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PARTICIPAÇÃO DA ARHGAP21 NA REGULAÇÃO DA HOMEOSTASE GLICÊMICA E ENERGÉTICA DE CAMUNDONGOS C57BL/6

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Biologia Funcional e Molecular, na área de Fisiologia

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Orientador: Helena Cristina de Lima Barbosa Sampaio

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RESUMO

A obesidade e o diabetes mellitus tipo 2 vem crescendo consideravelmente em todo mundo, principalmente devido a alterações no estilo de vida e consumo de dietas hipercalóricas. Essa condição está associada, principalmente, a prejuízos no metabolismo glicídico e energético em humanos e em modelos de experimentação animal. Frente a isto, diversas proteínas que modulam o metabolismo glicêmico e energético vêm sendo investigadas, a fim de elucidar possíveis alvos terapêuticos no tratamento destas enfermidades, dentre as quais destacamos as proteínas ativadoras de GTPase (GAPs). As GAPs regulam a atividade de proteínas G de pequeno tamanho molecular, em geral, acelerando seu retorno a um estado inativo, através da indução da hidrólise do GTP. Recentemente as GAPs da família Rho vem sendo exploradas no contexto metabólico, sendo que a redução do conteúdo da Rho-GAP ARHGAP21 (proteína ativadora de GTPase Rho 21), em ilhotas pancreáticas de neonatos, induz aumento na secreção de insulina frente a baixa concentração de glicose. No entanto, o papel da ARHGAP21 no controle da homeostase glicêmica e energética ainda não está claro. Assim, neste trabalho objetivamos avaliar o papel da ARHGAP21 na regulação do metabolismo. Para isto, camundongos C57BL/6 selvagens ou heterozigotos para ARHGAP21 foram alimentados com dieta padrão ou hiperlipídica por 10 semanas. Como esperado, os camundongos selvagens que receberam a dieta hiperlipídica apresentaram intolerância à glicose, resistência à insulina, hipersecreção de insulina, além de aumento no peso corporal e nos depósitos de gordura. Os animais heterozigotos para ARHGAP21, quando alimentados com dieta hiperlipídica foram protegidos dos efeitos deletérios da dieta e não apresentaram nenhuma das alterações supracitadas. Além disso, observamos que os animais heterozigotos para ARHGAP21 apresentaram aumento na expressão dos genes hipotalâmicos anorexigênicos POMC e CART, corroborando com a diminuição do consumo alimentar e aumento do gasto energético. Esses achados destacam a proteína Rho-GAP ARHGAP21 como um importante modulador do metabolismo energético e da glicose, e um possível candidato para o tratamento de alterações na homeostase glicêmica e energética.

ABSTRACT

Obesity and type 2 diabetes mellitus have been growing considerably worldwide, mainly due to changes in lifestyle and increased consumption of hypercaloric diets. These diseases are associated with impairment in glucose and energy metabolism, both in humans and animal experimental models. Therefore, several proteins that can modulate glycemic and energetic metabolism have been investigated in order to elucidate possible targets to therapeutic treatment of these diseases, among which there are the GTPase activating proteins (GAPs). GAPs regulate the activity of small G proteins, usually, accelerating their inactivation process through the induction of GTP hydrolysis. Recently the Rho-GAPs have been explored in the metabolic context, and the knockdown of the Rho-GAP ARHGAP21 in neonate's pancreatic islets induces increased insulin secretion in low glucose concentration. However, the role of ARHGAP21 in glycemic and energetic homeostasis remains unclear. Thus, we aimed to evaluate the role of ARHGAP21 on metabolism regulation. To achieve this, wild C57BL/6 mice or knockdown for ARHGAP21 were fed with chow or high-fat diet for 10 weeks. As expected, wild-type mice that received high-fat diet presented glucose intolerance, insulin resistance, insulin hypersecretion, and increased body weight and fat depots. ARHGAP21 knockdown mice fed with high-fat diet were protected from the deleterious effects of the diet and did not present any of the above-mentioned alterations. In addition, we observed that ARHGAP21 knockdown mice presented increased POMC and CART gene expression, both anorexigenic hypothalamic genes. This data corroborates with decreased food consumption and increased energy expenditure of these mice. These findings highlight the Rho-GAP ARHGAP21 protein as an important modulator of energy and glucose metabolism, and a possible candidate for the treatment of alterations in glycemic and energetic homeostasis.

LISTA DE ABREVIATURAS E SIGLAS

Alfa-MSH – Hormônio estimulador dos melanócitos alfa

ADP – Adenosina difosfato

AgRP – Proteína relacionada ao Agouti

AKT/PKB – Proteína quinase B

AMPc – Adenosina monofosfato cíclico

ARHGAP21 – proteína ativadora de GTPase Rho 21

AS160 – Proteína substrato da AKT de 160 kDa

ATP – Adenosina trifosfato

Ca^{2+} – Íon Cálcio

CAP – *Cbl associated protein*

CART - *Cocaine- and amphetamine-regulated transcript*

Cbl – *Casitas B-lineage lymphoma*

C/EBPα – *CCAAT-enhancer-binding proteins alfa*

CRH – Hormônio liberador de Corticotrofina

DLC1 – *Deleted in liver cancer-1*

DM2 – Diabetes Mellitus tipo 2

FOXO – *Forkhead box O*

GAPs – Proteínas ativadoras de GTPase

G6P – Glicose-6-fosfatase

GCK – Glicoquinase

GLUT2 – Transportador de Glicose do tipo 2

GLUT4 – Transportador de Glicose do tipo 4

GSK-3beta – *Glycogen synthase kinase 3 beta*

GTP – Guanosina trifosfato

Het – Animais heterozigotos para ARHGAP21 que receberam dieta padrão

Het-HFD – Animais heterozigotos para ARHGAP21 que receberam dieta hiperlipídica

IKK beta – *Inhibitor of nuclear factor kappa-B kinase subunit beta*

IRS – Substrato do receptor de insulina

JAK2 – *Janus kinase 2*

K^+ – Íon Potássio

MAPK – *Ras-mitogen-activated protein kinase* U

MC3R/MC4R – Receptores 3 e 4 de Melanocortina

MCH – Hormônio concentrador de Melanina

NFκB – *Factor nuclear kappa B*

NPY – Neuropeptídeo Y

ObR/LepR – Receptor de Leptina

PEPCK – Fosfoenolpiruvato carboxilase

PGC-1 alfa – *Peroxisome proliferator-activated receptor gamma coactivator-1 alpha*

PI3K – Fosfatidilinositol 3-quinase

POMC – Proopiomelanocortina

PPAR gama – *Peroxisome proliferator-activated receptor gamma*

PRDM16 – *PR Domain-containing protein16*

Rap1 – *Ras-proximate-1*

Rap1GAP - *RAP1 GTPase activating protein 1*

RE – Retículo Endoplasmático

RER – Relação de troca respiratória

STAT3 – *Signal transducer and activator of transcription 3*

TAB – Tecido Adiposo Branco

TAM – Tecido Adiposo Marrom

TBC1D1 – *TBC1 domain family member 1*

TBC1D4 – *TBC1 Domain family member 4*

TCGAP - *C10/cdc42 GTPase activating protein*

TRH – Hormônio liberador de Tireotrofina

TSC2 – *Tuberous sclerosis complex 2*

UCP1 – Proteína desaclopadora 1

VO_2 – Consumo de oxigênio

SUMÁRIO

1. Introdução

1.1 Homeostase glicêmica

O pâncreas é uma glândula mista composta por uma região exócrina e endócrina. A maior parte desse órgão secretor consiste de células acinares, que compõe a região exócrina, responsáveis pelas secreções de enzimas digestivas e bicarbonato. Em contraste, a região endócrina é composta por um pequeno número de células agrupadas, formando as ilhotas de *Langerhans* ou ilhotas pancreáticas, as quais secretam hormônios (Pan and Wright, 2011; Shih *et al.*, 2013). As ilhotas apresentam três grupos celulares principais: as células alfa, células beta e células delta, que se distinguem entre si devido à síntese e secreção de glucagon, insulina e somatostatina, respectivamente (Cabrera *et al.*, 2006). Esses hormônios ajudam a controlar as concentrações de glicose no sangue. Portanto, uma disfunção das células endócrinas pancreáticas resulta em prejuízos na homeostase glicêmica, podendo levar ao desenvolvimento do Diabetes Mellitus tipo 2 (DM2) (Campos, 2012; Srinivasan *et al.*, 2012).

Um dos principais hormônios secretados pelo pâncreas é a insulina, e a sua secreção é controlada continuamente de acordo com sinais hormonais, neurais e a concentração de nutrientes circulantes, em especial, a glicose (Komatsu *et al.*, 2013; Röder *et al.*, 2016). A glicose circulante é absorvida pelo transportador de glicose do tipo 2 (GLUT2) que está localizado na superfície das células beta (Rutter *et al.*, 2015). Uma vez no interior da célula, a glicose é fosforilada à glicose-6-fosfato pela enzima glicoquinase (GCK) e metabolizada gerando ATP, resultando em aumento da razão ATP/ADP intracelular. O aumento nesta razão leva ao fechamento de canais de K^+ sensíveis ao ATP presentes na membrana das células beta (Ashcroft *et al.*, 1984). Em condições não estimuladas, esses canais estão abertos garantindo a manutenção do potencial elétrico de repouso da membrana celular, através do efluxo de íons K^+. Com o fechamento desses canais, e a consequente diminuição do efluxo de K^+, o potencial de repouso é alterado levando à despolarização da membrana celular. Em seguida, ocorre a abertura de canais de Ca^{2+} dependentes de voltagem, permitindo o influxo desse íon. O aumento de concentrações do Ca^{2+} intracelular, eventualmente, desencadeia maior atividade da maquinaria exocítica, que leva à fusão de grânulos contendo insulina com a membrana e a subsequente liberação de seu conteúdo na corrente sanguínea (Rorsman, 1997; Yang and Berggren, 2006; Rutter *et al.*, 2015).

Quando a insulina chega às células-alvo, ela se liga e ativa um receptor proteico de

membrana. Esse receptor é constituído de quatro subunidades que se mantêm unidas por meio de ligações dissulfeto: duas subunidades alfa externas à membrana celular e duas subunidades beta que penetram através da membrana, projetando-se no citoplasma celular (Mosthaf *et al.*, 1990; Pessin and Saltiel, 2000). A insulina se liga às subunidades alfa do lado externo da célula, promovendo a autofosforilação das subunidades beta. Essa autofosforilação ativa a tirosina quinase local que, por sua vez, causa fosforilação de diversas outras moléculas intracelulares, incluindo os substratos do receptor de insulina (IRS) (White *et al.*, 1988; Patti and Kahn, 1998). Uma vez fosforilados, os IRS irão ativar duas outras proteinas: a fosfatidilinositol 3-quinase (PI3K) e a proteína quinase B (PKB) ou AKT. A AKT ativa irá fosforilar vários substratos em resíduos de Serina ou Treonina, incluindo: o fator de transcrição *Forkhead box O* (FOXO), *Tuberous Sclerosis Complex 2* (TSC2) , *Glycogen synthase kinase 3 beta* (GSK3-beta) e *TBC1 domain family member 4* (TBC1D4) também conhecida como proteína substrato da AKT de 160 kDa (AS160) (Haeusler *et al.*, 2018). Essas proteínas efetoras medeiam os efeitos da insulina na produção, utilização e captação de glicose, bem como a síntese de glicogênio, proteínas e lipídios. A via de sinalização celular induzida pela insulina também ativa a via da *Ras-mitogen-activated protein kinase* (MAPK), a qual exerce impacto sobre os mecanismos de controle do crescimento e diferenciação celular (Avruch, 1998; Fantin *et al.*, 2000).

A insulina age em vários tecidos periféricos, incluindo músculo, fígado e tecido adiposo, afim de reestabelecer a glicemia. No fígado, a insulina tem como função principal inibir a produção hepática de glicose, através da inibição das enzimas glicose-6-fosfatase (G6P) e fosfoenolpiruvato carboxilase (PEPCK). No músculo e no tecido adiposo, a ativação da via da insulina leva a translocação do transportador de glicose do tipo 4 (GLUT4) para a membrana celular, permitindo assim a captação de glicose e seu armazenamento na forma de substratos energéticos (Yamauchi *et al.*, 1996; Saltiel and Kahn, 2001; Lizcano and Alessi, 2002; Laviola *et al.*, 2006; Rask-Madsen and Kahn, 2012). Além disso, a insulina tem um importante papel central, agindo no hipotálamo, resultando na redução do consumo alimentar, e na inibição de neurônios orexígenos (Sisley and Sandoval, 2011), efeito associado à ativação de pelo menos duas vias de sinalização: AKT/FOXO1 e *Janus kinase 2/Signal transducer and activator of transcription 3* (JAK2/STAT3) (Saad *et al.*, 1996; Carvalheira *et al.*, 2001; Tsai *et al.*, 2003).

1.2 Homeostase energética

O hipotálamo desempenha um papel importante na regulação da homeostase energética corporal, controlando o comportamento alimentar e o metabolismo energético em mamíferos (Schwartz *et al.*, 2000). Nesse contexto, a regulação da massa corporal depende, em grande parte, do balanço entre a ingestão calórica e o gasto energético (Morton *et al.*, 2006). Falhas neste sistema podem alterar a massa corpórea, levando ao ganho ou redução do peso corporal (Schwartz *et al.*, 2000).

Para equilibrar a ingestão e o gasto calórico, mantendo constantes as reservas de energia, é necessário que o sistema nervoso seja capaz de avaliar o conteúdo de reserva energética corporal continuamente. A leptina é, sem dúvida, o mais importante sinal periférico responsável por estabelecer uma conexão entre os sítios de estoque de energia e o sistema nervoso central (Zhang *et al.*, 1994; Friedman and Halaas, 1998). A leptina é um hormônio polipeptídico produzido pelo tecido adiposo branco (TAB) e secretado na circulação, em concentrações proporcionais à massa deste tecido (Zhang *et al.*, 1994; Considine *et al.*, 1996; Friedman, 2009). Ela atravessa a barreira hemato-encefálica e se liga ao seu receptor (ObR ou LepR) (Tartaglia *et al.*, 1995), que se encontra constitutivamente ligado a proteína citosólica JAK2, que possui atividade tirosina quinase (Bjørbaek *et al.*, 1997). A ligação da leptina promove a dimerização do receptor, com fosforilação em tirosina da JAK2, que por sua vez catalisa a fosforilação de resíduos de tirosina na porção intracelular do receptor. Em seguida, há ativação de uma série de proteínas envolvidas na transdução do sinal da leptina, incluindo a STAT3, que é translocada para o núcleo e regula a expressão gênica de neurotransmissores e outras proteínas (Bäckhed *et al.*, 2004).

A leptina age no sistema nervoso central, principalmente no núcleo arqueado do hipotálamo, sobre duas subpopulações de neurônios. Na primeira, a leptina estimula neurônios que expressam a proopiomelanocortina (POMC), que é clivada dando origem ao hormônio estimulador dos melanócitos alfa (alfa-MSH). O alfa-MSH age nos receptores 3 e 4 da melanocortina (MC3R e MC4R) em neurônios hipotalâmicos de segunda ordem e/ou em outras regiões do cérebro produzindo efeitos catabólicos. A segunda subpopulação de neurônios tem sua atividade suprimida pela leptina e exerce funções anabólicas, sintetizando dois peptídeos orexigênicos: neuropeptídeo Y (NPY) e proteína relacionada ao Agouti (AgRP). O NPY atua em receptores Y estimulando a ingestão alimentar e o AgRP é um antagonista do MC3R e MC4R (Coll *et al.*, 2008). Por sua vez, núcleos hipotalâmicos específicos possuem neurônios de segunda ordem que expressam os receptores supracitados,

sendo: o hipotálamo lateral, que expressa o hormônio concentrador de melanina (MCH) e a orexina, com funções anti-termogênicas e orexigênicas e o núcleo paraventricular, cujas células produzem o hormônio liberador de tireotrofina (TRH) e o hormônio liberador de corticotrofina (CRH), com funções pró-termogênicas e anorexigênicas (Velloso *et al.*, 2008).

A transdução do sinal da leptina também sofre importante controle por vias paralelas de sinalização celular, sendo que, a insulina se destaca como o principal modulador do sinal da leptina (Carvalheira *et al.*, 2001; Carvalheira *et al.*, 2005). A insulina desempenha uma função intermediária entre o controle da adiposidade e o controle imediato da fome (saciedade). A concentração sanguínea desse hormônio oscila em função da ingestão imediata de alimentos, mas também em função da massa adiposa total do organismo. A insulina é considerada o segundo sinalizador periférico mais importante para o hipotálamo, não apenas por sua ação abrangente, mas também por atuar como potencializador do sinal da leptina (Flier, 2004; Velloso *et al.*, 2008).

Além do papel do hipotálamo na homeostase energética, o tecido adiposo marrom (TAM) também regula este processo através da termogênese. A termogênese é o processo que dissipa energia na forma de calor para manter a temperatura corporal, ocorrendo principalmente no TAM (Seale *et al.*, 2011). No passado, acreditava-se que o TAM estava presente apenas em crianças. No entanto, técnicas de tomografia por emissão de pósitrons revelou a presença de TAM em adultos. Os depósitos de TAM em humanos são distribuídos na área supraclavicular e nas áreas perivascular e perivisceral (ao redor do coração, vias aéreas, intestinos, fígado e glândulas adrenais), do tórax e do abdômen (Sacks and Symonds, 2013).

A termogênese do TAM é importante para manter a temperatura corporal em resposta à exposição ao frio, dissipando o excesso de energia após a ingestão de alto teor calórico. Como os substratos metabólicos, como a glicose e o ácido graxo, são consumidos durante a termogênese do TAM, isso pode afetar o peso e a massa de gordura corporal (Morrison and Madden, 2014). O cérebro regula a termogênese do TAM por meio da modulação do sistema nervoso simpático. A norepinefrina liberada dos terminais nervosos simpáticos age sobre os receptores beta 3-adrenérgicos nos adipócitos do TAM. Essa ativação de receptores adrenérgicos desencadeia sua sinalização via AMPc, que por sua vez aumenta a expressão da proteína desacopladora 1 (UCP1) na mitocôndria (Bartelt and Heeren, 2014).

Na regulação termogênica, o hipotálamo integra a sensação de temperatura corporal com a estimulação eferente simpática. Áreas hipotalâmicas, como o núcleo ventromedial, o

núcleo dorsomedial e o núcleo arqueado modulam a atividade termogênica influenciando o sistema nervoso simpático (Seoane-Collazo *et al.*, 2015). Além disso, sinais metabólicos hormonais e mediados por nutrientes podem influenciar a estimulação simpática para o TAM (Rothwell and Stock, 1981; Schwartz *et al.*, 1988; Rahmouni *et al.*, 2004; Lockie *et al.*, 2012).

Além do adipócito marrom clássico, existem as células beges, distribuídas discretamente no TAB, como no TAB inguinal em roedores (Wu *et al.*, 2012). Essas células também são conhecidas como adipócitos marrons induzíveis ou recrutáveis, pois podem ser induzidas a se diferenciar em TAM, processo conhecido como *browning* (Harms and Seale, 2013). Elas compartilham algumas características comuns com adipócitos marrons clássicos, como alto teor de mitocôndrias e expressão de UCP1 (Cousin *et al.*, 1992).

O *browning* do TAB é estimulado por uma interação hormonal complexa e vários fatores ambientais. O TAB é facilmente convertido em adipócitos beges com exposição prolongada ao frio ou tratamento com agonistas beta-adrenérgicos (Cousin *et al.*, 1992). O exercício também foi relatado sendo responsável pelo escurecimento do TAB e aumento no gasto energético (Handschin and Spiegelman, 2008). Dados crescentes também indicaram a importância do sistema nervoso central no escurecimento da gordura branca (Bi and Li, 2013; Mcglashon *et al.*, 2015), mostrando que o *knockdown* do NPY no hipotálamo dorsomedial promove o desenvolvimento de adipócitos marrons no TAB, aumentando a atividade do TAM e consequentemente o gasto energético (Chao *et al.*, 2011).

Em nível molecular, o escurecimento do TAB é regulado por múltiplos fatores e vias de sinalização, como *Peroxisome proliferator-activated receptor gamma coactivator-1 alpha* (PGC-1 alfa), *CCAAT-enhancer-binding proteins alpha* (C/EBP alfa), *Peroxisome proliferator-activated receptor gamma* (PPAR gama) e *PR Domain-containing protein16* (PRDM16) (Seale *et al.*, 2009). O frio induz a ativação de PGC-1 alfa no TAM (Puigserver *et al.*, 1998), que atua como regulador da biogênese mitocondrial e do metabolismo oxidativo em adipócitos, e pode induzir a expressão de UCP1 e outros componentes termogênicos. É importante ressaltar que o aumento do recrutamento de células ativas marrons e/ou bege no TAB promove o aumento do gasto energético e melhora na tolerância à glicose e a sensibilidade à insulina em modelo animal de DM2 (Coll and Yeo, 2013; Wang *et al.*, 2015; Brun *et al.*, 2017; Lapa *et al.*, 2017; Rabhi *et al.*, 2018). Dessa forma, fica evidente que alterações na homeostase energética estão relacionadas a alterações na homeostase glicêmica, podendo favorecer o desenvolvimento de algumas doenças metabólicas, como a obesidade e o DM2.

1.3 Obesidade e Diabetes Mellitus

A obesidade atingiu proporções epidêmicas, alcançando altos níveis de mortalidade e morbidade em todo o mundo (Hruby and Hu, 2015; Williams *et al.*, 2015; González-Muniesa *et al.*, 2017). A organização mundial da saúde estima que mais de 1,9 bilhões de pessoas estão acima do peso, e entre esses, 650 milhões são obesos, implicando em altos gastos anuais em saúde publica (Tsai *et al.*, 2011). A obesidade pode ser causada por fatores genéticos, metabólicos, endócrinos, neurais, ambientais, entre outros. Porém, o surgimento desta doença está relacionado principalmente com alterações no estilo de vida da população, como aumento na ingesta de dietas rica em gorduras e carboidratos, e redução ou substituição da atividade física por atividades sedentárias (Wilborn *et al.*, 2005; Barnes, 2012).

A obesidade é caracterizada pelo desenvolvimento de um estado inflamatório crônico de baixo grau. Nesse contexto, a expansão do tecido adiposo ocorre em resposta à sobrecarga calórica, e está associada ao aumento na infiltração de células imunes e uma subsequente resposta pró-inflamatória (Gregor and Hotamisligil, 2011). Este ambiente inflamatório afeta os tecidos periféricos, induzindo a resistência à insulina nos mesmos (Hotamisligil, 2003; Gregor and Hotamisligil, 2011), juntamente com hipersecreção de insulina pelas células beta pancreáticas (Bano, 2013; Abranches *et al.*, 2015). A própria hiperinsulinemia pode levar a disfunção na cascata de sinalização da insulina (Kanety *et al.*, 1994; Catalano *et al.*, 2014), contribuindo para uma alta demanda na secreção deste hormônio, podendo induzir a apoptose das células beta pancreáticas. Este efeito, associado com a resistência à insulina, resulta no desenvolvimento do DM2 (Bonner-Weir, 2000; Bano, 2013).

Além disso, a obesidade está associada à inflamação crônica de baixo grau e resistência à ação da leptina e da insulina no sistema nervoso central (Hotamisligil *et al.*, 1993; Könner and Brüning, 2012). Como um evento precoce no desenvolvimento da obesidade, a ingestão de uma dieta rica em ácidos graxos saturados induz uma resposta inflamatória em neurônios hipotalâmicos (Thaler *et al.*, 2012). Isso envolve a ativação das micróglias, os macrófagos residentes no sistema nervoso central. A inflamação local no hipotálamo médio-medial (envolvendo o núcleo arqueado, a parte anterior do núcleo paraventricular e a eminência mediana) promove o estresse do retículo endoplasmático (RE) nos neurônios hipotalâmicos, levando à resistência à insulina e à leptina (Kleinridders *et al.*, 2009; Pimentel *et al.*, 2014). Assim, a inflamação hipotalâmica prejudica os efeitos da insulina e da leptina, contribuindo não apenas para o desenvolvimento de hiperfagia e obesidade, mas também para a desregulação da homeostase da glicose. O aumento das vias de

sinalização associadas a estímulos inflamatórios como *Inhibitor of nuclear factor kappa-B kinase subunit beta/Factor nuclear kappa B* (IKK beta/NFκB) e o estresse do RE foram encontrados no hipotálamo de roedores obesos e mostraram-se prejudiciais à sinalização da leptina e da insulina hipotalâmica (Zhang *et al.*, 2008; Ozcan *et al.*, 2009).

Dessa forma, a patogênese da obesidade e do DM2 ocorre devido à desregulação central e periférica da homeostase energética e glicêmica. A redução da detecção de nutrientes e o comprometimento da sinalização de insulina e leptina no hipotálamo podem resultar em um balanço energético positivo e predispor o ganho de peso, causando resistência à insulina em órgãos metabólicos periféricos. Assim, a resistência à insulina associada à obesidade pode levar ao desenvolvimento do DM2, quando combinada com disfunção das células beta (Roh *et al.*, 2016).

1.4 Proteínas GAP's na regulação da homeostase glicêmica e energética: possível papel da ARHGAP21

Uma vez que as alterações na homeostase glicêmica e energética são importantes para o desenvolvimento da obesidade e DM2, existem inúmeros estudos explorando o papel de diferentes proteínas envolvidas na regulação dessas vias metabólicas (Röder *et al.*, 2016; Xu *et al.*, 2017). Entre elas, uma família de proteínas que tem sido estudada nesse contexto são as proteínas ativadoras de GTPase (GAPs).

As proteínas GAPs regulam a atividade de proteínas G de pequeno tamanho molecular, em geral, acelerando seu retorno a um estado inativo, através da indução da hidrólise do GTP (Bos *et al.*, 2007). As proteínas G de pequeno tamanho molecular são classificadas em cinco famílias principais: Ras, Rho, Rab, Arf e Ran, de acordo com seus subdomínios (Wennerberg *et al.*, 2005) e, por sua vez, cada família possui suas GAPs específicas (Bos *et al.*, 2007).

De maneira geral, essas proteínas controlam uma ampla variedade de processos celulares, desempenhando papel importante no rearranjo do citoesqueleto, tráfico de vesículas para a membrana e regulação do ciclo celular em vários tipos de células (Van Aelst and D'souza-Schorey, 1997; Hall, 1998; Bishop and Hall, 2000; Kowluru, 2010). Recentemente, algumas GAPs têm sido exploradas no contexto metabólico, demonstrando um papel importante sobre a homeostase glicêmica e energética.

Foi demonstrado que a deficiência da Rab-GAP *TBC1 domain family member 1* (TBC1D1) em células do músculo esquelético prejudica a captação de glicose (Szekeres *et al.*,

2012; Chadt *et al.*, 2015; Stöckli *et al.*, 2015). Além disso, o *knockdown* da TBC1D1 em cultura primária de células beta de ratos, leva ao aumento na secreção de insulina estimulada por glicose (Rütti *et al.*, 2014). Ainda, a deficiência de TBC1D1 reduz o peso corporal, diminui o quociente respiratório e aumenta o gasto energético, além de suprimir a obesidade induzida por dieta em modelo de experimentação animal (Chadt *et al.*, 2008; Dokas *et al.*, 2013; Hargett *et al.*, 2015; Dokas *et al.*, 2016; Hargett *et al.*, 2016). No entanto, em um estudo anterior, com enfoque em uma GAP da família Rho, foi demonstrado que a superexpressão da Rho-GAP - chamada *C10/cdc42 GTPase activating protein* (TCGAP) - diminui a captação de glicose em adipócitos (Chiang *et al.*, 2003). Esses dados reforçam a participação dessas proteínas na modulação da homeostase glicêmica e energética, e demonstram que sua função é dependente dos domínios específicos apresentados por cada uma delas (Bos *et al.*, 2007).

Em 2002, foi descoberta a existência de uma Rho-GAP chamada proteína ativadora de GTPase Rho 21 (ARHGAP21) (Bassères *et al.*, 2002). A ARHGAP21 contém 1958 aminoácidos e, além do seu domínio de atividade Rho-GAP, ela contém outros domínios, indicando que ela possui atividades que vão além da modulação das Rho GTPases (Bigarella *et al.*, 2009). De fato, a ARHGAP21 possui papel no tráfico de vesículas do Complexo de Golgi, na migração de mioblastomas (Bigarella *et al.*, 2009), e na migração e proliferação de carcinomas prostáticos (Lazarini *et al.*, 2013), indicando seu papel em diversos processos que envolvem o citoesqueleto.

O volume de estudos com a ARHGAP21 ainda é muito baixo, e os mecanismos moleculares pelos quais ela atua ainda não foram esclarecidos. Além disso, quase todos os trabalhos desenvolvidos investigaram seu papel majoritariamente em células cancerígenas. Porém, considerando o importante papel da ARHGAP21 no remodelamento de actina durante a secreção de insulina, é possível que esta proteína também exerça ações na célula beta pancreática. Em 2015, Ferreira e colaboradores comprovaram a expressão desta proteína em células beta pancreáticas de neonatos, e observaram que sua redução leva ao aumento na secreção de insulina (Ferreira *et al.*, 2015). No entanto, o possível efeito da ARHGAP21 na homeostase glicêmica e energética ainda não foi avaliado.

2. Objetivos

1. Investigar o papel da ARHGAP21 na homeostase glicêmica de animais heterozigotos para ARHGAP21 com obesidade induzida por dieta hiperlipídica (Artigo 01).

2. Investigar o papel da ARHGAP21 na homeostase energética de animais heterozigotos para ARHGAP21 com obesidade induzida por dieta hiperlipidica (Artigo 02).

3. Artigo 01

Whole body ARHGAP21 reduction improves glucose homeostasis in high-fat diet obese mice

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Keywords: ARHGAP21; Rho-GAP; glucose homeostasis; insulin secretion; obesity.

ABSTRACT

GTPase activating proteins (GAPs) are ubiquitously expressed, and their role in cellular adhesion and membrane traffic processes have been well described. TBC1D1, which is a Rab-GAP, is necessary for adequate glucose uptake by muscle cells, whereas increased TCGAP, which is a Rho-GAP, decreases GLUT4 translocation, and consequently glucose uptake in adipocytes. Here, we assessed the possible involvement of ARHGAP21, a Rho-GAP protein, in glucose homeostasis. For this purpose, wild type mice and ARHGAP21 transgenic whole-body gene-deficiency mice (heterozygous mice, expressing approximately 50% of ARHGAP21) were fed either chow (Ctl and Het) or high-fat diet (Ctl-HFD and Het-HFD). Het-HFD mice showed a reduction in white fat storage, reflected in a lower body weight gain. These mice also displayed an improvement in insulin sensitivity and glucose tolerance, which likely contributed to reduced insulin secretion and pancreatic beta cell area. The reduction of body weight was also observed in Het mice and this phenomenon was associated with an increase in brown adipose tissue and reduced muscle weight, without alteration in glucose-insulin homeostasis. In conclusion, the whole body ARHGAP21 reduction improved glucose homeostasis and protected against diet-induced obesity specifically in Het-HFD mice. However, the mechanism by which ARHGAP21 leads to these outcomes requires further investigation.

1 INTRODUCTION

GTPase activating proteins (GAPs) increase the intrinsic GTPase activity of small G proteins accelerating their return to an inactive state (Bos et al., 2007; Lamarche and Hall, 1994). Small G proteins are classified into five principal families: the Ras, Rho, Rab, Arf, and Ran, according to their sub-domains (Wennerberg et al., 2005) and, in turn, each family has specific GAPs (Bos et al., 2007). Indeed, these proteins play an important role in cytoskeletal rearrangements, membrane trafficking and cell cycle regulation in several cell types (Bishop and Hall, 2000; Hall, 1998; Kowluru, 2010; Van Aelst and D'Souza-Schorey, 1997). All these events suggest a possible role of GAP proteins in glucose homeostasis. However, few of them have been explored in this context.

The most studied GAP on glucose homeostasis is the Rab-GAP TBC1D1. Its inhibition in skeletal muscle cells impairs glucose uptake (Chadt et al., 2015; Stöckli et al., 2015; Szekeres et al., 2012). When it is inhibited in primary rat beta cells, meanwhile, glucose-stimulated insulin secretion increases (Rütti et al., 2014), reinforcing the importance of these proteins in glucose-insulin homeostasis.

Only recently, the GAPs from the Rho family have been explored in this context, e.g., the overexpression of TCGAP decreases glucose uptake in adipocytes (Chiang et al., 2003), and the inhibition of ARHGAP21 in islets from neonatal mice increases insulin secretion (Ferreira et al., 2015). The ARHGAP21 is a Rho-GAP that controls cell proliferation, migration (Bigarella et al., 2012), differentiation (Bassères et al., 2002), cell-cell adhesion (Perillo et al., 2015), and cell-cell junction remodeling (Barcellos et al., 2013; Sousa et al., 2005). Its function has been highlighted in cancer studies, where it acts as a tumor suppressor (Bigarella et al., 2009; Luo et al., 2016). However, the possible effect of ARHGAP21 on glucose homeostasis has not been yet determined. Thus, we used ARHGAP21-haplodeficient mice aiming to explore this question.

Here, we observed that whole body ARHGAP21 reduction in Het-HFD mice improved glucose tolerance and insulin sensitivity, which was associated with reduced insulin secretion. Body weight was also reduced in both, the Het and Het-HFD groups. However, it seems that the type of diet consumed affects the mechanism by which ARHGAP21 impacts body weight, since Het-HFD mice displayed reduced white adipose tissue, whereas Het mice showed increased brown adipose tissue and lower muscle weight.

Taken together, we confirmed the involvement of Rho-GAPs in glucose homeostasis, and described an as-yet unknown role for these proteins in controlling white and brown fat depots and muscle mass. These findings suggest the Rho-GAP ARHGAP21 protein as an

important modulator of glucose and energetic metabolism, supporting future investigations to explore the mechanism by which this phenomenon occurs.

2 MATERIAL AND METHODS

2.1 Ethics statement

All the experiments described herein were approved by the State University of Campinas Committee for Ethics in Animal Experimentation (approval number: 3783-1) and performed according to the “Principles of laboratory animal care” (NIH publication no.85-23, revised 1985).

2.2 Animals

The ARHGAP21 transgenic mice (Het) are a whole-body gene-deficiency model (heterozygous, expressing approximately 50% of ARHGAP21), maintained on the C57BL/6Unib genetic background, generated in-house from breeders originating from CEDEME-UNIFESP (São Paulo, SP). Male mice were genotyped by PCR and real-time PCR using the following primers within the beta-Geo vector: 5’-ggcgcctcatgaatattaacc-3’ and 5’-cactccaacctccgcaaactc-3’. Paired male wild type littermates served as controls (Ctl). All mice were maintained at standard housing conditions, on a 12-h light–dark cycle at 22 ± 1 ºC. At 1-mo-old, the mice were fed ad libitum with a chow diet (Ctl and Het) or a high-fat diet (Ctl-HFD and Het-HFD) for 10 weeks.

2.3 Body parameters and blood measurements

The body weight of all mice was evaluated during 10 weeks after the beginning of chow or high-fat diet introduction. At the end of this period, the mice (10 h fasting) were euthanised in a CO_2-saturated chamber followed by decapitation and the blood samples were collected. Afterward, these blood samples were centrifuged at 1,100 g, 4 ºC for 15 min, to obtain the serum, which was stored at -20 ºC to posterior insulin measurements by radioimmunoassay (RIA) (Scott et al., 1981). In addition, the perigonadal and subcutaneous fat pads, interscapular brown adipose tissue, and the gastrocnemius skeletal muscle were removed and weighed.

2.4 Intraperitoneal glucose and insulin tolerance tests (ipGTT and ipITT)

To perform ipGTT, mice were subjected to 10 h fasting and blood glucose was accessed by tail incision. Blood glucose was measured using glucose strips on an Accu-Check Performa II glucometer (Roche, Sao Paulo, BR). Glycemic level were measured (0 min) and 15, 30, 60, 90 and 120 min after the administration of glucose (2 g/kg body weight). The AUC of glucose during the ipGTT were calculated. For the ipITT, mice were subjected to 4 h fasting, and blood glucose was measured before (0 min) and 3, 6, 9, 12 and 15 min after the administration of insulin (0.75 U/kg body weight). The kITT (constant rate for glucose disappearance) was calculated as previously described (Akinmokun et al., 1992).

2.5 Glucose-stimulated insulin secretion in pancreatic islets

The pancreatic islets were isolated by the collagenase method (Boschero et al., 1995), and groups of 4 islets each were incubated in 0.5 mL of Krebs–Ringer bicarbonate (KRB) buffer (115 mM NaCl, 5 mM KCl, 10 mM NaHCO3, 2.56 mM CaCl2, 1 mM MgCl2 and 15 mM HEPES (Sigma-Aldrich Chemical, St Louis, MO, USA), supplemented with 5.6 mM glucose plus 0.3 % of BSA, and equilibrated with a mixture of 95 % O2/5 % CO2 to give pH 7.4) for 45 min at 37 °C. Subsequently, these islets were exposed to 1 ml KHBS containing 2.8 mM (low concentration) or 16.7 mM (high concentration) mmol/l glucose for 1 h at 37 °C. To measured insulin secreted we collected the supernatants that were stored at -20 ºC, and the remaining islets were homogenized in an alcohol/acid solution to measure the total insulin content by RIA (Scott et al., 1981).

2.6 Immunofluorescence

The pancreata were collected and fixed overnight in 4% paraformaldehyde solution at room temperature. Afterward, the dehydration and impregnation in paraffin was performed and these pancreata were cut into 5-µm-thick sections. The sections were rehydrated, permeabilized, blocked with 0,5% BSA solution and incubated with the primary (Polyclonal Guinea Pig Anti-Insulin, A0564, Dako, Glostrup, Copenhagen, Denmark) and secondary (FITC, F6261, Sigma-Aldrich, Saint Louis, Missouri, USA) antibodies (Barbosa-Sampaio et al., 2015). Finally, the slides were prepared using Mounting Medium for Fluorescence (Dako, Glostrup, Copenhagen, Denmark), and the pancreata were visualized by fluorescence microscopy (Leica CTR 6500, Wetzlar, Alemanha) using 20× objectives. The images were analyzed using the ImageJ software (National Institute of Health, Maryland, USA).

2.7 Statistical analysis

To analyze the data we used the Student's t-test (GraphPad Prism 5, La Jolla, CA, USA). The data were presented as means ± standard errors media (SEM), and the difference between the groups were considered statistically significant if $P \leq 0.05$.

3 RESULTS

3.1 Body parameters of ARHGAP21 Het mice

First, we characterized the ARHGAP21-haplodeficient mice by quantifying the gene expression of ARHGAP21 and, as expected, Het mice presented approximately 50% less ARHGAP21 than Ctl group (Fig 1A). We measured the body weight of mice once a week for 10 weeks. At the 7^{Th} week, Het mice displayed reduced body weight compared with Ctl, and this difference was observed until the end of the experiment (Fig 1B). Although the body weight was reduced, alteration in the weight of perigonadal and subcutaneous fat pads were not observed in Het mice (Figs 1C-D). Interestingly, brown adipose tissue was significantly increased in the Het mice (Fig 1E). In addition, these mice showed a decrease in gastrocnemius skeletal muscle weight (Fig 1F).

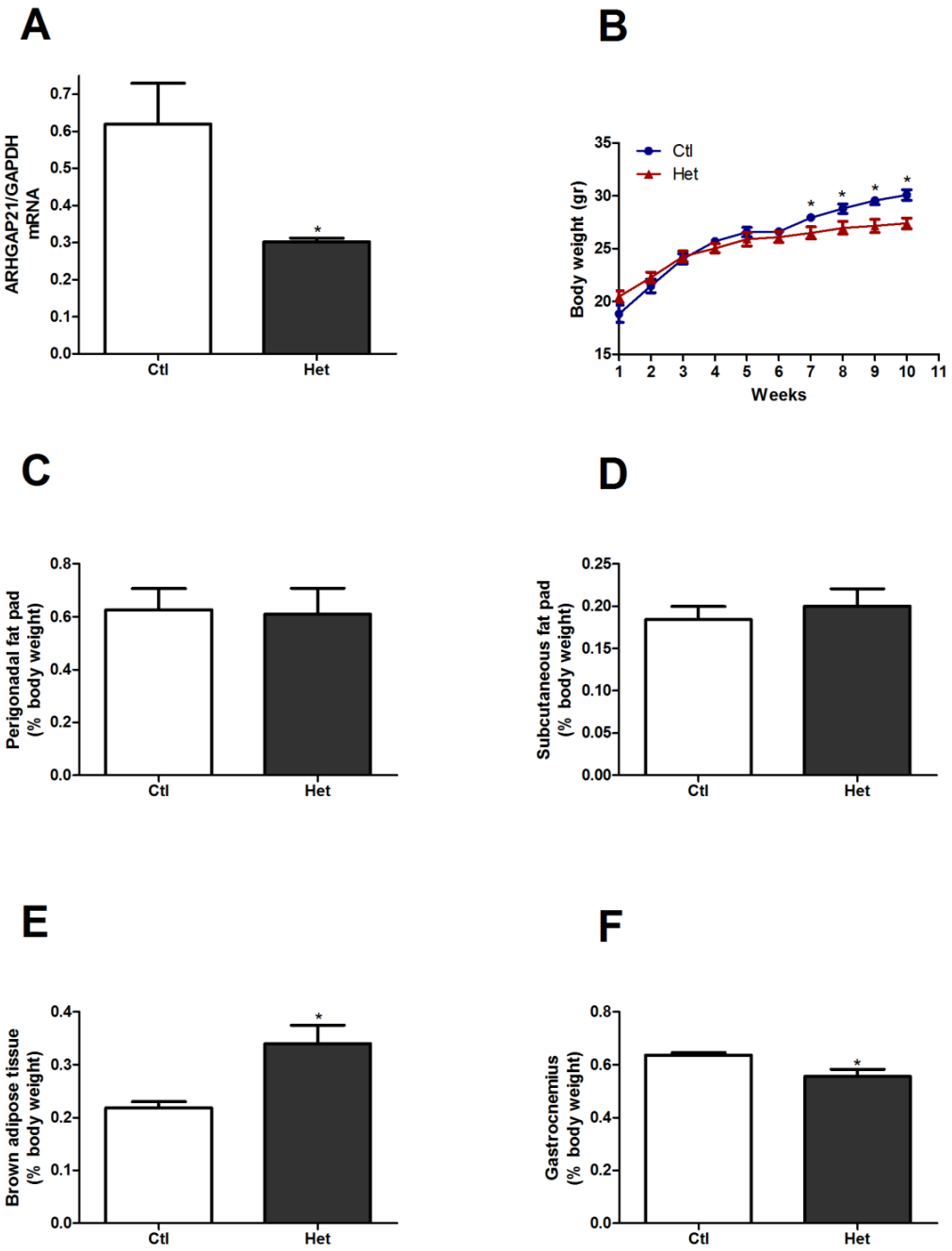

Figure 1. Body parameters of ARHGAP21 Het mice. ARHGAP21 expression was evaluated by qPCR (A). Body weight during the experimental period (B). Perigonadal (C) and subcutaneous fat pad (D), brown adipose tissue (E) and gastrocnemius (F) weights. Data are mean ± SEM (n=5-7). *P ≤ 0.05 (Student's-t-Test).

3.2 Glucose homeostasis of ARHGAP21 Het mice

Glucose homeostasis was similar between Het and Ctl groups. Insulin sensitivity, evaluated by ipITT, was similar between Ctl and Het groups (Figs 2C-D), which reflected in the same level of glucose tolerance, measured by glucose kinetics after a glucose challenge (ipGTT) (Figs 2A-B) as well as the fasted glycemia and insulinemia, which were similar between Ctl and Het groups (Figs 2E-F).

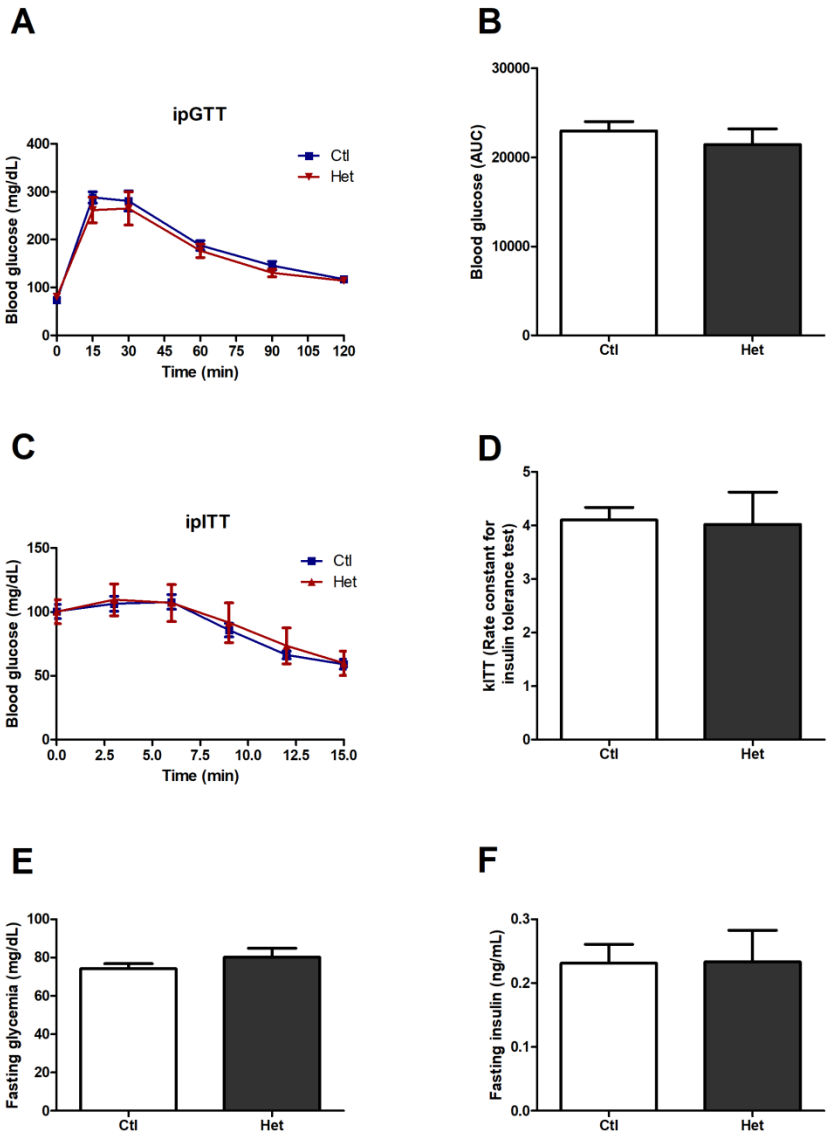

Figure 2. Glucose homeostasis of ARHGAP21 Het mice. Changes in blood glucose (A) and AUC of blood glucose (B) during ipGTT. Changes in blood glucose (C) and rate constant for glucose disappearance (kITT) (D) during the ipITT. Fasting glycemia (E) and insulinemia (F). Data are mean ± SEM (n=5-7). *P ≤ 0.05 (Student's-t-Test).

3.3 ARHGAP21 Het-HFD mice displayed reduced body and fat pads weight

We also investigated how the Het mice respond to a high-fat diet challenge. As shown in the Fig 3A, Het-HFD mice displayed a reduction in body weight at 4 weeks, and this was accompanied by a decrease of perigonadal and subcutaneous fat pad weight, compared with Ctl-HFD group (Figs 3B-C). Brown adipose tissue was similar between groups (Fig 3D) whereas Het-HFD mice showed a decrease in gastrocnemius skeletal muscle weight (Fig 3E).

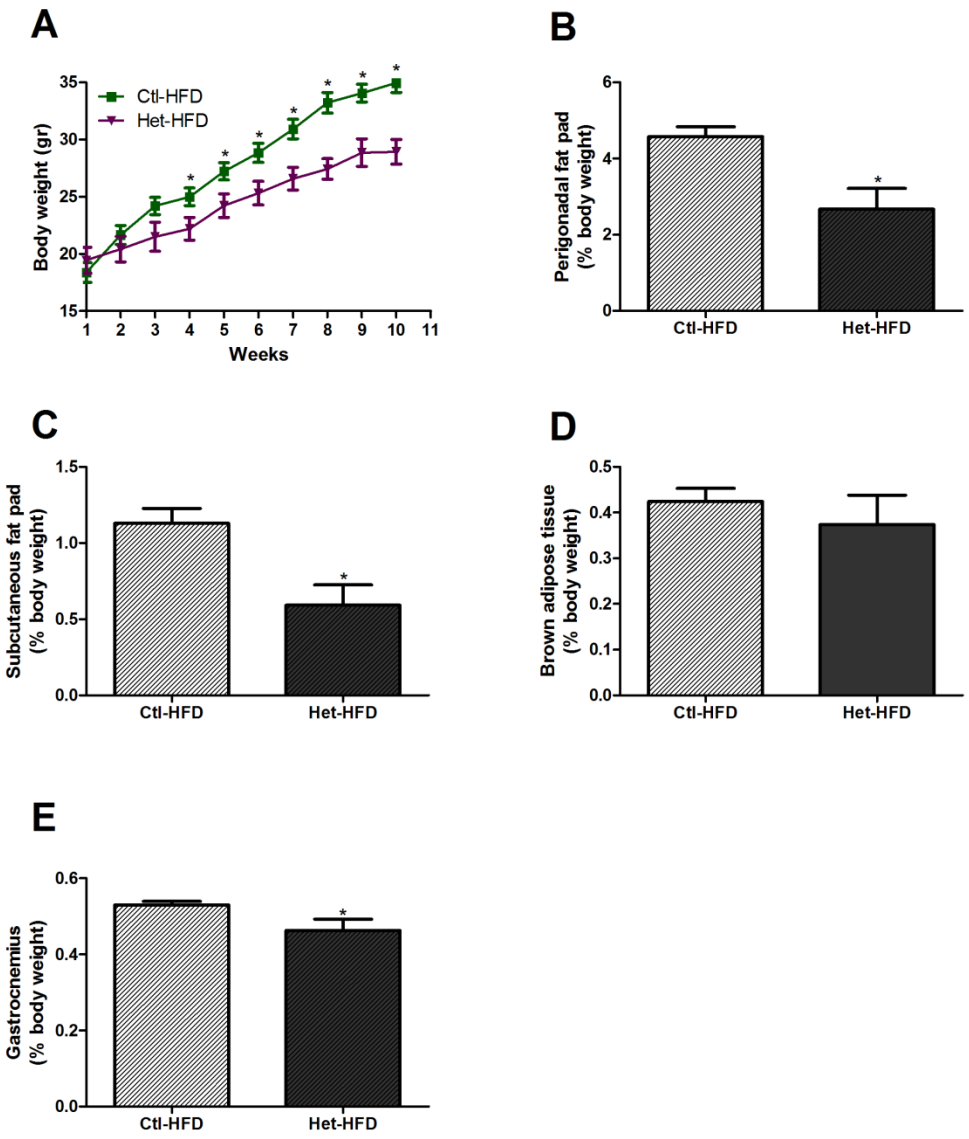

Figure 3. ARHGAP21 Het-HFD mice displayed reduced body and fat pads weight. Body weight during the experimental period (A). Perigonadal (B) and subcutaneous fat pad (C), brown adipose tissue (D) and gastrocnemius (E) weights. Data are mean ± SEM (n=4-9). *P ≤ 0.05 (Student's-t-Test).

3.4 ARHGAP21 Het-HFD mice showed an improvement in the glucose tolerance and insulin sensitivity

During the ipGTT, blood glucose was reduced at 60, 90 and 120 minutes (Fig 4A) in the Het-HFD, compared with Ctl-HFD mice, indicating an improvement in glucose tolerance, as confirmed by the AUC (Fig 4B). An improvement in insulin sensitivity also was observed, as judged by reduced blood glucose during ipITT and increased kITT (Figs 4C-D), in Het-HFD, compared with Ctl-HFD mice. However, fasting blood glucose was not different between groups (Fig 4E), and this could be explained by lower insulinemia in the Het-HFD, compared with Ctl-HFD group (Fig 4F).

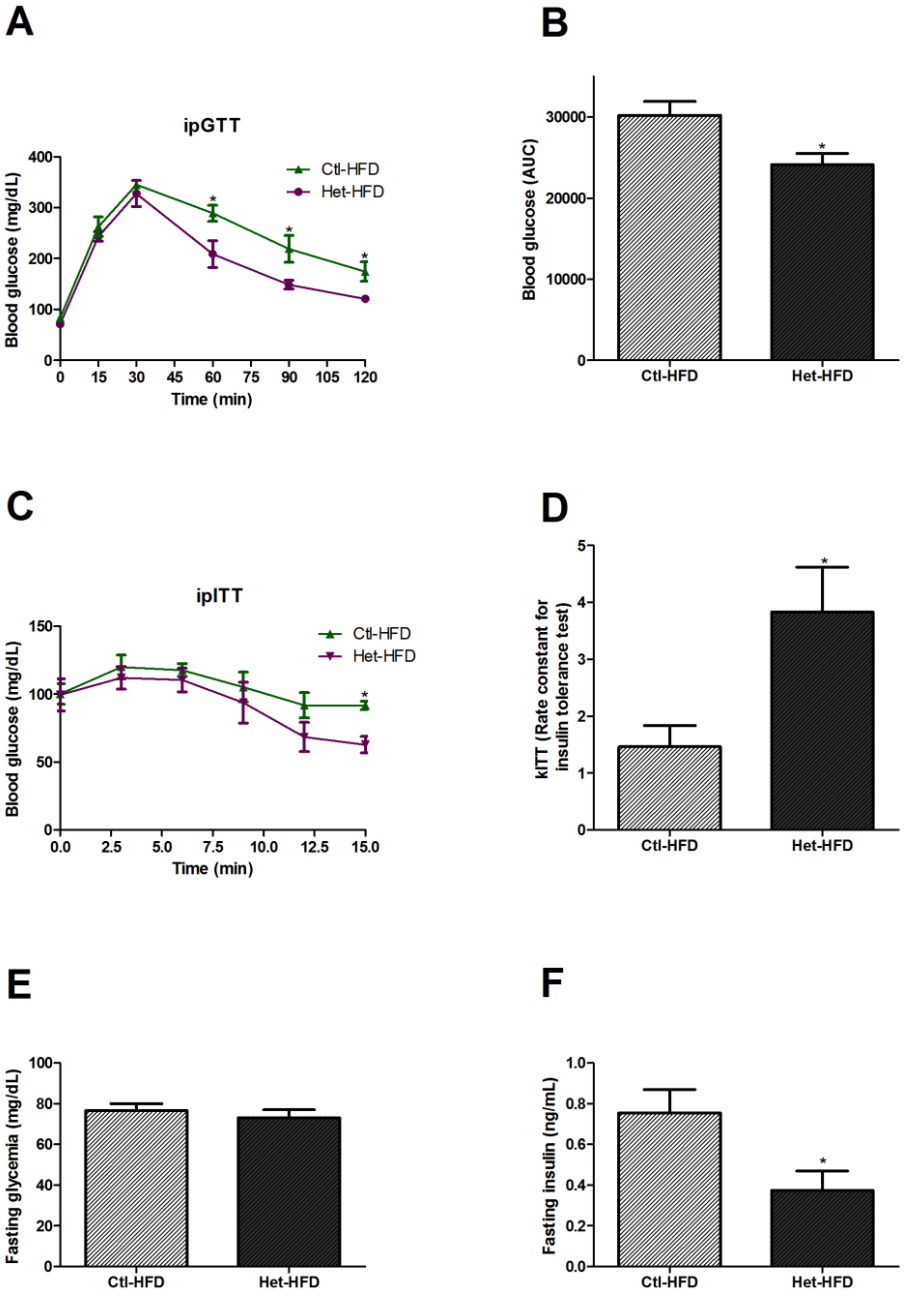

Figure 4. ARHGAP21 Het-HFD mice showed an improvement in the glucose tolerance and insulin sensitivity. Changes in blood glucose (A) and AUC of blood glucose (B) during ipGTT. Changes in blood glucose (C) and rate constant for glucose disappearance (kITT) (D) during the ipITT. Fasting glycemia (E) and insulinemia (F). Data are mean ± SEM (n=5-8). *P ≤ 0.05 (Student's-t-Test).

3.5 ARHGAP21 Het-HFD mice had reduced insulin secretion and beta cell area

To explain the lower insulinemia in the Het-HFD mice we analyzed the glucose stimulated insulin secretion (GSIS), in isolated pancreatic islets. At a high glucose concentration (16.7 mM), we observed-reduced insulin secretion in the islets from Het-HFD mice compared with the Ctl-HFD group; whereas the insulin secretion was not different between groups at a low glucose concentration (2.8 mM) (Fig 5A). The total insulin content was reduced in the islets from Het-HFD mice (Fig 5B), and this was associated with a lower beta cell area, compared with the Ctl-HFD mice (Figs 5C-D).

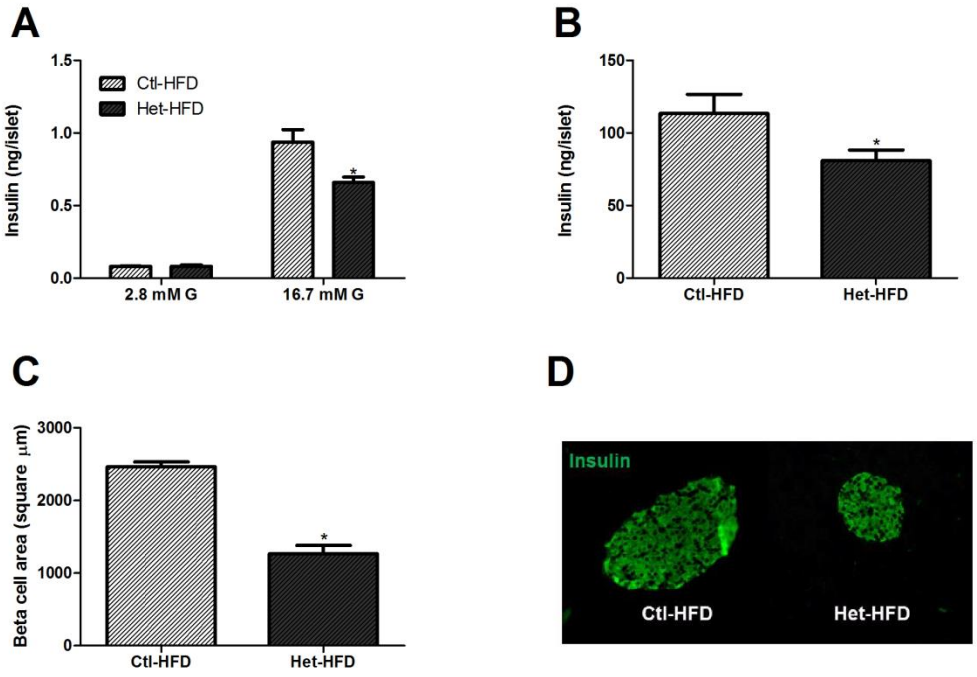

Figure 5. ARHGAP21 Het-HFD mice had reduced insulin secretion and beta cell area. Insulin secretion in isolated pancreatic islets stimulated with low (2.8 mM) and high (16.7 mM) glucose concentration (A). Total insulin content of islets (B). Beta cell area (C) and its representative images stained for insulin (D). Images were captured using a 20× objective. Data are mean ± SEM (n=3-8). *P ≤ 0.05 (Student's-t-Test).

4 DISCUSSION

GAP proteins have specific physiological roles in glucose homeostasis depending on their isoforms (Bos et al., 2007). The inhibition of a Rab-GAP, known as TBC1D1, decreases glucose uptake by skeletal muscle (Chadt et al., 2015; Stöckli et al., 2015; Szekeres et al., 2012), whereas the overexpression of TCGAP, a Rho-GAP protein member, decreases glucose uptake by adipose cells (Chiang et al., 2003). Here, we extend these findings, showing a beneficial effect of whole body reduction of ARHGAP21 in glucose homeostasis, as judged by an improvement in glucose tolerance and insulin sensitivity, associated with reduced insulin secretion. These effects were observed only in Het-HFD mice.

Our study is the first to show that Rho-GAP ARHGAP21 inhibition increased insulin sensitivity, resulting in glucose homeostasis improvement, suggesting similarities with other Rho-GAP TCGAP that, when overexpressed in adipocytes, impairs glucose uptake (Chiang et al., 2003). The mechanism by which TCGAP impacts the insulin-induced glucose uptake seems to involve a physical interaction with the non-canonical insulin signaling: CAP-Cbl pathway (Baumann et al., 2000; Chiang et al., 2003). As a consequence of insulin stimulation, TCGAP may expose its proline-rich domain, increasing the interaction with CrkII protein (a CAP-Cbl pathway member) (Chiang et al., 2001), habilitating CrkII to carry TCGAP toward the plasma membrane, activating it, and increasing GLUT4 translocation to the cell plasma membrane. However, when these adaptor proteins of the CAP-Cbl pathway are overexpressed, e.g. APS protein, a down regulation in the insulin-stimulated glucose uptake occurs, due to an excess of adaptor proteins over endogenous Cbl and insulin receptors, impairing glucose uptake (Chiang et al., 2003; Liu et al., 2002).

Since, Rho-GAP ARHGAP21 and TCGAP are proteins with different domains (Bos et al., 2007; Chiang et al., 2003), it is difficult to accept that they display a similar mechanism of action. Nevertheless, more studies are necessary to clarify the molecular mechanism by which ARHGAP21 inhibition improves peripheral insulin sensitivity, and glucose tolerance.

Probably, as a consequence of improved insulin sensitivity, the Het-HFD mice secrete less insulin and show a smaller pancreatic beta cell area, which helps to explain the lower body weight. Indeed, hyperinsulinemia may increase lipogenesis, as well as reduce fat acids oxidation, potentiating the deleterious effect of obesity (Jung and Choi, 2014; Vázquez-Vela et al., 2008).

Another possible explanation for the improvement in glucose homeostasis, observed in the Het-HFD mice, is as a direct effect of ARHGAP21 inhibition upon pancreatic beta cells. In fact, our group demonstrated that its inhibition, specifically in pancreatic islets from neonate mice, increased insulin secretion (Ferreira et al., 2015). However, these experiments were done in immature pancreatic islets, which present different physiological behavior, compared to those explored here (Carvalho et al., 2010; Mendonça et al., 1998).

Curiously, the Het mice also show lower body weight. However, this feature occurs by a different mechanism from that observed in Het-HFD mice, since they display reduced muscle weight instead of reduced white adipose tissue, and also an increased brown adipose tissue depot. These results are the first to point to the possible involvement of a Rho-GAP member in the control of body composition, decreasing white adipose tissue in Het-HFD mice, and increasing brown adipose tissue in Het mice. However, further investigation is necessary to figure out the mechanism by which this phenomenon occurs.

In summary, our results support that ARHGAP21 inhibition improves glucose tolerance by ameliorating insulin sensitivity and secretion, when mice are fed on a HFD. In addition, an as-yet undetermined role of a Rho-GAP member, related with body composition, was demonstrated here. Nevertheless, more studies are necessary to clarify the mechanism by which this occurs.

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CONFLICTS OF INTEREST

The authors declared that they have no conflicts of interest.

4. Artigo 02

WHOLE-BODY ARHGAP21-DEFICIENCY IMPROVES ENERGETIC HOMEOSTASIS IN LEAN AND OBESE MICE

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ABSTRACT

Inhibition of Rab-GAP TBC1 domain family member 1 (TBC1D1) reduces body weight and increases energy expenditure in mice. Here, we assessed the possible involvement of GTPase activating protein 21 (ARHGAP21), a Rho-GAP protein, in energy homeostasis. Wild-type and whole-body ARHGAP21-haplodeficient mice were fed either chow or high-fat diet for 10 weeks. These mice were analyzed for body weight, food intake, voluntary physical activity and energy expenditure by indirect calorimetry. Real-time PCR was performed to determine changes in the expression of hypothalamic-anorexic genes. Whole-body ARHGAP21-haplodeficient mice showed lower body weight and food intake associated with increased energy expenditure. These mice also showed higher expression of hypothalamic-anorexic genes such as POMC and CART. Our data suggest that the reduction in body weight of ARHGAP21-haplodeficient mice was related to alterations in the central nervous system. This suggests a new role for ARHGAP21 in energetic metabolism and prompts us to consider GAP protein members as possible targets for the prevention and treatment of obesity and related diseases.

1 INTRODUCTION

Energy homeostasis depends on a balance between food intake and energy expenditure regulated by complex physiological mechanisms. A disturbance in these processes can lead to obesity (1, 2). Obesity and overweight are pandemic, affecting more than 2 billion people worldwide (3, 4). The hypothalamus plays an important role in this context, controlling feeding behavior and energy metabolism through a complex network of neurons that express distinct neurotransmitters (5, 6).

Insulin and leptin signaling, as well as the POMC-NPY axis, are among the canonical molecular pathways that control energy intake and expenditure (7, 8). Insulin, just before a meal, inhibits food intake by activating anorexigenic genes in hypothalamus. On the other hand, leptin act regulating food intake and energy expenditure being secreted by adipose tissue, in order to estimate body energetic pads (9).

Actually, many proteins have been proposed to regulate food intake and energetic expenditure, and other less-studied molecules also appear to be involved (10). Of these, the GTPase activating proteins (GAPs) emerge as possible modulators of energy homeostasis. GAP proteins regulate the activity of small G proteins, in general, accelerating their return to an inactive state, through the induction of GTP hydrolysis (11). According to protein subdomains, small G proteins are classified into five families: Ras, Rho, Rab, Arf and Ran (12), and are mainly involved in cytoskeletal rearrangement and trafficking of vesicles to the membrane in various cell types (13, 14). Each small G protein has your own GAP, which regulates individual GTPase activity and function. In central nervous system, Rho GTPases regulates neuronal migration and growth as well as synaptic transmission (15), and recently, some GAP proteins have been explored in the metabolic context, demonstrating an important role in glycemic and energetic homeostasis.

Indeed, GAP TBC1D1-deficiency reduces body weight (16-20), decreases respiratory quotient (16-19) and increases energy expenditure (17-20) in addition to suppressing diet-induced obesity (16, 19). Recently, our group reported that reduction of ARHGAP21 (a Rho-GAP isoform) improved glucose tolerance and insulin sensitivity and reduced weight gain in mice fed on high-fat diet (21). However, the role of ARHGAP21 in hypothalamic appetite control and whole-body energy homeostasis remains unclear.

Here, we observed that whole-body ARHGAP21-deficiency reduced fat pad depots as well as body weight, probably increasing the expression of the anorexic genes POMC and CART in the hypothalamus, genes associated with reduction in food consumption and

increments of energy expenditure. These findings explain, at least in part, why Het-HFD mice did not become obese, highlighting GAP protein members as important targets for the prevention and control of obesity and associated diseases.

2 MATERIALS AND METHODS

2.1 ANIMALS

The haplodeficient mouse (Het) is a whole-body ARHGAP21 gene-deficiency model, expressing approximately 50% ARHGAP21. The generation and genotyping of ARHGAP21-haplodeficient mice were performed as previously described (22). Paired male wild-type littermates were used as controls (Ctl). All mice were maintained at 22 ± 1 °C on a 12-h light–dark cycle with free access to food and water. At 1 month of age, the mice received chow (Ctl and Het) or a high-fat diet, (Ctl-HFD and Het-HFD). This diet composition was described previously (23). Mice from all groups were allowed to feed and drink tap water for 10 weeks *ad libitum*. All experiments involving animals were approved by the Animal Care Committee at UNICAMP (approval number: 3783-1).

2.2 BODY PARAMETERS

The body weights of all mice were evaluated once a week during the 10 weeks of diet treatments. In addition, the perigonadal fat pad and the interscapular brown adipose tissue (BAT) were dissected and weighed. BAT and hypothalamus samples were separated for RNA extraction.

2.3 FOOD INTAKE

In the 9^{th} week of treatment, mice were maintained, individually, in home cages for 24 h of adaptation. After that, food consumption was measured during 3 consecutive days and was calculated by the difference between the food weight at 7 pm versus 7 am. Food intake was then determined as the mean food consumption of this period (24, 25).

2.4 INDIRECT CALORIMETRY

Metabolic rates were measured by indirect calorimetry using an open-circuit indirect calorimeter system, the Comprehensive Lab Animal Monitoring System: Oxymax-CLAMS (Columbus Instruments, Columbus, OH, USA). At the 10^{th} week of treatment, mice were acclimated for 24 hours in the system cages, and the Oxymax-CLAMS was calibrated as

recommended by the manufacturer. After the acclimation period, the rate of oxygen consumption (VO_2), respiratory exchange ratio (RER), heat rate (Kcal/h) and ambulatory activity (measured as total beam breaks which means, the sum of x, y and z axis) were measured during the light and dark periods (26). These data were acquired for 24 hours and were analyzed using Oxymax Windows software (Columbus Instruments, Columbus, OH, USA).

2.5 RNA ISOLATION AND REAL TIME QUANTITATIVE PCR

The total RNA content of the BAT and hypothalamus was extracted using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA), following phenol-chloroform RNA extraction, according to the manufacturer's recommendations. RNA concentration was measured by Nanodrop (Nanodrop Thermo scientific, Wilmington, DE, USA). cDNA was prepared using 1 μg RNA and MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA, USA). The SYBR-green master mix (Applied Biosystems, Foster City, CA, USA) was used in the PCR reactions. Quantification was performed using the 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The specificities of amplifications were verified by melting-curve analyses. The relative expression of mRNAs was determined after normalization with GAPDH, using the 2-ΔΔCt method. Primer sequences used for real-time PCR assays were as follows: ARHGAP21 forward: 5'-tcatgcctgtgtgcataccc-3', ARHGAP21 reverse: 5'-aagctcccaacagtgcaaac-3'; POMC forward: 5'-ggcttgcaaactcgacctc-3', POMC reverse: 5'-tgacccatgacgtacttccg-3'; CART forward: 5'-acctttgctgggtgcccgtg-3', CART reverse: 5'-tgcaacgcttcgatcagctcc-3'; NPY forward: 5'-tactccgctctgcgacacta-3', NPY reverse: 5'-tcttcaagccttgttctggg-3'; AgRP forward: 5'-gagttcccaggtctaagtctgaatg-3', AgRP reverse: 5'-atctagcacctccgccaaag-3'; UCP1 forward: 5'-ctgccaggacagtacccaag-3', UCP1 reverse: 5'-tcagctgttcaaagcacaca-3'; GAPDH forward: 5'-cctgcaccaccaactgctta-3', GAPDH reverse: 5'-gccccacggccatcacgcca-3'.

2.6 WESTERN BLOT ANALYSIS

The brown adipose tissue lysates were prepared using TissueLyser LT (Qiagen, Hilden, Germany) and then were placed in a 1.5 ml tube and mixed with a lysis/antiprotease buffer containing 7 mol/L urea, 2 mol/L thiourea, 100 mmol/L Tris pH 7.5, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L ethylenediamine tetraacetic acid (EDTA), 10 mmol/L sodium vanadate, 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1% Triton X100. The extracts were then centrifuged at 12,600 g at 4°C for 40 min to remove

insoluble materials. The protein concentration of the supernatants was assayed using the Bradford dye method (27), using bovine serum albumin (BSA) as a standard curve and the Bradford reagent (Bio-Agency Lab., São Paulo, Brazil). For SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis, all samples were treated with a Laemmli buffer containing dithiothreitol. After heating to 100 ºC for 5 min, proteins were separated by electrophoresis in a 12% polyacrylamide gel. The transfer to nitrocellulose membranes was performed in a Trans Blot transfer for 2 h in 100 V, with a tris/glycine buffer. After, the membranes were blocked with 5% BSA for 1 h and were then incubated with specific antibodies - UCP1 (#14670; Cell Signaling Technology, Danvers, MA), GAPDH (G9545; Sigma, St. Louis, Missouri, USA) - that were diluted 1:1,000 and subsequently detected by exposure to chemiluminescent substances (luminol and peroxidase). After incubation, the appropriate secondary antibody (dilution 1:10,000; Invitrogen, São Paulo, Brazil) was added for further luminescence detection followed by detection in Amersham Imager 600 (GE Healthcare Life Sciences, Buckinghamshire, UK). The quantification of the bands was performed by densitometry using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7 STATISTICAL ANALYSIS

The data were analyzed by Student's t-test (GraphPad Prism 5, La Jolla, CA, USA) and were presented as the means ± standard errors media (SEM). The differences between groups were considered statistically significant if $P \leq 0.05$.

3 RESULTS

3.1 ANOREXIGENIC EFFECTS OF WHOLE-BODY ARHGAP21 REDUCTION IN HET MICE

The body weight of mice was measured once per week for 10 weeks. At the 8th week, Het mice displayed lower body weight than did the Ctl mice until the end of the experimental period (Fig. 1A). The weight of the perigonadal fat pad of Het was similar to that of Ctl mice (Fig. 1B). Het mice showed lower food intake than did Ctl mice (Fig. 1C). Consistent with these findings, Het mice displayed significant increases in mRNA levels of the anorexigenic markers POMC and CART and reductions in the mRNA levels of the orexigenic markers NPY and agRP (Fig. 1D). We also found that the ARHGAP21 mRNA content was lower in the hypothalamus of Het mice (Fig. 1D).

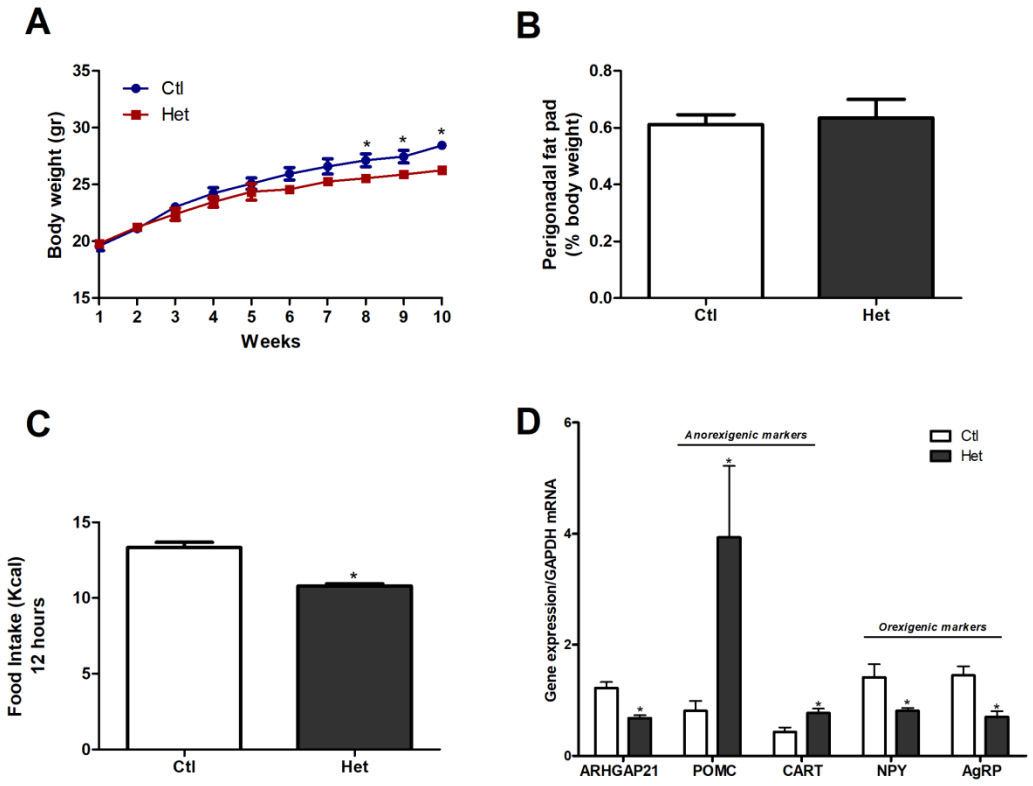

Figure 1. Anorexigenic effects of whole-body ARHGAP21 reduction in Het mice. Body weight curve (A). Perigonadal fat pad weight (B). Food intake over 12 hours (C). Real time PCR assay of hypothalamic ARHGAP21, POMC, CART, NPY and AgRP mRNA levels (D). Control mice (Ctl) and ARHGAP21-haplodeficient mice (Het) fed a chow diet for 10 weeks. Data are the mean ± SEM (n=3-6). *P ≤ 0.05 (Student's-t-Test).

3.2 ENERGY HOMEOSTASIS OF ARHGAP21 HET MICE

Het mice presented higher energy expenditure, as judged by the augmented VO_2 (Fig. 2A) and increased heat rate (Fig. 2B) during dark and light periods, than did Ctl mice. No difference was found in RER between groups (Fig. 2C). The ambulatory activity was significantly higher in Het than in the Ctl group in both periods (Fig. 2D-E). BAT weight (Fig. 2F), UCP1 mRNA expression (Fig. 2G) and protein content (Fig. 2H) were higher in Het mice than in Ctl mice. This accords with the higher energy expenditure observed in Het mice. A decrease in ARHGAP21 mRNA content in the BAT of Het mice was also observed (Fig. 2G).

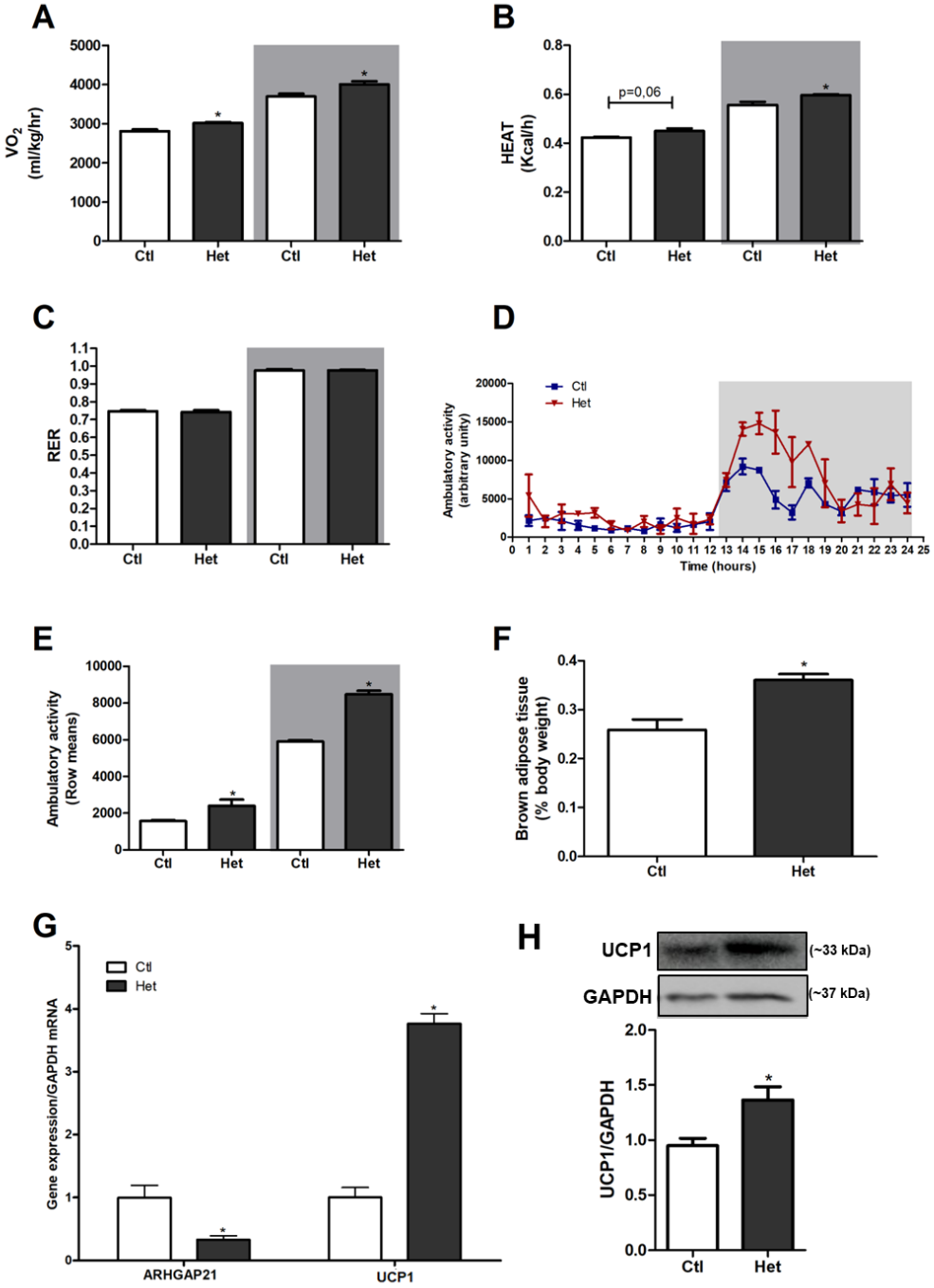

Figure 2. Energy homeostasis of ARHGAP21 Het mice. VO2 (A), heat rate (B) and respiratory exchange ratio (RER) (C). Ambulatory activity for 24 hours during light and dark periods (D) and mean of light and dark periods (E). BAT weight (F). Real-time PCR assay of ARHGAP21 and UCP1 mRNA levels in BAT (G). Brown adipose tissue UCP1 protein content (H). Control mice (Ctl) and ARHGAP21-haplodeficient mice (Het) fed a chow diet for 10 weeks. Data are the mean ± SEM (n=3-6). *P ≤ 0.05 (Student's-t-Test).

3.3 ANOREXIGENIC EFFECTS OF WHOLE-BODY ARHGAP21 REDUCTION IN HET-HFD MICE

We also challenged Het mice to a high-fat diet. As shown in Fig. 3A, Het-HFD mice displayed lower body weight from the 3rd week until the end of the experimental period, accompanied by a decrease in perigonadal fat pad weight (Fig. 3B), than did the Ctl-HFD group. In addition, Het-HFD mice had less food intake (Fig. 3C) and presented higher levels in the mRNA of anorexigenic markers (POMC and CART) than did Ctl-HFD mice (Fig. 3D). However, NPY and AgRP mRNA levels were not different between groups (Fig 3D). Again, the expression of ARHGAP21 mRNA in the hypothalamus of Het-HFD mice was lower than in Ctl-HFD mice (Fig 3D).

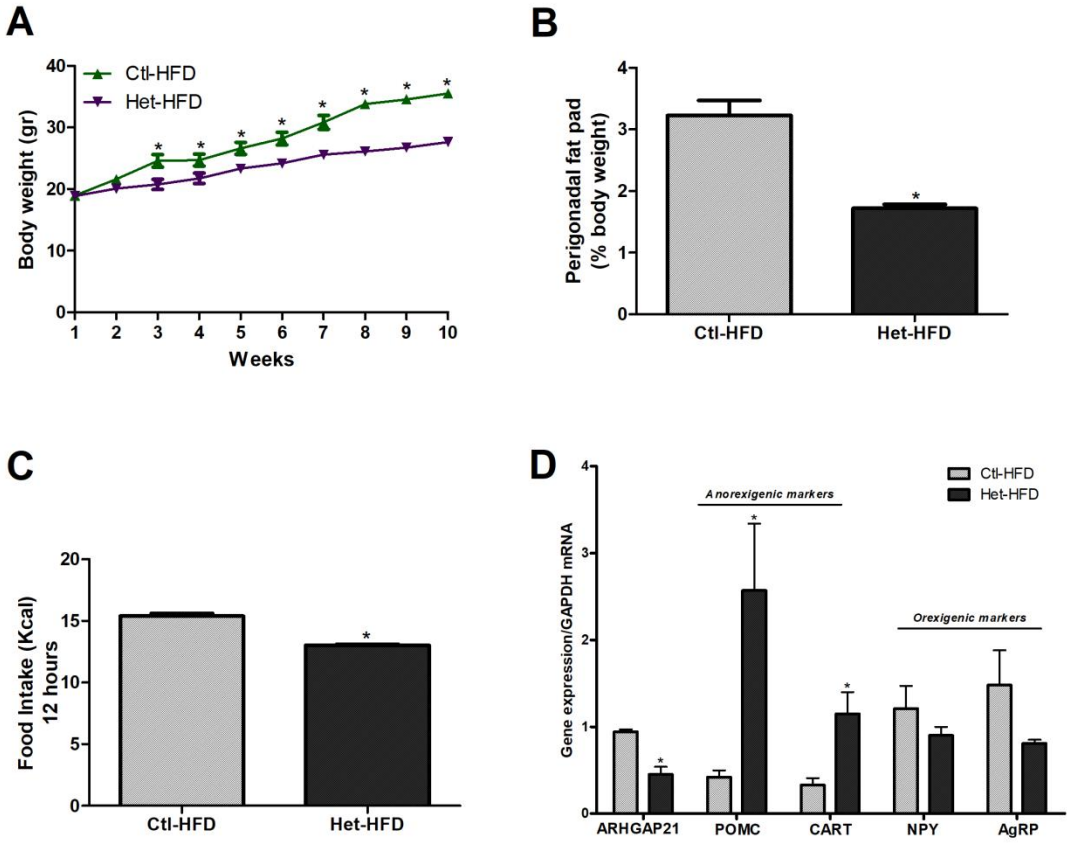

Figure 3. Anorexigenic effects of whole-body ARHGAP21 reduction in Het-HFD mice. Body weight curve (A). Perigonadal fat pad weight (B). Food intake over 12 hours (C). Real-time PCR assay of hypothalamic ARHGAP21, POMC, CART, NPY and AgRP mRNA levels (D). Control mice (Ctl) and ARHGAP21-haplodeficient mice (Het) fed a high-fat diet for 10 weeks. Data are the mean ± SEM (n=3-6). *P ≤ 0.05 (Student's-t-Test).

3.4 ENERGY HOMEOSTASIS OF ARHGAP21 HET-HFD MICE

The VO_2 (Fig. 4A) and heat rate (Fig. 4B) were higher in Het-HFD than in Ctl-HFD during dark and light periods. Moreover, the RER of Het-HFD mice was higher during the dark phase, suggesting that they predominantly used carbohydrate oxidation in this period, as opposed to the Ctl-HFD mice that displayed metabolic inflexibility (Fig. 4C). In addition, the ambulatory activity was significantly higher in the Het-HFD mice than in the Ctl-HFD mice (Fig. 4D-E). BAT weight was similar between the groups (Fig. 4F); however, we observed higher UCP1 mRNA expression (Fig. 4G) and protein content (Fig. 4H) in BAT of Het-HFD than in the Ctl-HFD group. Finally, Het-HFD mice had lower ARHGAP21 mRNA levels in the BAT than did the Ctl-HFD group (Fig. 4G).

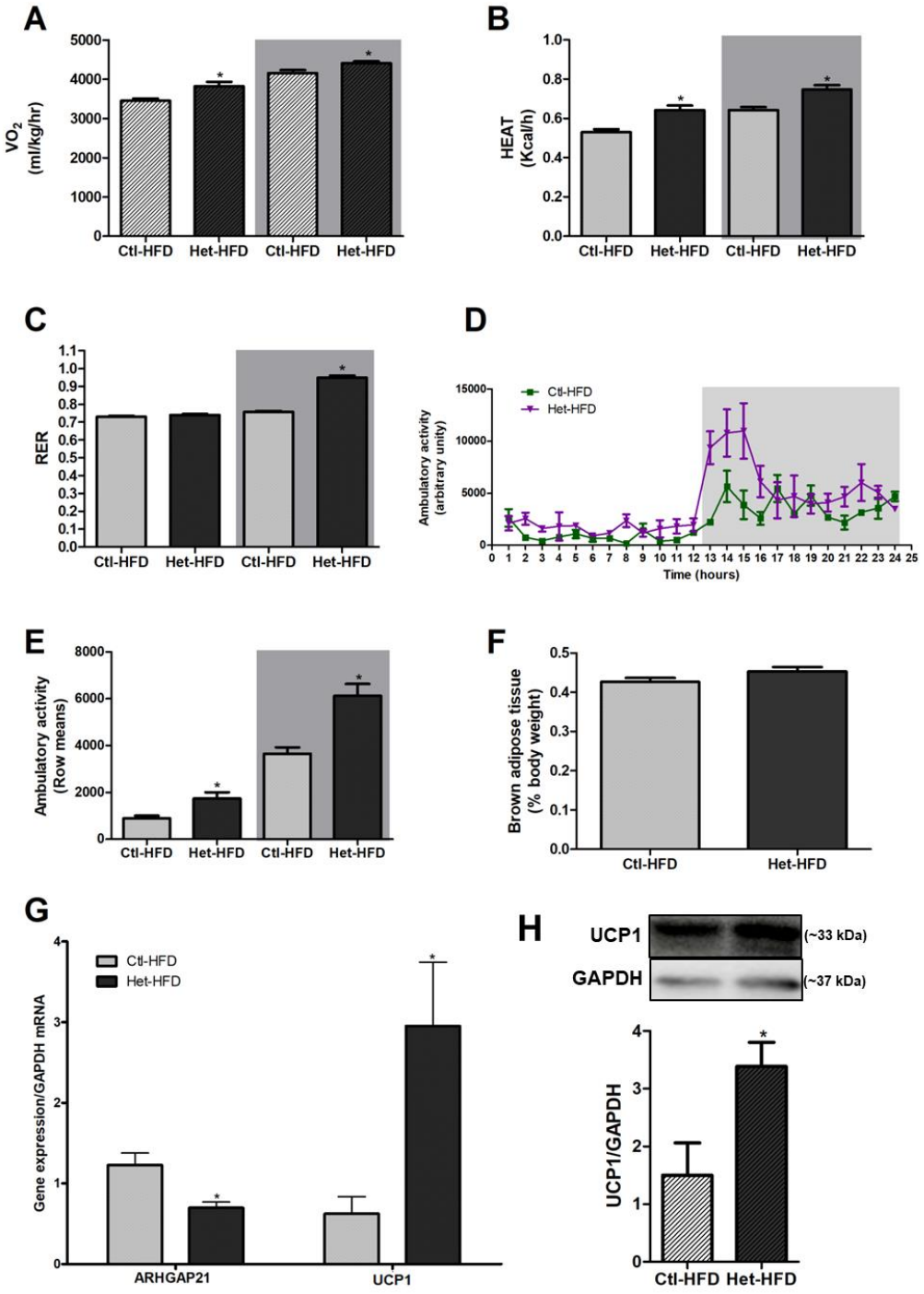

Figure 4. Energy homeostasis of ARHGAP21 Het-HFD mice. VO2 (A), heat rate (B) and respiratory exchange ratio (RER) (C). Ambulatory activity for 24 hours during light and dark periods (D) and mean of light and dark periods (E). BAT weight (F). Real-time PCR assay of ARHGAP21 and UCP1 mRNA levels in BAT (G). Brown adipose tissue UCP1 protein content (H). Control mice (Ctl) and ARHGAP21-haplodeficient mice (Het) fed a high-fat diet for 10 weeks. Data are the mean ± SEM (n=3-6). *P ≤ 0.05 (Student's-t-Test).

4 DISCUSSION

The inhibition of GAP TBC1D1 reduced the body weight and increased the energy expenditure in mice (16-20). Here, we extended these findings, showing a beneficial effect of whole-body reduction of ARHGAP21 in energy homeostasis, as judged by the increased energy expenditure and reduced food intake in both control and high-fat diet groups. These results were the first to point to possible involvement of a GAP family member in the control of food intake via increasing the expression of mRNA of the hypothalamic anorexigenic markers (POMC/CART). All these phenomena favor the reduction in body weight observed in ARHGAP21-haplodeficient mice.

We previously showed that ARHGAP21 inhibition decreased body weight in mice (21), but the mechanisms involved remain unknown. It is well established that body weight and appetite control are complex and that central mechanistic disturbances can lead to hyperphagia or anorexia depending on the balance between the expression of anorexic and orexic genes in the hypothalamus (7, 28). Here, we observed reduced food intake in Het and Het-HFD mice, corroborated by higher mRNA POMC and CART expression in the hypothalamuses of these mice.

POMC and CART peptides also stimulate energy expenditure (29). In fact, genetic ablation of POMC and CART in obese rodents was associated with reduced physical activity and energy expenditure (30). Conversely, intra-cerebroventricular (ICV) administration of CART in rodents induced an opposite effect (31). In agreement with these findings, we found that ARHGAP21-haplodeficient mice increased ambulatory activity and consequently energy expenditure, as judged by higher VO_2 and heat rates than their respective controls.

The hypothalamic expression of POMC and CART is also known to increase energy expenditure independent on their effect on ambulatory activity. These peptides stimulate specific central neurons that, via the efferent sympathetic branch, stimulate thermogenesis by increasing mitochondrial uncoupling in adipose tissue (32-34). Accordingly, here, we observed increased UCP1 mRNA levels and protein content in the BAT of Het and Het-HFD mice. Our results reinforce the previously described involvement of a Rho-GAP family protein, DLC1, as a regulator of the adipocyte phenotype (35).

Moreover, we evaluated the RER, widely utilized to evaluate the metabolic flexibility. The RER was calculated by measuring the amount of carbon dioxide (CO_2) produced in comparison to the amount of oxygen (O_2) used, and it is possible to predict which substrate is

being oxidized as a fuel. During the light period, when mice are at rest and fasting, the RER is ~0.6, indicating a predominant use of fatty acid. On the other hand, during the dark period, when they are more active and fed, the RER is ~1.0, suggesting that they are using predominantly carbohydrate oxidation (36, 37). In some pathologies, such as, obesity and diabetes, the organism display metabolic inflexibility due to the incapacity to adjust uptake of macronutrients according to the metabolic needs (37, 38). In our study, as expected, mice submitted to high-fat diet presented metabolic inflexibility (Figure 4C). In fact, Het-HFD mice presented increased RER during the dark phase, suggesting an improvement in glucose-insulin homeostasis, as previously shown (21). These data support the hypothesis that ARHGAP21 reduction is able to boost the nutrient handling, energetic homeostasis, and metabolic flexibility (36, 37).

In summary, our study provides evidence supporting the beneficial effects of ARHGAP21 reduction upon energetic homeostasis, reducing food intake and increasing energy expenditure. Altogether, these events contributed to a reduction in body weight even in mice fed a high-fat diet. Thus, ARHGAP21 protein emerges as an important candidate to be considered for the prevention and treatment of obesity and associated diseases.

5 CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 AUTHOR CONTRIBUTIONS

G.M.S. AND H.C.B.S. CONCEIVED AND DESIGNED RESEARCH; G.M.S. AND L.Z. PERFORMED EXPERIMENTS; G.M.S., L.Z., J.M.C.J., J.F.V., AND H.C.B.S. ANALYZED DATA; G.M.S., L.Z., J.M.C.J., J.F.V., AND H.C.B.S. INTERPRETED RESULTS OF EXPERIMENTS; G.M.S. AND H.C.B.S. PREPARED FIGURES; E.M.C. AND A.C.B. CONTRIBUTED TO REAGENTS/MATERIALS/ANALYSIS TOOLS. S.T.S. PROVIDED THE KNOCKDOWN ANIMALS USED IN THE EXPERIMENTS; G.M.S., J.M.C.J., J.F.V., DRAFTED MANUSCRIPT; G.M.S., A.C.B., AND H.C.B.S. EDITED AND REVISED MANUSCRIPT; G.M.S., L.Z., J.M.C.J., J.F.V., E.M.C., S.T.S., A.C.B., AND H.C.B.S. APPROVED FINAL VERSION OF MANUSCRIPT.

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5. Discussão

As GAPs apresentam papéis fisiológicos específicos na homeostase da glicose, dependendo de suas isoformas (Bos *et al.*, 2007). Neste trabalho, nós demonstramos que a redução da ARHGAP21 provoca um efeito benéfico na homeostase da glicose, conforme observado por uma melhora na tolerância à glicose e sensibilidade à insulina, associada à redução da secreção de insulina. Esses efeitos foram observados apenas em camundongos heterozigotos para ARHGAP21 que receberam a dieta hiperlipídica (Het-HFD).

Nosso estudo é o primeiro a mostrar que a redução do conteúdo da Rho-GAP ARHGAP21 aumenta a sensibilidade à insulina, resultando na melhoria da homeostase da glicose, sugerindo semelhanças com outra Rho-GAP – TCGAP - que, quando superexpressa em adipócitos, prejudica a captação da glicose (Chiang *et al.*, 2003). O mecanismo pelo qual o TCGAP afeta a captação da glicose induzida pela insulina parece envolver uma interação física com a sinalização da via não-canônica da insulina: via *Cbl associated protein/Casitas B-lineage lymphoma* (CAP/Cbl) (Baumann *et al.*, 2000; Chiang *et al.*, 2003). Como as Rho-GAPs ARHGAP21 e TCGAP são proteínas com diferentes domínios (Chiang *et al.*, 2003; Bos *et al.*, 2007), é difícil presumir que elas exibam um mecanismo de ação semelhante. Dessa forma, mais estudos são necessários para esclarecer o mecanismo molecular pelo qual o heterozigotos *da* ARHGAP21 melhora a sensibilidade periférica à insulina e a tolerância à glicose.

Provavelmente, como consequência da melhora na sensibilidade à insulina, os camundongos Het-HFD apresentaram redução na secreção de insulina e menor área de células beta pancreáticas. Outra possível explicação para a melhora na homeostase da glicose, observada nos camundongos Het-HFD, é um efeito direto da redução da ARHGAP21 nas células beta pancreáticas. De fato, nosso grupo demonstrou que sua redução parcial, especificamente em ilhotas pancreáticas de camundongos neonatos, aumentou a secreção de insulina expostas à concentração baixa de glicose (Ferreira *et al.*, 2015). Entretanto, esses experimentos foram realizados em ilhotas pancreáticas imaturas, que apresentam comportamento fisiológico diferente, quando comparadas às exploradas aqui (Mendonça *et al.*, 1998; Carvalho *et al.*, 2010). Frente a isso, a redução da ARHGAP21 previne alterações morfofuncionais oriundas da dieta hiperlipídica no pâncreas endócrino de camundongos, evitando a hipersecreção de insulina.

Além da melhora na homeostase glicêmica, animais do grupo Het-HFD apresentaram diminuição do peso corporal e dos depósitos de gordura. Curiosamente, os camundongos

heterozigotos para ARHGAP21 que receberam a dieta padrão (Het) também apresentam menor peso corporal. No entanto, esta característica ocorre, provavelmente, por um mecanismo diferente daquele observado em camundongos Het-HFD, uma vez que eles apresentam redução do tecido muscular ao invés de redução do TAB, e também aumento do depósito de TAM. Dessa forma, avaliamos o metabolismo energético desses animais e observamos um efeito benéfico associado à redução da ARHGAP21 na homeostase energética, a julgar pelo aumento do gasto energético e pela redução do consumo alimentar em ambos os grupos heterozigotos para ARHGAP21, recebendo dieta controle ou hiperlipídica.

A GAP mais estudada na homeostase energética é a Rab-GAP TBC1D1. Seu *knockout* reduz o peso corporal, diminui o quociente respiratório e aumenta o gasto energético, além de suprimir a obesidade induzida por dieta em modelo de experimentação animal (Chadt *et al.*, 2008; Dokas *et al.*, 2013; Hargett *et al.*, 2015; Dokas *et al.*, 2016; Hargett *et al.*, 2016). Em camundongos obesos deficientes em leptina, a deleção de TBC1D1 não tem impacto sobre o comportamento alimentar ou a ingestão calórica, mas resulta em aumento do gasto energético, alteração da preferência do substrato energético com aumento da oxidação de ácidos graxos e supressão da obesidade (Dokas *et al.*, 2016). Especula-se que a TBC1D1 possa estar envolvida em uma via ainda desconhecida que controla a homeostase energética, presumivelmente regulando a oxidação de ácidos graxos no músculo esquelético, entretanto, seu papel potencial na regulação central do metabolismo energético ainda não foi totalmente esclarecido (Chadt *et al.*, 2008).

O controle do peso corporal e do apetite é derivado de mecanismos complexos, e distúrbios nesses mecanismos centrais podem levar à hiperfagia ou anorexia, dependendo do equilíbrio entre a expressão de genes anorexigênicos e orexigênicos no hipotálamo (Benarroch, 2010; Kim *et al.*, 2018). Aqui, observamos uma redução na ingestão alimentar em camundongos Het e Het-HFD, corroborada pela maior expressão gênica hipotalâmica de POMC e CART. Nossos resultados foram os primeiros a apontar para o possível envolvimento de um membro da família GAP no controle da ingestão alimentar através do aumento da expressão gênica de marcadores anorexígenos hipotalâmicos.

Os peptídeos POMC e CART também estimulam o gasto energético (Thorburn and Proietto, 2000). De fato, a deleção de POMC e CART em roedores obesos foi associada à redução da atividade física e do gasto energético (Butler and Cone, 2001). Enquanto a administração intra-cerebroventricular (ICV) de CART em roedores induziu um aumento do gasto energético (Kimmel *et al.*, 2000). De acordo com esses achados, observamos que os

animais heterozigotos para ARHGAP21 apresentaram aumento na atividade locomotora e, consequentemente, no gasto energético, além de maiores taxas de VO_2 e aumento na produção de calor, comparados aos seus respectivos controles.

A expressão hipotalâmica de POMC e CART também é conhecida por aumentar o gasto energético, independente de seu efeito na atividade locomotora. Esses peptídeos estimulam neurônios centrais específicos que, via ramo simpático eferente, estimulam a termogênese pelo aumento do desacoplamento mitocondrial no TAM (Fedorenko *et al.*, 2012; Morrison and Madden, 2014; Diniz and Bittencourt, 2017). Assim, aqui, observamos aumento na expressão gênica da UCP1 no TAM dos grupos Het e Het-HFD. Nossos resultados reforçam o envolvimento de Rho-GAPs na termogênese, como previamente descrito para uma proteína da mesma família, a *Deleted in liver cancer-1* (DLC1), como um regulador do fenótipo dos adipócitos (Sim *et al.*, 2017).

Dessa forma, todos os fenômenos citados acima favorecem a redução do peso corporal observada em camundongos heterozigotos para ARHGAP21. Assim, acreditamos que a redução na expressão de ARHGAP21 pode regular a expressão gênica de POMC e CART, resultando na modulação do comportamento alimentar e, consequentemente, do peso corporal. As informações sobre a ARHGAP21 e o hipotálamo ainda são escassas, e o papel dessa proteína no hipotálamo e na regulação da ingestão alimentar ainda não foi explorado. Um estudo recente demonstrou o efeito de uma proteína G de pequeno tamanho molecular, *Ras-proximate-1* (Rap1), na ingestão de alimentos e no gasto energético. A Rap1 é expressa em múltiplos núcleos do hipotálamo que regulam o metabolismo corporal. Esta proteína pode ser regulada pela *RAP1 GTPase activating protein 1* (Rap1GAP), uma GAP que age de forma semelhante a ARHGAP21. A deleção de Rap1 protege os animais de alterações em genes hipotalâmicos induzidas pela dieta, protegendo-os da obesidade. Este efeito está associado ao aumento da expressão de POMC e aumento da sinalização de insulina e leptina no hipotálamo, juntamente com a redução da ingestão de alimentos e ganho de peso (Kaneko *et al.*, 2016). Assim, especula-se que a modulação da atividade de proteínas G de pequeno tamanho molecular, através de suas proteínas reguladoras, como Rap1GAP ou ARHGAP21, pode alterar a expressão dos genes hipotalâmicos anorexígenos, o gasto energético e o comportamento alimentar.

Além disso, a relação de troca respiratória (RER) de camundongos Het-HFD encontra-se aumentada durante o ciclo escuro, sugerindo uma recuperação da flexibilidade metabólica. Animais sadios oxidam, preferencialmente, glicose durante o ciclo escuro (estado alimentado) e ácidos graxos durante o ciclo claro (estado de jejum) (Randle, 1986; Goodpaster and Sparks,

2017). Na obesidade, essa flexibilidade se torna prejudicada, caracterizando a inflexibilidade metabólica (Goodpaster and Sparks, 2017). Aqui, mostramos que a redução do conteúdo da ARHGAP21 foi capaz de melhorar o metabolismo energético dos animais submetidos à dieta hiperlipídica, provavelmente devido à normalização da sensibilidade à insulina, permitindo adequada captação e oxidação da glicose. Estes dados reforçam a hipótese de que a redução do conteúdo da ARHGAP21 é capaz de impulsionar o manuseio de nutrientes, a homeostase energética e a flexibilidade metabólica (Randle, 1986; Goodpaster and Sparks, 2017).

6. Conclusão

A redução do conteúdo da ARHGAP21 contribuiu para diminuição do peso corporal e reestabelecimento da homeostase glicêmica, mesmo em camundongos alimentados com uma dieta rica em gordura. Esses achados sugerem a proteína Rho-GAP ARGHAP21 como um importante modulador do metabolismo glicêmico e energético, apoiando futuras investigações para explorar o mecanismo pelo qual esse fenômeno ocorre.

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8. Anexos

8.1. Comitê de ética para experimentação animal

UNICAMP

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Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o projeto "**Participação da atividade colinérgica e proteínas G de pequeno tamanho molecular em mecanismos envolvidos no transporte do grânulo exocítico e secreção de insulina**" (protocolo nº **3783-1**), sob a responsabilidade de **Profa. Dra. Helena Cristina de Lima Barbosa Sampaio / Gabriela Moreira Soares**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **18 de março de 2015**.

Campinas, 18 de março de 2015.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

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8.2. Publicação do artigo 01

ORIGINAL RESEARCH ARTICLE

WILEY Journal of Cellular Physiology

Whole body ARHGAP21 reduction improves glucose homeostasis in high-fat diet obese mice

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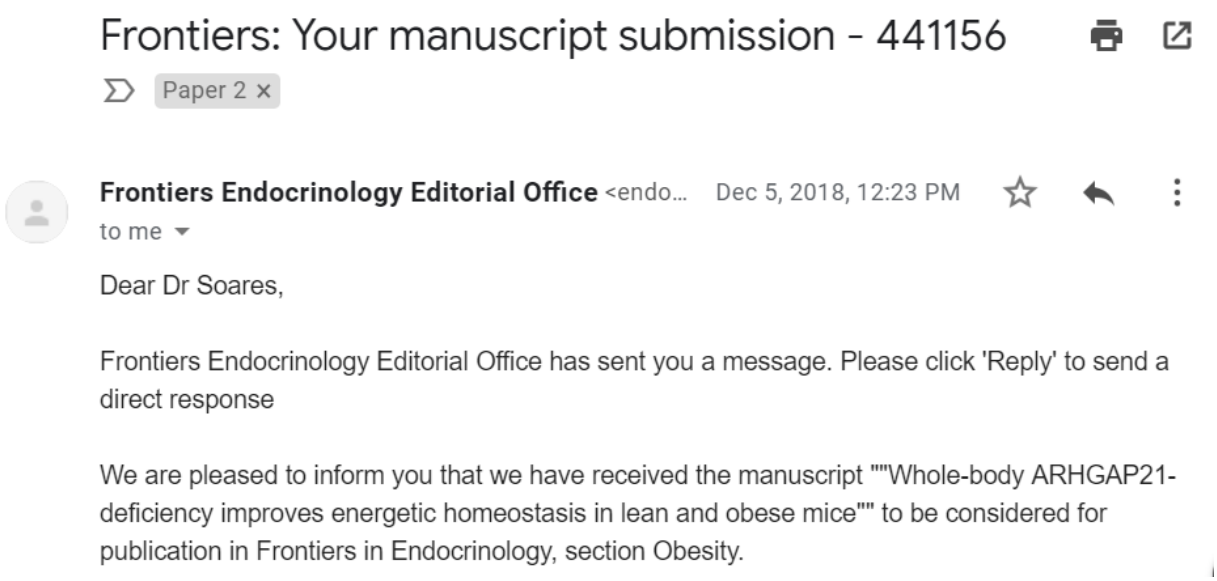
GTPase activating proteins (GAPs) are ubiquitously expressed, and their role in cellular adhesion and membrane traffic processes have been well described. TBC1D1, which is a Rab-GAP, is necessary for adequate glucose uptake by muscle cells, whereas increased TCGAP, which is a Rho-GAP, decreases GLUT4 translocation, and consequently glucose uptake in adipocytes. Here, we assessed the possible involvement of ARHGAP21, a Rho-GAP protein, in glucose homeostasis. For this purpose, wild type mice and ARHGAP21 transgenic whole-body gene-deficiency mice (heterozygous mice, expressing approximately 50% of ARHGAP21) were fed either chow (Ctl and Het) or high-fat diet (Ctl-HFD and Het-HFD). Het-HFD mice showed a reduction in white fat storage, reflected in a lower body weight gain. These mice also displayed an improvement in insulin sensitivity and glucose tolerance, which likely contributed to reduced insulin secretion and pancreatic beta cell area. The reduction of body weight was also observed in Het mice and this phenomenon was associated with an increase in brown adipose tissue and reduced muscle weight, without alteration in glucose-insulin homeostasis. In conclusion, the whole body ARHGAP21 reduction improved glucose homeostasis and protected against diet-induced obesity specifically in Het-HFD mice. However, the mechanism by which ARHGAP21 leads to these outcomes requires further investigation.

KEYWORDS
ARHGAP21, glucose homeostasis, insulin secretion, obesity, Rho-GAP

8.3. Submissão e resposta do Artigo 02



Frontiers: Your manuscript submission - 441156

Paper 2 ×

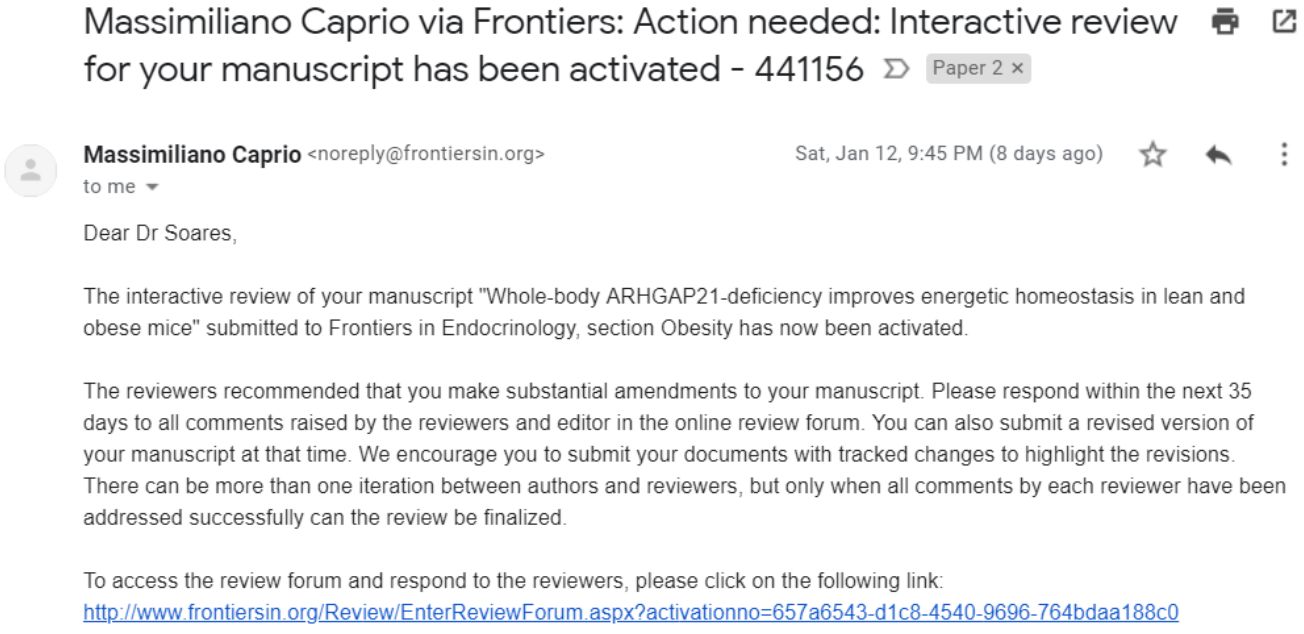
Frontiers Endocrinology Editorial Office <endo… Dec 5, 2018, 12:23 PM

to me

Dear Dr Soares,

Frontiers Endocrinology Editorial Office has sent you a message. Please click 'Reply' to send a direct response

We are pleased to inform you that we have received the manuscript ""Whole-body ARHGAP21-deficiency improves energetic homeostasis in lean and obese mice"" to be considered for publication in Frontiers in Endocrinology, section Obesity.



Massimiliano Caprio via Frontiers: Action needed: Interactive review for your manuscript has been activated - 441156

Paper 2 ×

Massimiliano Caprio <noreply@frontiersin.org> Sat, Jan 12, 9:45 PM (8 days ago)

to me

Dear Dr Soares,

The interactive review of your manuscript "Whole-body ARHGAP21-deficiency improves energetic homeostasis in lean and obese mice" submitted to Frontiers in Endocrinology, section Obesity has now been activated.

The reviewers recommended that you make substantial amendments to your manuscript. Please respond within the next 35 days to all comments raised by the reviewers and editor in the online review forum. You can also submit a revised version of your manuscript at that time. We encourage you to submit your documents with tracked changes to highlight the revisions. There can be more than one iteration between authors and reviewers, but only when all comments by each reviewer have been addressed successfully can the review be finalized.

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8.4. Direitos Autorais

Declaração

Declaro que as cópias dos artigos de minha autoria e co-autoria, publicados e/ou submetidos para publicação em revistas científicas ou anais de congresso sujeitos a arbitragem, que constam na minha Tese de Doutorado intitulada: "**Participação da ARHGAP21 na regulação da homeostase glicêmica e energética de camundongos C57BL/6**" não infligem os dispositivos da Lei nº9610/98, nem o direito autoral de qualquer editor.

Campinas, 19 de março de 2019

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