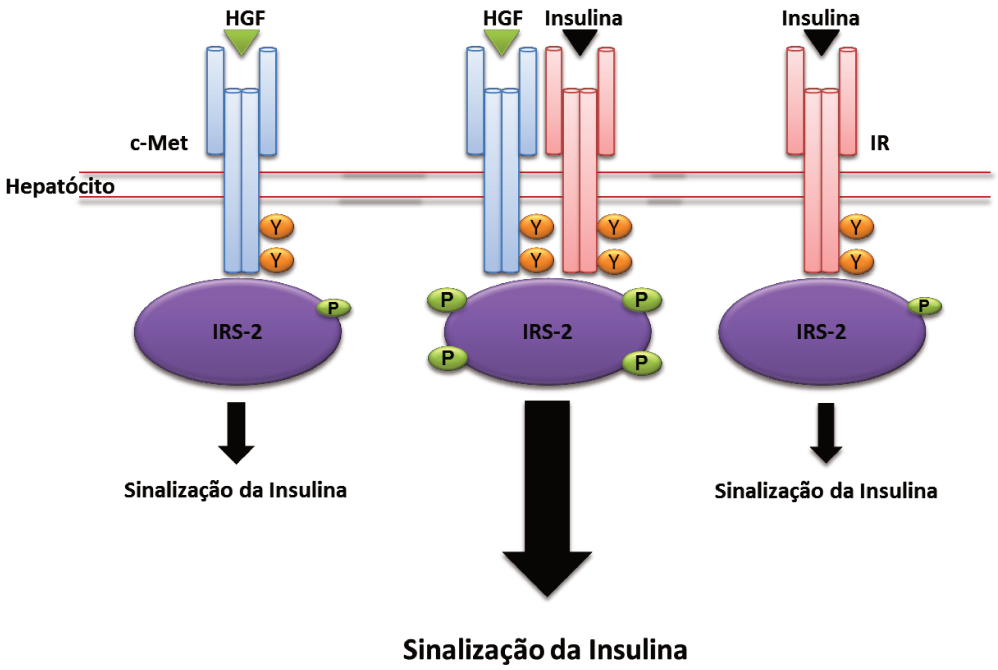

Figura 3. Modelo proposto para o *crosstalk* entre o c-Met e o receptor de insulina (IR). Esquema demonstra o papel do HGF em amplificar a sinalização da insulina.

HGF estimula a hiperplasia das ilhotas e a secreção de insulina

Dor e cols. (2004) (Dor, Brown et al. 2004), usando métodos de rastreamento celular em pâncreas de camundongos, determinaram que apesar de baixa, a proliferação de células β desempenha um papel central na manutenção da massa deste tipo celular. Dentre os reguladores da proliferação celular merecem destaque os complexos ciclinas tipo D e as cinases dependentes de ciclinas 4 e 6 (Cdk-4/D e Cdk-6/D, respectivamente). Camundongos nocaute para Cdk-4/D apresentaram hipoplasia de células β pancreáticas, levando à diabetes e cetoacidose (Rane, Dubus et al. 1999). Já, a superexpressão das proteínas Cdk-4 e ciclina D em ilhotas de camundongos, ratos e humanos resultaram no aumento da proliferação de células β (Cozar-Castellano, Weinstock et al. 2006). Assim, agindo juntas as Cdk-4/D e Cdk-6/D estimulam a progressão do ciclo celular. Entretanto, duas famílias de proteínas apresentam modulação negativa sobre o complexo Cdk-4/-6/ciclinas D: são as cinases inibitórias (INKs) e proteínas inibitórias de ciclinas (CIPs) ou proteínas inibitórias de cinases (KIPs). Portanto, as proteínas INKs/CIPs/KIPs apresentam papel fundamental sob controle da progressão do ciclo celular. Ainda, é de grande importância a regulação de outros fatores presentes no ciclo celular de células β, tais como, o PDX-1 e o MafA. O PDX-1 (*pancreatic and duodenal homeobox-1*) é um fator de transcrição pancreática que apresenta um papel crucial na formação do pâncreas, diferenciação de célula β e manutenção da função da célula β madura. O MafA é um fator de transcrição específico para célula β, recentemente isolado, que age como um potente ativador da transcrição do gene para insulina (Kaneto, Miyatsuka et al. 2008).

Várias descobertas apontam para importância da sinalização da insulina no controle da massa de células β. Camundongos nocaute para o substrato do receptor do substrato de insulina 2 (IRS-2) apresentaram redução na massa de células β (Withers, Gutierrez et al.

1998). Já, os níveis de mRNA do IR e de IRS-2 estão diminuídos em ilhotas de pacientes com resistência insulínica (Gunton, Kulkarni et al. 2005). Em camundongos nocaute específico para IR na célula β (βIRKO) foi observado que os mesmos são incapazes de promover hiperplasia compensatória de células β (Okada, Liew et al. 2007). Ainda, a ativação da via da Akt (Elghazi, Balcazar et al. 2006) e da via da MAPK (Beith, Alejandro et al. 2008) são essenciais no controle da proliferação de células β. Desta forma há um grande interesse em identificar os reguladores endógenos dos fatores que controlam a expansão da massa de célula β bem como aumento na secreção de insulina.

Otonkoski e cols. (1994), investigaram em culturas de células β diversos fatores de crescimento possuidores de atividades morfogênicas e mitogênicas, e observaram pela primeira vez a atividade insulinotrópica do HGF. Hayek e cols. (1995) (Hayek, Beattie et al. 1995), concluíram que a partir da adição de HGF ao meio de cultura é possível induzir a proliferação de células β *in vitro*, assim fortalecendo os resultados obtidos por Otonkoski e cols. (1994). Ainda, foi observado que RNAm que codificam HGF e receptor c-Met, são altamente expressos em células β durante a morfogênese do pâncreas e mantêm-se a níveis reduzidos durante a puberdade e a vida adulta (Otonkoski, Cirulli et al. 1996). Esses estudos despertaram o interesse de pesquisar o papel do HGF como potencial candidato a estimulador de hiperplasia de células β, com posterior hiperinsulinemia.

Garcia-Ocaña e cols. (2000) estudaram o papel da HGF em ilhotas *in vivo*, e desenvolveram um camundongo transgênico com superexpressão do HGF nas ilhotas pancreáticas. Eles concluíram que a superexpressão de HGF nas ilhotas *in vivo* resulta em proliferação de células β, aumento do número de ilhotas, na massa de células β e na produção de insulina. A combinação desses efeitos resultou num animal que apresentava hipoglicemia moderada e hiperinsulinemia. Em 2005, dois estudos que utilizaram um

modelo de camundongo nocaute para o receptor c-Met em células β pancreáticas, inibindo o papel fisiológico do HGF para tal tecido, apresentaram resultados que não foram similares (Dai, Huh et al. 2005; Roccisana, Reddy et al. 2005). Roccisana e cols. (2005) demonstraram que os animais nocaute especifico para o receptor c-Met em células β apresentavam redução na secreção de insulina e também diminuição da tolerância à glicose acompanhada de redução da expressão de GLUT-2 em células β. Também, foi observada ausência de alterações na massa absoluta e proliferação das células β, bem como na morfologia das ilhotas. Entretanto, Dai e cols. (2005) demonstraram que o tamanho da ilhota nesse modelo animal mostrou-se diminuído. Tais resultados contraditórios reforçam a idéia de que a sinalização e o papel fisiológico da HGF, bem como os mecanismos moleculares envolvidos, necessitam serem melhores estudados nas ilhotas.

Recentemente, um estudo demonstrou um novo papel fisiológico para o HGF, este relacionado ao desenvolvimento das células β no processo gestacional. Foi demonstrado que a sinalização do HGF/c-Met é essencial para adaptação das células β durante a gravidez. Portanto, a falta ou atenuação desta sinalização leva ao desenvolvimento do diabetes gestacional (Demirci, Ernst et al. 2012).

Via PI3K/AKT e sua relação com o HGF

Análises iniciais do mecanismo de sinalização intracelular do HGF correlacionado com o efeito mitogênico em células β indicam que a ativação da via PI3-quinase-Akt representa um importante papel nesse efeito (Gahr, Merger et al. 2002). Em células β, a insulina e o IGF-I ativam a Akt, também, a glicose pode ativar diretamente a sinalização de

Akt depois de uma estimulação prolongada, porém esse mecanismo não está claramente definido (Dickson and Rhodes 2004).

Em camundongos transgênicos com redução na atividade de Akt em células β foi observado diminuição na secreção de insulina e nenhuma alteração na massa das células β (Bernal-Mizrachi, Fatrai et al. 2004). Além disso, encontrou-se redução da captação de glicose estimulada por insulina quando se utilizou camundongo nocaute para o gene da Akt (Cho, Mu et al. 2001). Entretanto, quando a atividade da Akt foi superexpressa em células β de camundongo transgênico obteve-se aumento da massa de células β através do aumento do tamanho e proliferação das células β (Bernal-Mizrachi, Wen et al. 2001). Também, foi observada nos níveis de glicose sanguínea e o desenvolvimento de hiperinsulinemia (Fiaschi-Taesch, Stewart et al. 2007). Esses estudos indicam que a ativação de Akt pode resultar em proliferação de células β *in vivo* e hiperinsulinemia.

Papel do HGF na sobrevivência das células β

Vários estudos tem demonstrado o papel do HGF na sobrevivência das células beta. Primeiramente, foi demonstrado que o HGF seria um fator de pró-sobrevivência à estreptozotocina (agente reconhecido como diabetogênico e citotóxico) (Garcia-Ocana, Takane et al. 2000; Dai, Li et al. 2003; Garcia-Ocana, Takane et al. 2003). Ainda, o HGF apresenta atividade anti-apoptótica em situações de hipóxia e escassez de nutrientes presentes nas primeiras horas de transplante de ilhotas (Nakano, Yasunami et al. 2000; Garcia-Ocana, Vasavada et al. 2001; Garcia-Ocana, Takane et al. 2003; Lopez-Talavera, Garcia-Ocana et al. 2004; Fiaschi-Taesch, Berman et al. 2008). Além disso, também foi demonstrado *in vitro* que o HGF protege células de insulinoma de ratos contra o ácido

graxo livre (palmitato), este conhecido por induzir apoptose (Santangelo, Matarrese et al. 2007). Recentemente, o eixo HGF/c-Met foi caracterizado como essencial na sobrevivência das células beta através da atenuação da ativação do NF-κB (fator nuclear kappa B). Assim, foi sugerido que a falta da ativação do eixo HGF/c-Met aumenta a morte de células beta pancreática e acelera o desenvolvimento do diabetes (Mellado-Gil, Rosa et al. 2011).

O HGF na obesidade

A obesidade está associada com diversas alterações metabólicas, tais como: a hiperinsulinemia e a resistência insulínica (Selig 2006). A resistência insulínica do tecido adiposo pode estar envolvida na resistência insulínica em geral e nas alterações hepáticas (Shiota, Umeki et al. 1995; Marchesini, Bugianesi et al. 2003). O tecido adiposo branco pode estar associado com essas complicações por meio da secreção de diferentes peptídeos bioativos e proteínas, conhecidos como adipocitocinas (Trayhurn and Beattie 2001). Esse tecido é fonte de hormônios, citocinas, proteínas de fase aguda e fatores de crescimento, esse último que inclui o fator de crescimento epididimal, fator de crescimento endotelial vascular, fator de crescimento de transformação, fator de crescimento de nervo, e também o fator de crescimento do hepatócito (HGF) (Bertola, Bonnafous et al. 2007). De fato, o HGF é expresso e secretado por adipócitos 3T3-L1 de camundongos (Rahimi, Saulnier et al. 1994) e tecido adiposo humano (Fain, Madan et al. 2004; Bell, Ward et al. 2006; Rittig, Dolderer et al. 2012). Esse HGF sintetizado no tecido adiposo humano pode apresentar um importante papel na dinâmica da obesidade (Hiratsuka, Adachi et al. 2005; Tsukagawa, Adachi et al. 2012). Entretanto, o papel biológico da HGF não é totalmente entendido.

Os níveis de HGF têm sido demonstrados estar correlacionado com medidas antropométricas da obesidade, tais como: a circunferência abdominal, o índice de massa corporal e a massa gorda corporal (Vistoropsky, Trofimov et al. 2008). Por outro lado a perda de peso depois da intervenção por gastroplastia têm sido associada com a redução dos níveis de HGF plasmático em pacientes obesos (Swierczynski, Korczynska et al. 2005; Bell, Ward et al. 2006; Tsukagawa, Adachi et al. 2012). Em pacientes com síndrome metabólica observou-se uma significante associação entre o HGF e a insulina plasmática, sugerindo uma ligação fisiopatológica entre eles (Hiratsuka, Adachi et al. 2005; Tsukagawa, Adachi et al. 2012).

Justificativa

Em resumo, o HGF preenche alguns critérios de bom candidato a fator circulante que conecta a resistência à insulina ao aumento da massa de células beta, tais como: 1) o HGF é predominantemente produzido pelo fígado, 2) tanto *in vitro* quanto *in vivo* o HGF estimula a secreção de insulina e o aumento da massa da ilhota e 3) os níveis deste fator estão elevados na situação de resistência insulínica mais comum, que é a obesidade. Entretanto, essa vinculação ainda não foi estabelecida.

Objetivos

O objetivo geral do presente estudo é estabelecer a vinculação citada acima, através dos seguintes objetivos específicos:

1- Investigar em situação de aumento endógeno do HGF (hepatectomia parcial em ratos), a regulação da massa de células beta.

2- Investigar o efeito da infusão de HGF recombinante *in vivo* na regulação da massa de células beta.

3- Investigar em modelo animal de obesidade induzida por dieta cafeteria, a evolução temporal (1 – 8 semanas) dos níveis circulantes insulinêmicos e de HGF, bem como a massa de células beta.

4- Investigar em modelo animal de obesidade induzida por dieta cafeteria, o efeito de um bloqueador do receptor de HGF (c-Met) na regulação da massa de células beta e na sinalização de HGF na ilhota.

Capítulo

Em anexo, artigo científico produto do presente trabalho, este publicado no periódico *Endocrinology*.

Campinas, 12 de Dezembro de 2012

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Hepatocyte Growth Factor Plays a Key Role in Insulin Resistance-Associated Compensatory Mechanisms

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Insulin resistance is present in obesity and in type 2 diabetes and is associated with islet cell hyperplasia and hyperinsulinemia, but the driving forces behind this compensatory mechanism are incompletely understood. Previous data have suggested the involvement of an unknown circulating insulin resistance-related β-cell growth factor. In this context, looking for candidates to be a circulating factor, we realized that hepatocyte growth factor (HGF) is a strong candidate as a link between insulin resistance and increased mass of islets/hyperinsulinemia. Our approach aimed to show a possible cause-effect relationship between increase in circulating HGF levels and compensatory islet hyperplasia/hyperinsulinemia by showing the strength of the association, whether or not is a dose-dependent response, the temporality, consistency, plausibility, and reversibility of the association. In this regard, our data showed: 1) a strong and consistent correlation between HGF and the compensatory mechanism in three animal models of insulin resistance; 2) HGF increases β-cell mass in a dose-dependent manner; 3) blocking HGF shuts down the compensatory mechanisms; and 4) an increase in HGF levels seems to precede the compensatory response associated with insulin resistance, indicating that these events occur in a sequential mode. Additionally, blockages of HGF receptor (Met) worsen the impaired insulin-induced insulin signaling in liver of diet-induced obesity rats. Overall, our data indicate that HGF is a growth factor playing a key role in islet mass increase and hyperinsulinemia in diet-induced obesity rats and suggest that the HGF-Met axis may have a role on insulin signaling in the liver. (***Endocrinology*** **153: 5760–5769, 2012)**

In individuals with insulin resistance, there is an increase in pancreatic β-cell mass as a compensatory response (1). This compensatory response of β-cells is the first step in developing diabetes by inducing hyperinsulinemia. In fact, insulin-resistant animals and human subjects present islet hyperplasia and/or hyperinsulinemia before the onset of detectable hyperglycemia, suggesting the existence of as yet unknown mechanisms enhancing compensatory expansion of β-cell mass in response to obesity-related insulin resistance. Although some studies have indicated that a neural component is involved in triggering this response (2–4), others have also suggested the involvement of an unknown circulating insulin resistance-related β-cell growth factor (1, 5).

In this regard, some data have indicated (1, 2, 5, 6) that this hypothetical circulating growth factor is produced by the liver. In addition, it has been demonstrated that the ERK pathway in the liver is involved in this compensatory response (2). In this sense, considering candidates for a circulating factor that could have such features, hepatocyte growth factor (HGF) presents a strong candidate, having at least four characteristics that mark the pathophysiological link between insulin resistance and islet hyperplasia/hyperinsulinemia: 1) HGF is mainly produced by the liver (7); 2) it is under regulation by the ERK pathway (8); 3) HGF stimulates insulin secretion and increased islet mass both *in vitro* and *in vivo* (9–13); and 4) levels of

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Abbreviations: Akt^{ser473}, Protein kinase B serine 473; DIO, diet-induced obesity; DIO-30D, DIO for 30 d; GTT, glucose tolerance test; HFD, high-fat diet; HGF, hepatocyte growth factor; IRS, insulin receptor substrate; Met, HGF receptor; rHGF, recombinant HGF; TUNEL, terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling.

this factor are elevated in obesity associated-insulin resistance (14, 15).

Taken together, HGF fulfills at least some of the criteria of a good candidate for a circulating factor that connects insulin resistance with increased β-cell mass. Therefore, the aim of this study was to evaluate whether HGF is the driving force behind this compensatory mechanism.

Research Design and Methods

Animal studies

Diet-induced obese (DIO) rats

First, male Wistar rats (9-wk-old; obtained from the State University of Campinas Central Breeding Center) were housed in grouped cages under a 12-h light, 12-h dark cycle in a controlled environment (room temperature, 22 ± 3 C; humidity, 55 ± 5%) and also were randomly assigned to two diet groups: either standard rodent chow and water *ad libitum* or a cafeteria diet, for 30 d [DIO for 30 d (DIO-30D)] or DIO-60D. The cafeteria diet group received soft drinks *ad libitum*, instead of water, alternated daily (Coca-Cola and Guaraná Antarctica), and were fed a pellet made of 37.5% standard rodent chow, 25% peanuts, 25% chocolate, and 12.5% cookies, offered together with palatable food items comprising wafer, snacks, cakes, and biscuits, totaling 4.41 kcal/g of gross energy (43.1% from carbohydrates, 12.1% from proteins, and 46.9% from fats) as opposed to the 2.63 kcal/g of gross energy of the standard chow diet Nuvilab CR-1 (Nuvital, Colombo, Paraná, Brazil) (5, 16). Second, beginning the sixth week of the cafeteria and chow diet, the rats were treated with a pharmacological inhibitor of HGF receptor (SU11274; Tocris Bioscience, Bristol, UK) at a concentration of 0.5 mg/kg·d, ip, for 16 d. On the other hand, for treatment of obese animals with recombinant HGF (rHGF), it was administered to rats from 6 wk after the start of the cafeteria diet. The protein was injected into the tail vein at a dose of 0.5 μg/kg·d diluted in saline for 16 d; the control received only saline. Six rats were used per group (n = 6).

ob/ob *and* Swiss *mice*

Six-week-old *Swiss* and *ob/ob* mice were obtained from the State University of Campinas Central Breeding Center. Animals were housed in individual cages with free access to water and rodent chow or high-fat diet (HFD) under a 12-h light, 12-h dark cycle in a controlled environment (room temperature, 22 ± 3 C; humidity, 55 ± 5%). The *Swiss* mice were fed with HFD (consisted of 55% of calories derived from fat, 29% from carbohydrates, and 16% from protein) for 2 months, as previously described (17, 18). The *ob/ob* mice were fed on a chow diet for 2 months.

Dose-response evaluation

To perform the dose-response evaluation of treatment with purified recombinant active mouse HGF (rHGF) (Peprotech, Inc., Rocky Hill, NJ), *Swiss* mice at 6 wk were treated for 5 d with rHGF at concentrations of 0.05, 0.5, or 5.0 μg/kg·d. Mice were randomly distributed into four groups, each group having six mice. The rHGF was injected into the tail vein and diluted in saline (50 μl).

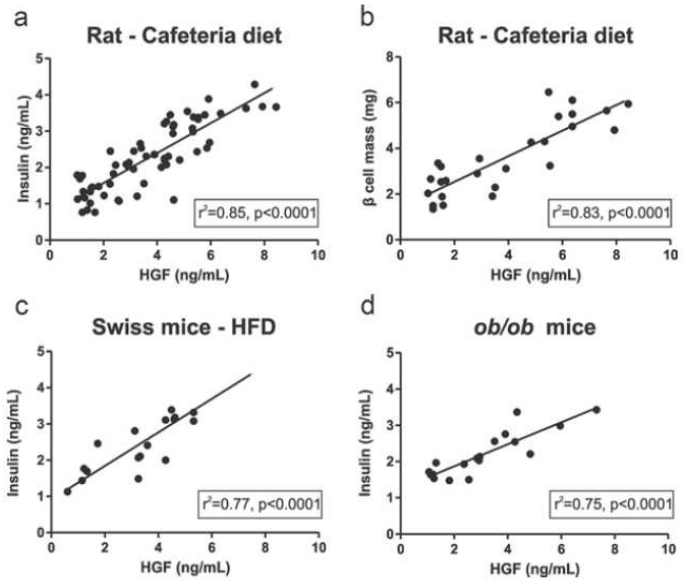

FIG. 1. Strong correlation between circulating HGF and insulin levels in three different models of insulin resistance. Correlation between fasting plasma HGF and insulin levels (A) and between fasting plasma HGF levels and β-cell mass (B) in DIO (DIO-60D) rats. Correlation between fasting plasma HGF and insulin levels in *Swiss* mice fed on HFD (C) and in *ob/ob* mice (D). Correlations between parameters were tested by linear regression analysis.

70% Partial hepatectomy (Higgins procedure)

Additionally, to conduct a study of another model that shows an increase of endogenous HGF (19), we used the technique of partial hepatectomy 70%. Wistar rats were anesthetized with ketamin 5% (30 mg/kg) and xylazine 2% (30 mg/kg) ip. Rats were divided into two groups (n = 6). Under strict sterile conditions, 70% (two thirds) partial hepatectomy was performed according to the method of Higgins and Anderson (20). In brief, the left lateral and median hepatic lobes, constituting approximately 70% of the total liver weight, were ligated and resected. In sham-operated controls, the livers were briefly removed from the peritoneal cavity but not tied or excised.

During the surgery, animals were warmed by a halogen light (45 W, 127 V), and corporeal temperature was monitorized by a rectal digital thermometer (YSI Precision 4000A Thermometer; YSI Temperature, Dayton, OH) and kept around 37 C. Animals were allowed to spontaneous ventilation with an oxygen-enriched mixture (40%) during all the procedure

(21). All animal studies were approved by the Animal Care and Use Committee at the State University of Campinas and are in accordance with the guidelines for the Care and Use of Laboratory Animals.

Assays

Rats and mice were fasted overnight, and the blood samples were withdrawn from the retrobulbar intraorbital capillary plexus. The plasma was separated by centrifugation at 2500 × *g*/5 min, in tubes containing EDTA (1 mg/ml, disodium salt). Glucose was measured from whole venous blood with a glucose monitor (glucometer; Bayer Diagnostics, New York, NY). Readouts of fasting blood insulin, C-peptide, and HGF levels were determined by ELISA [insulin and C-peptide were from Millipore (Bedford, MA) and HGF was from Abcam, Inc. (Cambridge, MA)]. The glucose tolerance test (GTT) was performed as follows. After a 6-h fasting, rats were anesthetized by an ip injection of sodium amobarbital (15 mg/kg body weight), and the experiments were initiated after the loss of corneal and pedal reflexes. After collection of an unchallenged sample (time 0), a bolus of 2.0 g/kg body weight of glucose was administered into the peritoneal cavity. Blood samples were collected from the tail tip for determination of glucose and insulin concentrations (17, 18, 22).

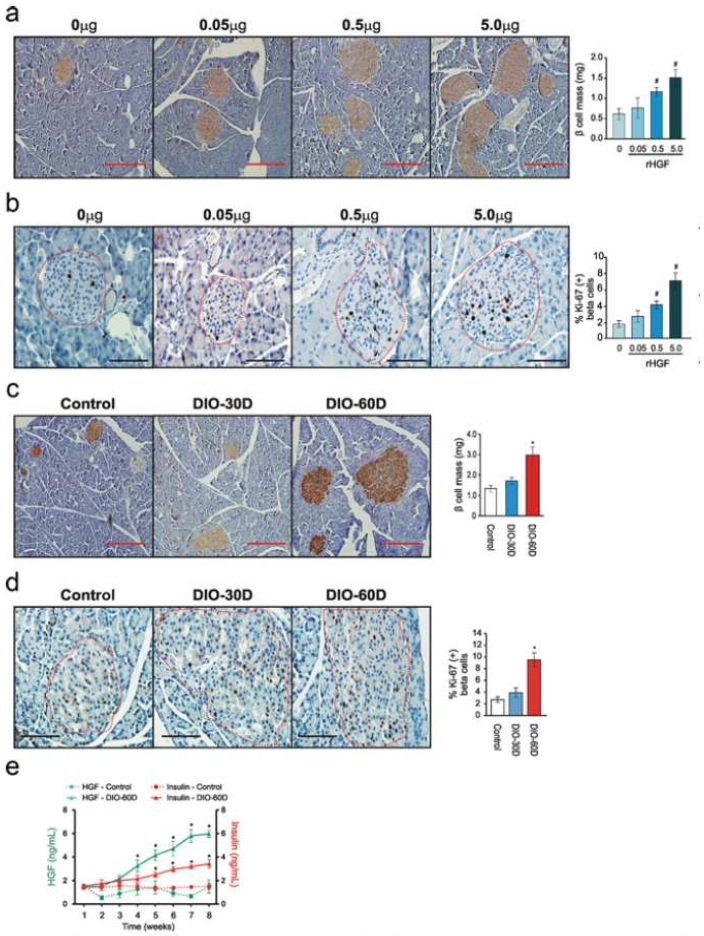

FIG. 2. Effect of HGF on insulin resistance-associated compensatory mechanisms. A, β-Cell mass and representative images of pancreatic islets stained for insulin, as well as the percentage of Ki-67-positive cell in β-cells and representative images of islets stained for Ki-67 (B) from mice after receiving different concentrations of rHGF (0.05, 0.5, and 5.0 μg/kg·d) for 5 d. C, β-Cell mass determinations and their respective representative images, plus the percentage of Ki-67-positive cell in β-cells and representative images of islets stained for Ki-67 (D) in control, DIO-30D, and DIO-60D rats. E, Temporal evolution of circulating HGF and insulin levels in control and DIO-60D rats for 8 wk. *Scale bars*, 100 μm (*black*) and 400 μm (*red*). Data are presented as means ± SEM from six mice or six rats per group in experiments that were repeated at least three times. *, $P < 0.05$ *vs.* control; #, $P < 0.05$ *vs.* dose 0.

Stereologic estimation of β-cell

To study the morphometric parameters of endocrine pancreas, six pancreases from each group were excised and processed according to a previous description (23). The cellular distribution of insulin was analyzed as described previously (23). β-Cell mass was determined by point-counting morphometry on each pancreas section immunostained for insulin according to previous descriptions (23). Each section was systematically scored with a grid of 100 points. The β-cell relative volume was calculated by dividing the intercepts over β-cells by the intercepts over the total pancreatic tissue; the β-cell mass was then estimated by multiplying the β-cell relative volume by the total pancreas weight. A minimum of 500 fields/pancreas was counted.

Average β-cell proliferation was obtained by counting total islet cell nuclei stained for insulin and Ki-67, a well-established proliferation marker, using the image analysis software ImageJ (http://rsbweb.nih.gov/ij/). At least 50 islets per group were sampled. The β-cell proliferation was estimated by the percentage of Ki-67-positive cells from the total of insulin-positive cells (24). On the other hand, average β-cell death was obtained by counting total islet β-cell nuclei stained for insulin and terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling (TUNEL), an *in situ* DNA end-labeling method. The TUNEL staining was done by a commercial apoptosis detection kit (Roche Diagnostics, Indianapolis, IN), according to the recommendations of the manufacturer. At least 50 islets per group were sampled. Using the same software aforementioned, the β-cell death was estimated by the percentage of TUNEL-positive cells from the total of insulin-positive cells (25).

Islet isolation and culture of pancreatic islets

Islets were isolated from rats after injection of collagenase through the pancreatic duct, then selected with a micropipette un-

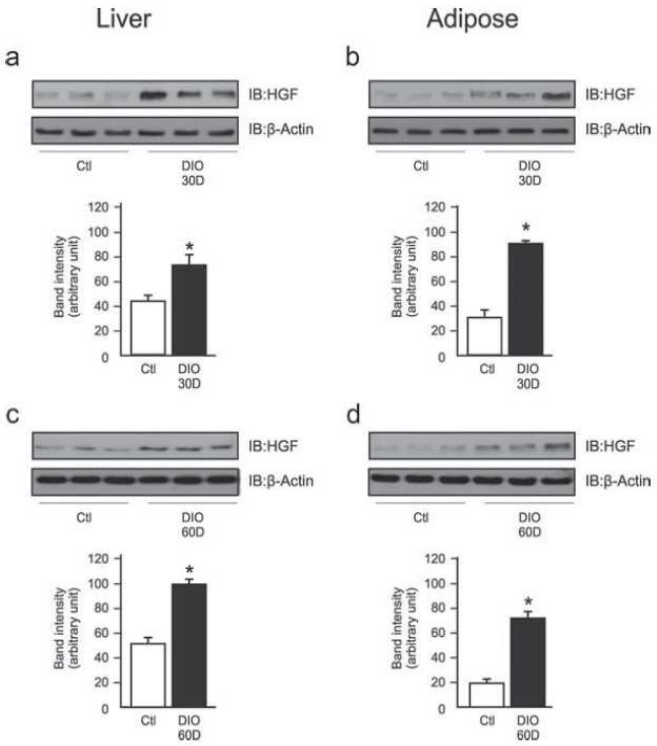

FIG. 3. DIO rats showed an increase in HGF protein content in the liver and adipose tissue. A–D, Representative blots show HGF protein expression in liver and epididymal adipose tissue of control (Ctl), DIO-30D, and DIO-60D rats. Data are presented as means ± SEM from six rats per group in experiments that were repeated at least three times. *, $P < 0.05$ *vs.* control. IB, Immunoblot.

der a microscope to exclude any contaminating tissues. The selected 300- to 400-rat islets were first incubated in Krebs-Ringer bicarbonate buffer equilibrated with 95% O_2-5% CO_2 (pH 7.4) and afterward treated with rHGF for 15 and 30 min (Peprotech, Inc.). Then, at least 300 clean islets from each experimental group were transferred to an Eppendorf and homogenized (Eppendorf, Hamburg, Germany), by sonication 15 sec, in 200 μl of solubilization buffer. The samples (total islets extracts) were treated with Laemmli buffer containing 100 mmol/liter dithiothreitol, heated in a boiling water bath for 4 min, and subjected to SDS-PAGE and immunoblotted, as previously described (26).

Tissue extraction and immunoblotting

Rats were anesthetized (anesthesia was ensured by the loss of pedal and corneal reflexes). The abdominal cavity was opened, the portal vein was exposed, and 0.1 ml of normal saline was injected with or without insulin (10^{-6} mol/liter). At 30 and 90 sec after insulin injection, the liver and epididymal adipose tissue were removed, minced coarsely, and homogenized immediately in extraction buffer, as previously described (18). The whole-tissue extracts were subjected to SDS-PAGE and immunoblotted, as previously described (18, 22). All antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), with the exception of β-actin and antiphospho-Met, which were obtained from Cell Signaling Technology (Beverly, MA).

Statistical analysis

The results of the experiments are displayed as mean ± SEM of a least three independent experiments. The results of blots are presented as direct comparisons of bands or spots in autoradiographs, quantified by optical densitometry (UN SCAN IT gel; Silk Scientific, Inc., Orem, UT). Multiple comparisons were tested by one-way ANOVA, followed by Tukey's *post hoc* test, with the significance level set at $P < .05$ using SPSS software (SPSS for Windows, version 16.0; SPSS, Chicago, IL). Correlations between parameters were tested by linear regression analysis using the GraphPad Prism software program (GraphPad, San Diego, CA).

Results

Increasing levels of HGF correlate with increased islet mass/hyperinsulinemia in DIO animals

In DIO-60D rats, there is a strong correlation between circulating HGF levels and hyperinsulinemia and islet mass increase, suggesting that an association exists between these events (Fig. 1, A and B). Investigating the nature of this correlation, we observed that in different models of insulin resistance (three different animal models of obesity), a strong and significant correlation between HGF and insulin levels exists. The others models used were *Swiss* mice on a HFD (Fig. 1C) and *ob/ob* mice (Fig. 1D).

HGF increases β-cell mass in a dose-dependent manner

Treating *Swiss* mice for 5 d with three different doses of HGF revealed that a strong correlation exists between the dose of infused HGF and islet cell hyperplasia and hyperinsulinemia, indicating that the compensatory mechanism responds to HGF in a dose-dependent manner (Fig. 2A). In addition, this mechanism was confirmed by the marked β-cell proliferation as judged by distribution of Ki-67-positive nuclei (Fig. 2B).

The rise in HGF is an early event in the compensatory response of insulin resistance and also to hepatectomy

We observed that weekly analysis of islet mass, insulin, and circulating HGF levels in DIO-60D rats revealed that

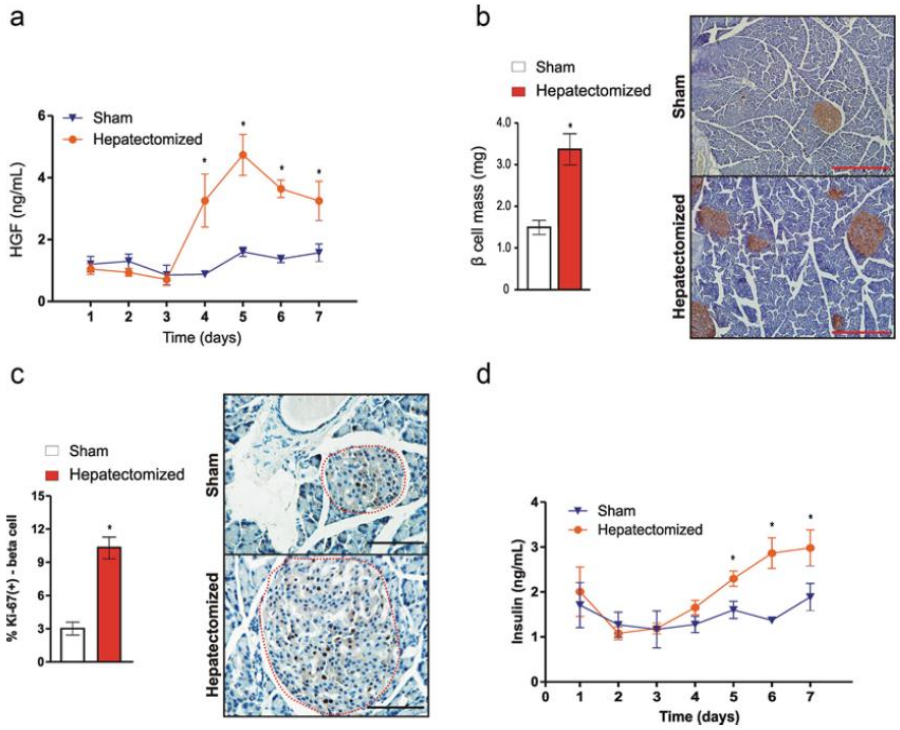

FIG. 4. Partial-hepatectomized (70%) model shows a correlation between HGF levels and β-cell mass. A, Temporal evolution of fasting plasma HGF levels in partial-hepatectomized (70%) and sham-operated rats during 7 d of experiment. B, β-Cell mass and representative images of pancreatic islets stained for insulin, besides the percentage of Ki-67-positive cell in β-cells and representative images of islets stained for Ki-67 (C) from partial-hepatectomized (70%) and sham-operated rats at seventh day. D, Temporal evolution of fasting plasma insulin levels in partial-hepatectomized (70%) and sham-operated rats during 7 d of experiment. *Scale bars*, 100 μm (*black*) and 400 μm (*red*). Data are presented as means ± SEM from six rats per group in experiments that were repeated at least three times. *, $P < 0.05$ *vs.* sham operated.

incremental increases in islet mass, as well as β-cells proliferation, and insulin levels followed an increase in HGF levels (Fig. 2, C–E). Additionally, the DIO-60D rats showed an increase in HGF protein content in the liver and adipose tissue from the fourth week on with the diet (Fig. 3, A–D). These data indicate that a sequential mode occurs, in which an increase in HGF levels seems to precede the compensatory response associated to insulin resistance.

Next, we investigated hepatectomized rats, which carry higher levels of endogenous HGF, and observed the temporal evolution of plasma HGF and insulin levels and β-cell mass, as well as β-cell proliferation, for 7 d. In this study, we noted that from the fourth day after partial hepatectomy (70%), the HGF levels rise and remain high until the seventh day of the experiment (Fig. 4A). This change was followed by incremental changes in islet mass along with augment on Ki-67-positive nuclei of β-cells at seventh day (Fig. 4, B and C). Consequently, we also observed an increase in plasma insulin level from the fifth day after the surgery (Fig. 4D).

Pharmacologic inhibitor of HGF receptor blunts the compensatory mechanism response associated with insulin resistance

SU11274 is a pyrrole indolinone compound, which acts as a HGF receptor (Met) kinase inhibitor thought to specifically block HGF-dependent Met activation. The Met blockage has been associated with inhibition of downstream signaling and biological events typical to Met activity. Indeed, this compound presented low IC_{50} values for Met inhibition; thus, suggesting its high specificity (27). In this regard, when we blocked the action of HGF in DIO-60D rats by using SU11274 for 2 wk (d 45–60), a reduction in islet mass was observed (Fig. 5A). We further hypothesized that a decrease in β-cell proliferation accompanied by increase in β-cell death would likely explain the diminished β-cell mass in DIO-60D rats treated with SU11274. In accordance, here, it was detected a decrease in β-cell proliferation (accessed by Ki-67-stained β-cell). Indeed, we stained pancreas section with TUNEL to detect fragmented DNA and observed that islets from obese rats treated with SU11274 showed increased levels of β-cell

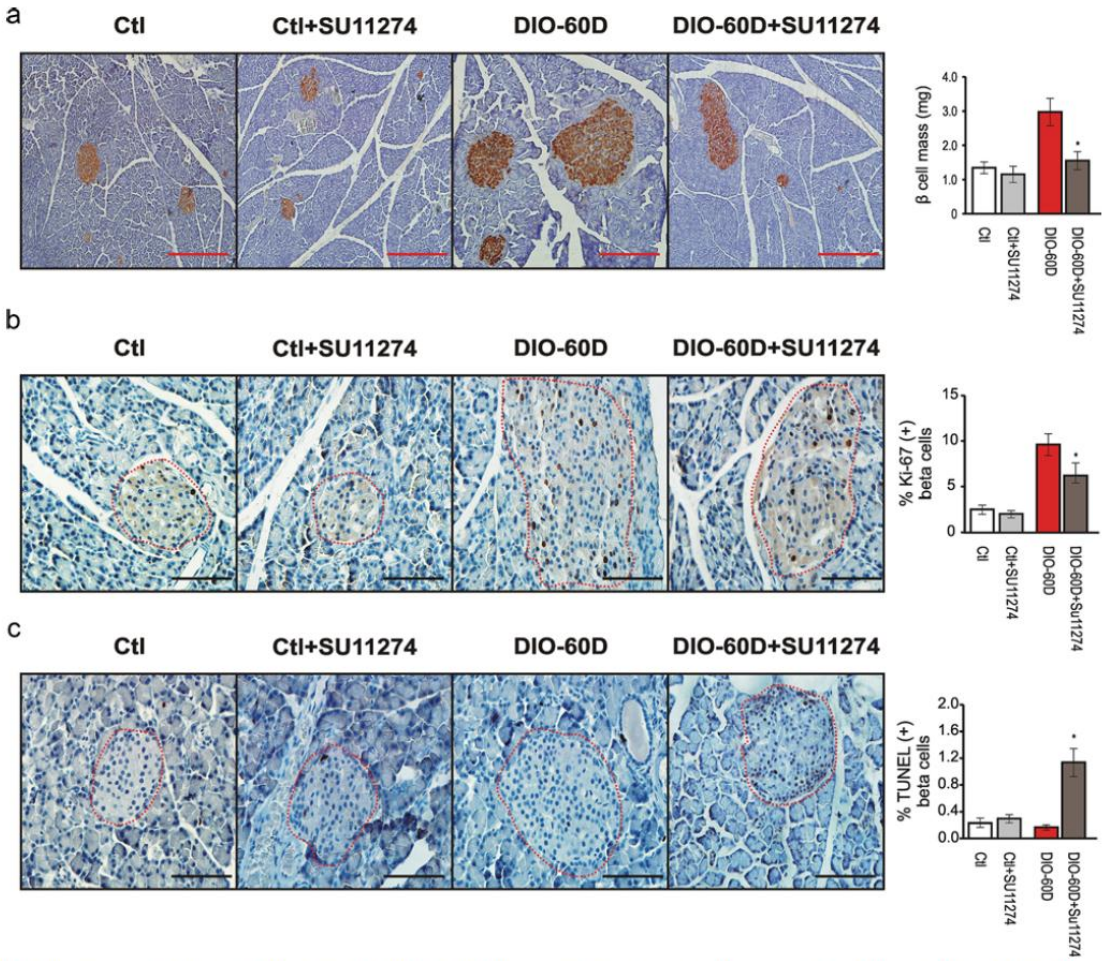

FIG. 5. Pharmacologic inhibitor of HGF receptor blunts the link between insulin resistance and increased mass of islets. A, Effects of the HGF receptor pharmacological inhibitor (SU11274) on β-cell mass, accompanied by representative images of pancreatic islets stained for insulin from DIO rats. B, Percentage of Ki-67-positive cell in β-cells and representative images of islets stained for Ki-67, in addition to (C) percentage of TUNEL-positive cell in β-cells and representative images of islets stained for TUNEL in DIO-60D rats treated with SU11274. *Scale bars*, 100 μm (*black*) and 400 μm (*red*). All experiments were conducted in triplicate (six rats each), and results are expressed as mean ± SEM. *, $P < 0.05$ *vs.* DIO-60D. Ctl, Control.

death (Fig. 5, B and C). Along with these results, an incremental change on insulin levels was no longer observed, suggesting that the compensatory mechanism was shut down (Fig. 6A).

We also measured plasma C-peptide levels to investigate *de novo* insulin production. In accordance with reduction in plasma insulin levels, obese animals treated with SU11274 also showed a decrease in plasma C-peptide concentrations, therefore demonstrating a reduction in *de novo* synthesis of insulin in these animals (Fig. 6B). Additionally, DIO-60D rats treated with SU11274 were more glucose intolerant (Fig. 6C) than untreated DIO-60D rats. This effect in the treated rats was also accompanied by a reduction in insulin levels during the GTT (Fig. 6D).

The animals under a chow diet and treated with SU11274 for the same time showed no change in fasting insulin, glucose, or C-peptide levels or in glucose tolerance. It is important to mention that SU11274 did not change the body weight of the rats on cafeteria or chow diet (Fig. 6E), as well as fat mass (epididymal fat pad content) (data not shown).

HGF signaling in isolated β-cells and in liver

HGF treatment of isolated β-cells from rats for 15 and 30 min resulted in increased levels of insulin receptor sub-

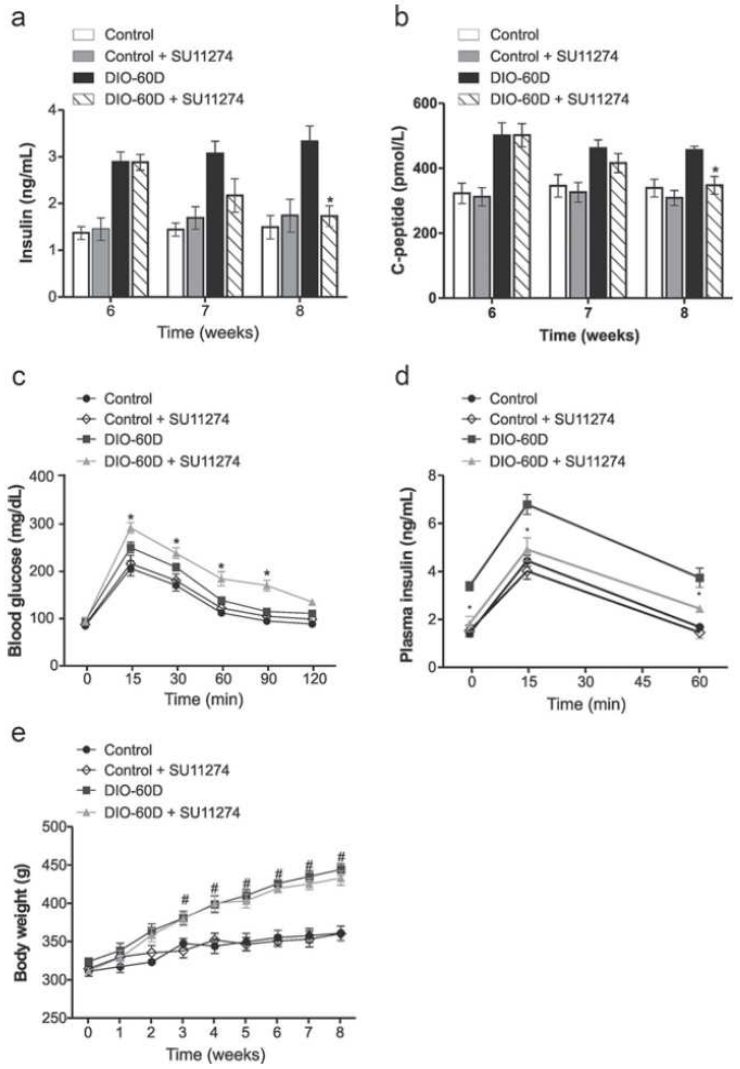

FIG. 6. SU11274 treatment and metabolic parameters of obese and nonobese rats. Fasting plasma insulin (A) and C-peptide levels (B) of obese rats treated with SU11274. The treatment with SU11274 started in the sixth week of the cafeteria or chow diet and lasted until the eighth week. All groups of animals received the respective diet throughout the treatment (*i.e.* from wk 6 to 8). Blood glucose (C) and plasma insulin (D) during ip GTT from control, control + SU11274, DIO-60D, and DIO-60D + SU11274 groups in the eighth week of the experiment. E, Temporal evolution of body weight from control, control + SU11274, DIO-60D, and DIO-60D + SU11274 groups for 8 wk. All experiments were conducted in triplicate (six rats each), and results are expressed as mean ± SEM. *, $P < 0.05$ *vs.* DIO-60D; #, $P < 0.05$ *vs.* control.

strate IRS-2 tyrosine phosphorylation, and also protein kinase B serine 473 (AKT^{ser473}) phosphorylation and ERK phosphorylation (Fig. 7A). In liver, as previously described (5, 17), insulin-induced IR and AKT^{ser473} phosphorylation were reduced in DIO-60D rats, but the infusion of HGF together with insulin significantly induce IR and AKT^{ser473} phosphorylation. On the other side, the blockage of HGF signaling was able to decrease even more the already reduced insulin-induced IR and AKT^{ser473} phosphorylation (Fig. 7B).

Discussion

In most situations of insulin resistance, β-cells compensate for the insulin resistance with an increase in β-cell mass. However, the driving forces behind this compensatory mechanism are incompletely understood. Although it has been demonstrated that there is a neuronal component in pancreatic β-cell proliferation in obesity (2–4), there is also clear evidence that circulating growth factors have a determinant role in this pathophysiological mechanism (1, 28, 29).

It has been assumed that glucose, independent of other factors, can induce an increase in islet mass, and it is known that glucose stimulates β-cell replication (29). However, in most situations of insulin resistance, the increase in islet mass precedes the increase in blood glucose, and also, there is no correlation between the blood glucose levels and islet hyperplasia (1, 30), indicating that other circulating factors independent of glucose likely contribute to the islet growth. Among circulating growth factors, GH, IGF-I, prolactin, placental lactogen, leptin, and HGF can induce islet mass hyperplasia (1, 9, 10, 31, 32). Prolactin and placental lactogen are candidates that can contribute to explain the compensatory response to insulin resistance in pregnancy. Leptin has also been suggested to promote β-cell growth (33); but in genetic leptin-deficient mouse, which also has insulin resistance and islet hyperplasia, this could not be a factor (34). Our data, in accordance with previous data, showed that GH and IGF-I are either normal or low in DIO mice or rats, indicating that these growth factors are unlikely candidates (35, 36). In the present study, we focus on HGF, as previously described, on the basis that it is increased in the most prevalent situation of insulin resistance-obesity, it is produced by the liver under the control of ERK (8), and it is able to

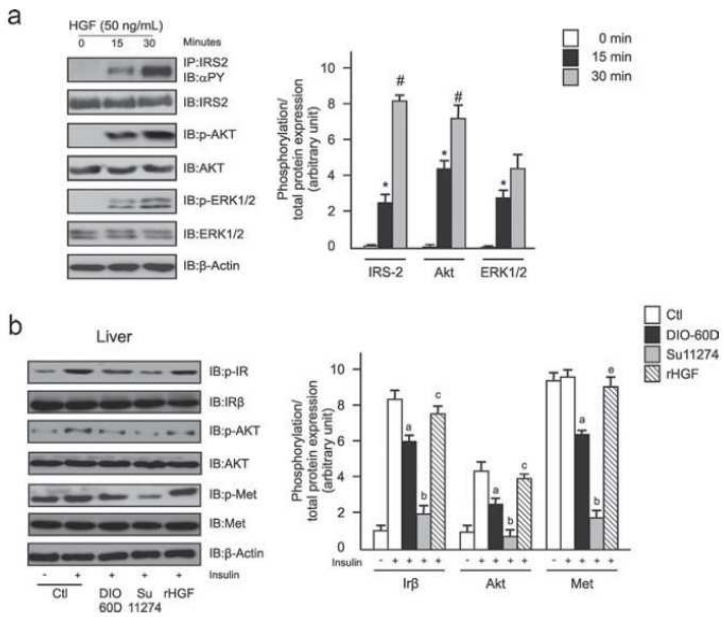

FIG. 7. HGF induces insulin signaling proteins in pancreatic islets and liver of rats. A, The effects of rHGF treatment for 15 or 30 min on phosphorylation levels of IRS-2, AKT^{ser473}, and ERK1/2 in isolated rat islet. B, Insulin-induced phosphorylation of IRβ, AKT^{ser473}, and Met in liver of control (Ctl), DIO-60D, SU11274-treated, and rHGF-treated obese rats. With the exception of the control group, all groups of animals received the cafeteria diet throughout the treatment (*i.e.* from wk 6 to 8). All experiments were conducted in triplicate (six rats each), and results are expressed as mean ± SEM. *, $P < 0.05$ *vs.* 0 min; #, $P < 0.05$ *vs.* 15 min; a, $P < 0.05$ *vs.* control; b, $P < 0.01$ *vs.* DIO-60D; c, $P < 0.05$ *vs.* SU11274-treated group; and e, $P < 0.05$ *vs.* DIO-60D and SU11274-treated group. IB, Immunoblot; IP, immunoprecipitation.

induce islet hyperplasia. Our approach aimed to show a possible cause-effect relationship between increase in circulating HGF levels and compensatory islet hyperplasia/hyperinsulinemia by showing the strength of the association, whether or not is a dose-dependent response, and the temporality, consistency, plausibility, and reversibility of the association.

Our data showed that in different models of insulin resistance, a strong and significant correlation between HGF and islet hyperplasia/insulin levels exists, indicating that this association is consistent. In addition, we observed that an increase in HGF levels seemed to precede the compensatory response associated with insulin resistance and also after partial hepatectomy and that there was a clear dose response for the effect of HGF on increased islet mass.

It is important to mention that in an animal model of insulin resistance, we observed an increase in HGF protein expression in liver and in adipose tissue, which are two tissues very well characterized in insulin-resistant situations. We can then hypothesize that somehow insulin resistance in these tissues might induce an increase in HGF, which will contribute to trigger a compensatory response.

HGF exerts its effects through Met, which is the tyrosine kinase cell surface receptor for HGF. It is important to mention that this receptor is structurally related to the insulin receptor, which is also a tyrosine kinase. Very recently, Fafalios *et al.* (37) demonstrated that Met activation induces a Met-insulin receptor complex that uses substrates of the insulin receptor and amplifies the signal. It is well established that the insulin signaling pathway has an important role in islet cell hyperplasia and increases in islet mass. Our data, in accordance with very recent data obtained in mice liver (37), showed that HGF treatment of isolated β-cells from rats resulted in increased levels of IRS-2 tyrosine phosphorylation and also of AKT^{ser473} and ERK phosphorylation. This indicates that the correlation between HGF and β-cell mass increase may involve these proteins from insulin signaling pathways, which may represent the molecular mechanism by which HGF induces the compensatory response associated with insulin resistance.

Taken together, these data suggest the association between HGF and islet hyperplasia may be a cause-effect phenomenon. This is supported by the importance and consistency of the association, as well as by its dose responsiveness and its temporal component. There is also a molecular mechanism through the insulin signaling pathway that can contribute to explaining this association, and equally important, by blocking the Met receptor we can completely reverse islet hyperplasia and in this way impair glucose tolerance, confirming the reversibility of the phenomenon. In addition, several studies have demonstrated the antiapoptotic effect of HGF (38–40), and our data suggest that such effect was blunted by blockage of the Met receptor.

Another important point in our study is the demonstration that the compensatory effect of HGF is not only related to islets. At least in liver, blocking Met impaired the already reduced insulin-induced insulin signaling in DIO-60D rats. These data strongly support the idea that HGF signaling has a protective role in insulin resistance.

Overall, our data indicate that HGF is a growth factor performing a key role in islet mass increase and hyperinsulinemia in DIO animals and suggest that the HGF-Met axis has an important role on insulin signaling in the liver.

Our study provides additional evidence for a role of HGF in insulin signaling in liver and may greatly contribute toward a better understanding of insulin resistance mechanisms associated with obesity and type 2 diabetes mellitus.

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Discussão

Na maioria das situações de resistência à insulina, as células β compensam a resistência à insulina com um aumento da sua massa, no entanto, as forças por traz deste mecanismo de compensação não são completamente compreendidas. Embora tenha sido demonstrado que há um componente neuronal na proliferação de células β do pâncreas na obesidade (Lam, Schwartz et al. 2005; Uno, Katagiri et al. 2006; Imai, Katagiri et al. 2008), também há provas claras de que fatores circulantes de crescimento tem um papel determinante neste mecanismo fisiopatológico (Bonner-Weir and Smith 1994; Mauvais-Jarvis, Virkamaki et al. 2000; Flier, Kulkarni et al. 2001). Tem sido assumido que a glicose, independente de outros fatores pode induzir um aumento da massa das ilhotas pancreáticas, pois sabe-se que a mesma estimula a proliferação de células β (Bonner-Weir and Smith 1994). Entretanto, na maioria das situações de resistência à insulina o aumento da massa das ilhotas precede o aumento da glicose no sangue, além disso, não foi demonstrada nenhuma correlação entre os níveis de glicose no sangue e a hiperplasia das ilhotas (Bruning, Winnay et al. 1997; Flier, Kulkarni et al. 2001), indicando que outros fatores circulantes independentes da glicose provavelmente contribuem para o crescimento das ilhotas. Entre os fatores circulantes de crescimento, podemos citar o hormônio de crescimento, o IGF-I, a prolactina, o lactogênio placentário, a leptina e o HGF, os quais podem induzir um aumento da massa das ilhotas (Otonkoski, Beattie et al. 1994; Otonkoski, Cirulli et al. 1996; Rhodes 2000; Flier, Kulkarni et al. 2001; Wu, Liu et al. 2011). O lactogênio placentário e a prolactina são candidatos que podem contribuir para explicar a resposta compensatória relacionada a resistência à insulina durante a gravidez. A leptina também foi sugerida como promotora do crescimento de células β (Tanabe, Okuya

et al. 1997), mas no animal geneticamente deficiente de leptina, que também tem a resistência à insulina e hiperplasia das ilhotas, este não pode ser um fator (Zhang, Proenca et al. 1994). Nossos dados estão de acordo com dados anteriores que mostraram que o hormônio de crescimento e o IGF-I estão normais ou baixos em ratos obesos induzidos por dieta, o que indica que estes fatores de crescimento são candidatos pouco prováveis (Cascieri, Slater et al. 1989; Pete, Fuller et al. 1999).

No presente estudo, nós focamos no HGF, como descrito anteriormente com base em que o mesmo está aumentado na situação de resistência à insulina mais comum, a obesidade. Ainda, o HGF é produzido pelo fígado sob o controle da Erk (Motoki, Sugiura et al. 2008), e é capaz de induzir hiperplasia na ilhota. Nossa abordagem teve como objetivo mostrar uma relação de causa-efeito entre o possível aumento dos níveis circulantes de HGF e a hiperplasia compensatória da ilhota/hiperinsulinemia, mostrando a força da associação, se a mesma é ou não é uma resposta dose-dependente, sua temporalidade, e ainda a consistência, a plausibilidade, e a reversibilidade da associação.

Nossos dados mostraram que em diferentes modelos de resistência à insulina, se observa uma correlação forte e significante entre os níveis de HGF circulante e a hiperplasia das ilhotas/níveis circulantes de insulina, indicando que esta associação é consistente. Além disso, observou-se que o aumento nos níveis de HGF circulante parecia preceder a resposta compensatória associada com a resistência à insulina e também após a hepatectomia parcial, e ainda que havia uma clara relação de dose-resposta do HGF sobre o aumento na massa de ilhotas. É importante mencionar que, no modelo animal de resistência à insulina, observou-se um aumento da expressão da proteína do HGF no fígado e no tecido adiposo, que são dois tecidos muito bem caracterizados em situações de resistência à

insulina. Assim, podemos então a sugerir que a resistência à insulina de alguma forma nestes tecidos pode induzir um aumento no HGF, o que de certa forma irá contribuir para desencadear uma resposta compensatória.

O HGF exerce os seus efeitos através de seu receptor (c-Met), este que é um receptor tirosina quinase de superfície celular. É importante mencionar que este receptor está estruturalmente relacionado com o receptor de insulina, que é também uma tirosina quinase. Recentemente, Fafalios et al. (Fafalios, Ma et al. 2011) demonstraram que a ativação do c-Met induz a formação de um complexo, receptores c-Met-insulina, o qual utiliza substratos do receptor de insulina e amplifica a sinalização da mesma. Está bem estabelecido que a via de sinalização da insulina tem um papel importante na hiperplasia e no aumento da massa das ilhotas. Nossos dados, de acordo com dados recentes, obtidos em fígado de ratos (Fafalios, Ma et al. 2011), demonstraram que o tratamento com o HGF em ilhotas isoladas de ratos resultou em um aumento dos níveis de fosforilação em tirosina do IRS-2, e também da fosforilação da Akt^{ser473} e da Erk. Isto indica que a correlação entre o HGF e o aumento da massa de células β pode envolver estas proteínas de vias de sinalização da insulina, desta forma, representando o mecanismo molecular pelo qual o HGF induz a resposta compensatória associada com a resistência à insulina.

Em conjunto, estes dados sugerem que a associação entre o HGF e a hiperplasia das ilhotas pode ser um fenômeno de causa-efeito. Isto é suportado pela importância e consistência da associação, bem como pela sua capacidade de resposta a dose e a sua temporalidade. Há também um mecanismo molecular através da via de sinalização da insulina que pode contribuir para explicar esta associação e, igualmente importante, o qual através do bloqueio do receptor (c-Met) podemos reverter completamente a hiperplasia das

ilhotas e, deste modo diminuir a tolerância à glicose, o que confirma a reversibilidade do fenômeno. Além disso, vários estudos têm demonstrado o efeito anti-apoptótico do HGF (Dai, Li et al. 2003; Garcia-Ocana, Takane et al. 2003; Santangelo, Matarrese et al. 2007), e os nossos dados sugerem que este efeito foi anulado pelo bloqueio do receptor c-Met. Outro ponto importante em nosso estudo é a demonstração de que o efeito compensatório do HGF não é apenas relacionado com ilhotas. Pelo menos, no fígado de ratos obesos induzidos por dieta, onde a sinalização da insulina já se encontra reduzida, é observado que após o tratamento destes animais com o bloqueador do c-Met ocorre uma piora nesta sinalização. Estes dados suportam a idéia de que a sinalização do HGF tem um papel protetor na resistência à insulina.

Conclusão

No geral, os nossos dados indicam que o HGF é um fator de crescimento que desempenha um papel chave no aumento da massa das ilhotas/hiperinsulinemia em animais obesos induzidos por dieta, e também sugerem que o eixo HGF/c-Met tem um papel importante na sinalização da insulina no fígado. Nosso estudo fornece evidências adicionais para um papel do HGF na sinalização da insulina no fígado, e também pode contribuir significativamente para uma melhor compreensão dos mecanismos de resistência à insulina associada à obesidade e diabetes mellitus tipo 2.

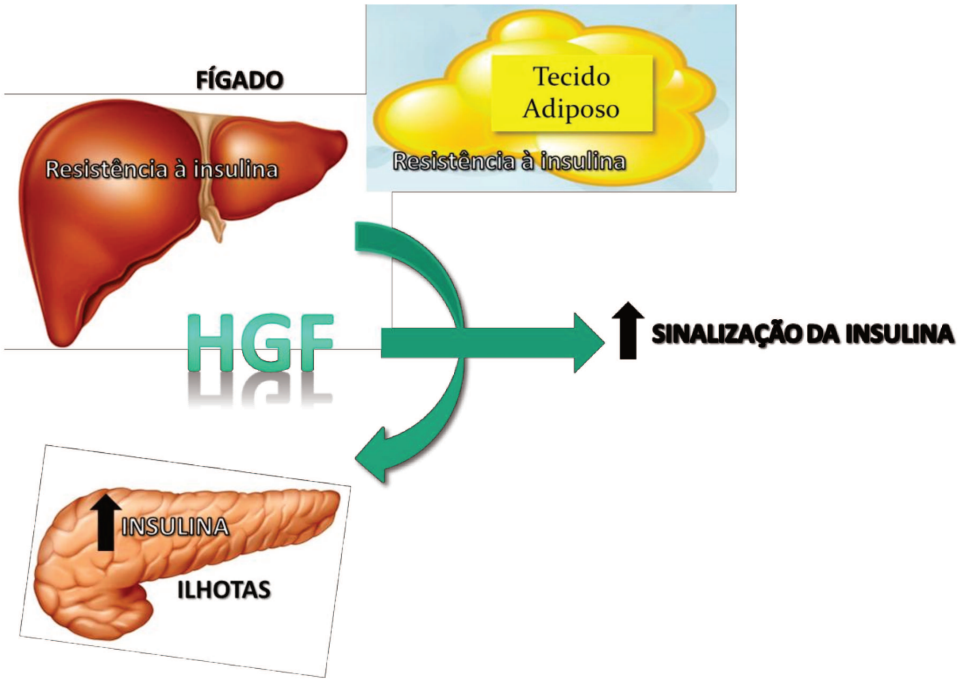

Figura 4. Modelo proposto para o papel do HGF como elo entre a resistência à insulina e a resposta compensatória das células beta.

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ORIGINAL ARTICLE

Liver regeneration following partial hepatectomy is improved by enhancing the HGF/Met axis and Akt and Erk pathways after low-power laser irradiation in rats

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Abstract A simple, easy, and safe procedure aiming to improve liver regeneration could be of great clinical benefit in critical situations such as major hepatectomy, trauma, or hemorrhage. Low-power laser irradiation (LPLI) has come into a wide range of use in clinical practice by inducing regeneration in healthy and injured tissues. However, the effect of LPLI on the process of liver regeneration, especially those related to the molecular mechanisms, is not fully understood. Thus, the aim of the present study was to investigate the main molecular mechanisms involved in liver regeneration of partially hepatectomized rats exposed to LPLI. We used Wistar male rats, which had their remaining liver irradiated or not with LPLI (wavelength of 632.8 nm and fluence of 65 mW/cm^2) for 15 min after a 70 % hepatectomy. We subsequently investigated hepatocyte growth factor (HGF), Met, Akt, and Erk 1/2 signaling pathways through protein expression and phosphorylation analyses along with cell proliferation (proliferating cell nuclear antigen (PCNA) and Ki-67) using immunoblotting and histological studies. Our results show that LPLI can improve liver regeneration as shown by increased HGF protein expression and the phosphorylation levels of Met, Akt, and Erk 1/2 accompanied by higher levels of the PCNA and Ki-67 protein in the remnant livers. In summary, our results suggest that LPLI may play a clinical role as a simple, fast, and easy-to-perform strategy in order to enhance the liver regenerative capacity of a small liver remnant after hepatectomy.

Keywords Hepatectomy · Laser · Liver regeneration · HGF · Met · Proliferation · Rats

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Introduction

The capacity for the liver to regenerate has been long recognized and is related to a sequence of molecular and cellular events that result in the induction of DNA synthesis, cell cycle progression, mitosis, and cell division [1, 2]. Several strategies to accelerate these events have been tested with controversial results [3–8]. A simple, easy, and safe procedure aiming to prepare the liver to regenerate could be of great clinical benefit in critical situations such as major hepatectomy, trauma, or hemorrhage.

In the clinical setting, preparing the liver parenchyma for regeneration during surgery is very interesting. Theoretically, it could lead to a better postoperative outcome in cases with a small liver section remaining following liver resection or living-related liver transplantation. In the past,

several strategies generically known as "pre/post-conditioning" were tested in order to stimulate liver regeneration before massive resection and in situations when the liver remnant may be too small [3, 4, 9].

Different techniques for conditioning the liver remnant can be pharmacological or mechanical. Ischemic preconditioning, intermittent clamping of the hilum, and stutter clamping release are examples of mechanical strategies, but their clinical effectiveness is still under investigation [3–6]. Inhibitors of PPAR-gamma, *n*-acetyl cysteine, and pentoxifiline are examples of pharmacological approaches that have also been used in the past in order to improve liver capacity to respond to ischemic injury. However, these drugs may interfere in important homeostatic systems, such as coagulation, that make their usage very restricted [7, 8]. There is some evidence showing that low-power laser irradiation (LPLI), especially red and near infrared light, may have an important role towards inducing regeneration in healthy and injured tissues [10, 11].

LPLI has come into a wide range of use in clinical practice especially for presenting a very important physiological effect: the induction of cell proliferation [10]. This biostimulatory effect has been shown in many cell type studies in vitro, including keratinocytes [12], fibroblasts from different systems [13–15], human osteoblasts [16], lymphocytes [17], mesenchymal stem cells, cardiac stem cells [18], aortic smooth muscle cells [19], and venous endothelial cells [20]. In particular, LPLI is well known to promote a stimulating effect on cell regeneration accompanied by a consequent improvement in ischemic or mechanical problems in organs such as skeletal muscle, heart, brain, and liver [11, 21–26]. Regarding the main molecular mechanisms involved in the liver regeneration, it is well established that hepatocyte growth factor (HGF) is a mesenchymal-derived pleiotropic cytokine that regulates cell proliferation, anti-apoptosis, motility, morphogenesis, and anti-inflammatory process in hepatic regeneration [27]. These multifaceted events occur via activation of its receptor, a transmembrane tyrosine kinase called Met. Activation of the Met receptor by HGF leads to autophosphorylation on tyrosine residues, followed by the phosphorylation of downstream signaling molecules, including phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathway proteins [27]. However, the effect of LPLI on the process of liver regeneration, specifically related to the molecular mechanisms, is not yet fully understood. The aim of the present study was to investigate the main molecular mechanisms involved in liver regeneration of partially hepatectomized rats (70 % hepatectomy) exposed to LPLI.

Materials and methods

Study subjects

All experimental protocols were approved by the Animal Care and Use Committee at the State University of Campinas (no. 1962-1) and were in accordance with the guidelines for Care and Use of Laboratory Animals. Five-week-old male *Wistar* rats weighing 200–250 g were obtained from the State University of Campinas Central Breeding Center. The animals were maintained under a controlled room temperature (23±2 °C) under a 12/12-h light and dark cycle and were fed with standard laboratory chow and water ad libitum.

All animals were randomly assigned to three groups which consisted of six rats each: group sham-operated controls (sham); group PHx (submitted to partial hepatectomy (70 %)); and group PHx + Laser (submitted to partial hepatectomy (70 %) and laser therapy).

Seventy percent partial hepatectomy (Higgins procedure)

Animals were anesthetized with ketamine 5 % (30 mg/kg) and xylazine 2 % (30 mg/kg) intraperitoneally. Under strict sterile conditions, two thirds partial hepatectomy was performed according to the method of Higgins and Anderson [28]. Briefly, the left lateral and median hepatic lobes, constituting approximately 70 % of the total liver weight, were ligated and resected. In sham-operated controls that were anesthetized as described above, the livers were briefly removed from the peritoneal cavity, but were not tied or excised.

During the surgery, animals were kept warm with a halogen light (45 W, 127 V) and corporeal temperature was monitored by a rectal digital thermometer (YSI Precision 4000A Thermometer) and maintained around 37 °C. Animals were allowed spontaneous ventilation with an oxygen-enriched mixture (40 %) during the entire procedure. To improve the survival rate post-surgery, 20 % glucose was added to the rat's drinking water [29].

Laser treatment

Before closing the surgical wound, the laser group was treated by direct irradiation of the remnant liver with an fluence of 65 mW/cm^2 for a period of 15 min at a distance of 10 cm, with a He–Ne Laser model 3184H V1.0, wavelength of 632.8 nm, and 3.30 mW (Hughes® Aircraft Company Electron Dynamics Division, Culver City, CA, USA). The laser beam was optically expanded to match the entire size of the remaining liver to guarantee uniform exposure. Recordings from the thermocouple placed under the irradiated area showed no elevation of temperature in the abdomen of the rats.

Tissue extraction and immunoblotting

Forty-eight hours after the partial hepatectomy and laser exposure, rats were anesthetized and used 10–15 min later, as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened and any remaining liver tissue was removed and homogenized immediately in extraction buffer at 4 °C (1 % Triton X-100, 100 mM Tris–HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM phenylmethylsulfonyl fluoride, and 0.1 mg of aprotinin/ml) with a Polytron PTA 20 S generator (model PT 10/35; Brinkmann Instruments). Insoluble material was removed by centrifugation for 30 min at 9,000×*g* in a 70 Ti rotor (Beckman, Fullerton, CA, USA) at 4 °C. The protein concentrations of the supernatants were determined by the Bradford dye-binding method [30–32]. In direct immunoblot experiments, protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-HGFα, anti-phospho-Met, anti-phospho-Akt, anti-phospho-Erk 1/2, and anti-PCNA. The homogeneity of gel loading was evaluated by blotting the membranes with antibodies against β-actin, Met, Akt, and Erk 1/2 as appropriate.

Immunohistochemistry and histology

To detect Ki-67, microwave postfixation was carried out by a domestic microwave oven which was applied to slides immersed in 0.01 mol/L citrate buffer, pH 6.0, in two 7-min doses separated by a 2-min break. Sections were then incubated at 4 °C overnight with primary monoclonal mouse antihuman Ki-67 clone MIB-1 from Dako (diluted 1:100). The slides were then incubated with avidin–biotin complex LSAB + Kit from DakoCytomation for 30 min followed by the addition of diaminobenzidine tetrahydrochloride as a substrate–chromogen solution. After hematoxylin counterstaining and dehydration, the slides were mounted in Entellan from Merck® [32, 33].

Outcome variables

The outcome variables in this study were changes in the expression levels (arbitrary units) of the proteins (HGF and PCNA), as well as changes in the phosphorylation levels (arbitrary units) of the proteins (Met, Akt, and Erk). These outcomes were obtained by immunoblotting technique. Another outcome variable was obtained by immunohistochemistry method, which consists of the number of cells stained for Ki-67 per field analyzed.

Materials

Antibodies against β-actin (mouse monoclonal, sc-8432), HGFα (rabbit polyclonal sc-7949), phospho-Erk 1/2 (mouse monoclonal, sc-7383), Erk 1/2 (mouse monoclonal, sc-93), and PCNA (mouse monoclonal, sc-25280) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho (Tyr1234/1235)-Met (rabbit monoclonal, #3077), Met (rabbit monoclonal, #8198), phospho (Ser 473)-Akt (rabbit polyclonal, #9271S), and Akt (rabbit polyclonal, #9272) were obtained from Cell Signaling Technology (Beverly, MA, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless specified otherwise.

Statistical analysis

Data are displayed as mean ± standard error of the mean (SEM). The results of blots are presented as direct comparisons of bands or spots in autoradiographs and quantified by optical densitometry (UN SCAN IT gel®, Silk Scientific Inc., Orem, UT, USA). Multiple comparisons were tested by one-way ANOVA, followed by Tukey's post hoc test, with the significance level set at $p<0.05$ using SPSS software (SPSS for Windows, version 16.0, Chicago, IL, USA).

Results

Laser exposure improves the HGF/Met axis in hepatectomized rats

In this paper, we realized to determine how the HGF expression behaves after laser exposure in hepatectomized rats. As shown in Fig. 1a, the HGF protein expression in the remnant liver of PHx group was increased when compared to the sham group, and the PHx + Laser group exhibited a more pronounced increase when compared with the PHx group.

Since the phosphorylation at tyrosine residues of Met receptor is a hallmark of its activation, we examined the phosphorylation levels of Met. The results showed that tyrosine phosphorylation levels of Met were higher in PHx group compared to the sham group and that the laser exposure induced a more pronounced increase in Met phosphorylation (Fig. 1b). We did not observe differences in Met expression amongst any study groups (Fig. 1b, lower panel).

Laser exposure enhanced Akt and Erk 1/2 signaling along with higher PCNA expression in hepatectomized rats

The downstream pathways of Met mainly related to cell proliferation and growth, such as PI3K/Akt and MAPK pathway, were analyzed. Animals submitted to partial hepatectomy showed higher phosphorylation levels of Akt and Erk 1/2 when compared to the sham group (Fig. 2a, b).We also observed that the PHx + Laser group exhibited a

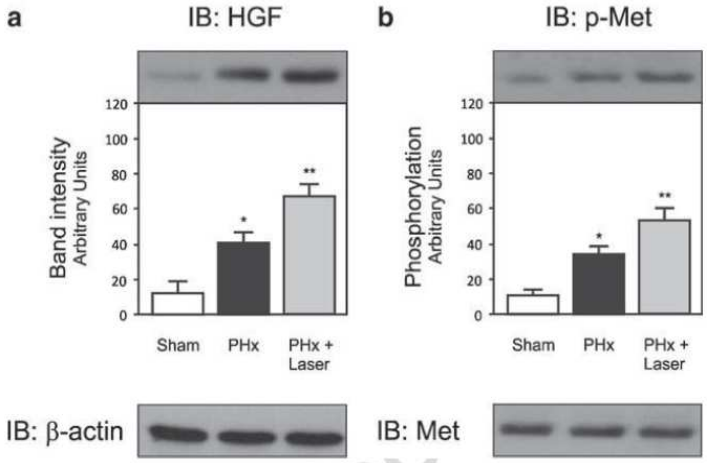

Fig. 1 Representative blottings show HGF content (**a**) and Met activation (**b**) in remaining livers of partial hepatectomized rats exposed or nonexposed to the laser. Total protein expression of Met (**b**). Western blots were quantified after standardization with β-actin. Data were representative of three independent experiments. The values represent the mean ± SEM (n=6). *p<0.05 vs. sham; **p<0.05 vs. PHx. *IB*, immunoblot

significant increase in phosphorylation levels of Akt and Erk 1/2 in comparison with the PHx group (Fig. 2a, b). No changes in Akt and Erk 1/2 protein expression were observed among groups (Fig. 2a, b—lower panels).

In order to strengthen the role of the HFG/Met axis in cell proliferation, we considered PCNA protein expression. The results showed that PCNA expression was increased in the PHx group when compared to sham animals, and that the laser treatment was able to induce an additional increase in PCNA protein expression (Fig. 2c).

Ki-67-positive cells are increased by laser treatment in hepatectomized rats

After 48 h of laser exposure, remaining livers from partially hepatectomized rats were also evaluated by Ki-67 immunohistochemistry to contribute towards data on hepatic regeneration. As expected, we observed a strong increase in the number of positive Ki-67 staining cells from the PHx group in comparison with the sham group (Fig. 3). Indeed, when the partially hepatectomized rats were submitted to laser exposure, the number of positive Ki-67 staining cells was higher than those observed in the PHx group (Fig. 3).

Discussion

Looking for an easy, safe, and intraoperative strategy in order to improve post-hepatectomy liver regeneration, the intraoperative application of LPLI on the remnant liver parenchyma in animal model may be an interesting option. Several studies have demonstrated that LPLI improves hepatic regeneration post-hepatectomy [11, 23–26]. However, the molecular mechanisms behind this effect are not well understood. This paper helps to demonstrate the key

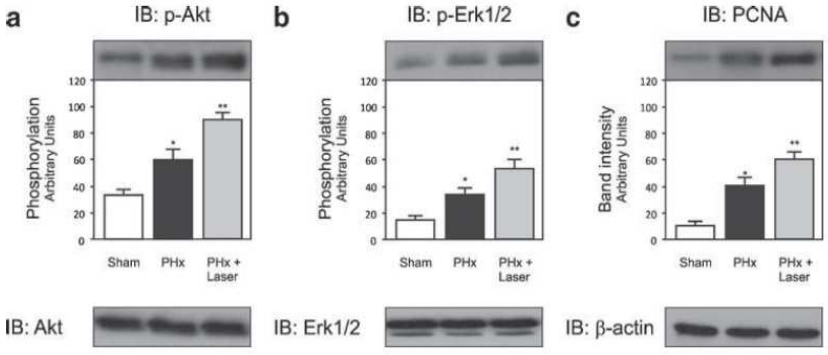

Fig. 2 Representative blottings show Akt (**a**) and Erk 1/2 (**b**) phosphorylation levels, as well as PCNA expression (**c**) in remaining livers of partial hepatectomized rats exposed or nonexposed to the laser. Total protein expression of Akt and Erk 1/2 (**a**, **b**). β-actin was used as a normalization housekeeping protein. Data were representative of three independent experiments. The values represent the mean ± SEM (n=6). *p<0.05 vs. sham; **p<0.05 vs. PHx. *IB*, immunoblot

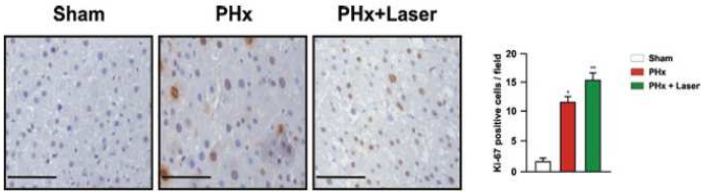

Fig. 3 Representative microphotographs of Ki-67 immunopositive staining hepatocytes on liver and remnant livers of sham, PHx, and PHx + Laser rats. The *graph* indicates the number of Ki-67-positive cells per field; five fields per section. *Scale bars*, 200 μm. The values represent the mean ± SEM (n=6). *p<0.05 vs. sham; **p<0.05 vs. PHx

molecular pathways involved in the LPLI effect on liver regeneration.

In the current study, we used a robust and traditional model of liver regeneration in rodents called the "Higgins procedure," which consists of a two thirds partial hepatectomy [28, 34]. This induces an intense and sustained hyperplasia of the remnant lobes, which reach the original size in about 6–7 days [34]. Furthermore, justifying the approaches chosen in this study, previous research shows that in the model of the partial hepatectomy, the hepatocytes undergo a well-orchestrated cell cycle. DNA synthesis begins 12–16 h after hepatectomy and peaks at 24–48 h. The beginning of mitosis occurs 6–8 h after the surgery, reaching its maximum 48 h later [35]. Based on this information, we studied the remaining livers 48 h after partial hepatectomy surgery and exposure to laser.

With regard to liver regeneration, HGF signaling is a major mechanism that controls cell proliferation and regeneration [35, 36]. When HGF binds to its receptor (Met), this receptor becomes phosphorylated on tyrosine residues, which in turn stimulates the phosphorylation of downstream signaling molecules, including PI3K and MAPK pathway proteins [27]. The PI3K/Akt signaling pathway produces a diversity of the biological effects of HGF, such as proliferation, anti-apoptosis, and migration [37, 38]. Similarly, the Erk 1/2 signaling pathway is also related to mediating proliferation, anti-apoptosis, and differentiation processes [39]. Our data show that after partial hepatectomy, the above-mentioned pathways were most active. It was observed that hepatectomized animals exhibited an increase in the expression of HGF followed by increases in phosphorylation levels of Met and its downstream signaling molecules (Akt and Erk 1/2).

Previous research has focused on the various mechanisms related to the biological effects of LPLI. Studies have demonstrated that the gene or protein expressions of several growth factors, such as transforming growth factor-β, vascular endothelial growth factor, platelet-derived growth factor, insulin-like growth factor 1, and others were increased by LPLI [10]. Accompanied with increased expressions of these growth factors, LPLI may induce improved cellular proliferation and differentiation induced by LPLI. Our data have built upon this previous research and demonstrate that HGF expression is increased in rat liver remnants exposed to the laser.

Additionally, LPLI presented mitogenic effects through the activation of specific receptors that are in the "right energetic state" to accept the laser energy, leading to their autophosphorylation and downstream effects [40]. LPLI (632.8 nm) has been reported to induce the phosphorylation of Met receptor and consequently activated downstream signaling molecules [41]. Accordingly, our study demonstrates that the remnant liver of rats exposed to the laser showed an increased phosphorylation of Met accompanied by an augmentation in Akt and Erk 1/2 phosphorylation. In corroboration with our results, studies carried out in myoblasts demonstrated that Akt phosphorylation is augmented when stimulated by LPLI, and when Wortmannin, a specific

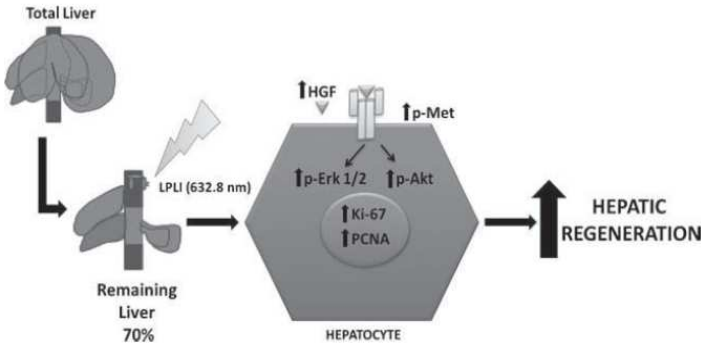

Fig. 4 Schematic representation showing an overview of the LPLI effect on the remaining liver of partially hepatectomized rats. LPLI can improve liver regeneration as evidenced by increasing HGF protein expression and the phosphorylation levels of Met, Akt, and Erk 1/2, along with higher levels of PCNA and Ki-67

inhibitor for the PI3K/Akt pathway was used, the LPLI-induced cell proliferation was attenuated [42]. In addition, another study performed in skeletal muscle cells demonstrates that LPLI also has a role in the MAPK/Erk pathway [41].

In order to confirm and emphasize our results, it is important to consider previously established proliferating markers. PCNA and Ki-67 fulfill this role well, but there are significant differences in expression of these two proteins. PCNA is evident from the M phase to G0 and/or G1, with a much longer half-life. On the other hand, Ki-67 is expressed in mid-G1, through S and G2, and reaches its peak expression in M phase [43]. In our study, in response to the signaling increased on aforementioned pathways (Met → Akt and Erk 1/2), the PCNA expression on the remnant liver of partially hepatectomized rats was increased. Indeed, when the remnant liver was exposed to the laser, PCNA expression presented an additional augmentation. This observation is in accordance with a previous study that also shows the upregulation of PCNA following LPLI stimulation in primary rat satellite cells [44]. In addition, the changes related to improvement in hepatocytes proliferation were consistent with our immunohistochemical study. In this study, we observed a significant increase in the number of hepatocytes stained with Ki-67 in remnant liver exposed to the laser. This approach has reinforced the important effect of the laser in the improvement of liver regeneration.

From a clinical perspective, it is well known that the small liver remnant is an important problem in a liver surgeon's daily practice that may lead to catastrophic postoperative course. It is difficult to predict when the liver remnant is big enough or too small to keep the patient alive in the postoperative period. In this sense, important strategies such as partial portal vein embolization have been proposed in order to avoid this situation, since it improves the volume of the liver remnant [3, 4, 6]. When portal blood flow is redirected to only one half of the liver (the remnant lobe), there is a substantial hyperplasia due to the presence of hepatotropic elements (e.g., insulin) in portal blood [3, 4]. However, this portal embolization must be performed preoperatively and one should wait some weeks (4–5 weeks) for a substantial improvement on the liver remnant. Nowadays, there are not any intraoperative alternative to improve liver regenerative capacity when the surgeon realizes that the liver remnant seems to be too small. This situation often causes a tremendous distress on surgical team. Since it is not possible to improve liver remnant size intraoperatively, an alternative to activate regenerative molecular pathways may be useful in order to avoid postoperative liver failure. To the best of our knowledge, laser has never been used to improve liver remnant regenerative potential. Since low potency laser application is safe and easy to perform and the activation of regenerative molecular pathways does not rely on the depth of laser penetration, thus it is reasonable to propose the utilization of laser during liver resection as a useful therapy in clinical practice.

In conclusion, our results show that LPLI can improve liver regeneration as evidenced by increasing HGF protein expression and the phosphorylation levels of Met, Akt, and Erk 1/2, along with higher levels of PCNA and Ki-67 in the remnant liver of partially hepatectomized rats (Fig. 4). Future research may help to show that LPLI may play a clinical role as a simple, fast, and easy-to-perform strategy in order to enhance liver regenerative capacity of a small liver remnant after hepatectomy.

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Conflict of interest No potential conflicts of interest relevant to this article were reported.

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